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Polyamines in Experimental Taurodeoxycholate-Induced and Clinical Acute Pancreatitis

ACADEMIC DISSERTATION
To be presented, with the permission of
the Faculty of Medicine of the University of Tampere,
for public discussion in the Main Auditorium of Building M,
Pirkanmaa Hospital District, Teiskontie 35,
Tampere, on October 30th, 2009, at 12 o'clock.

UNIVERSITY OF TAMPERE

ACADEMIC DISSERTATION
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www.uta.fi/taju
http://granum.uta.fi

Cover design by
Juha Siro

Acta Universitatis Tamperensis 1442
ISBN 978-951-44-7803-1 (print)
ISSN-L 1455-1616
ISSN 1455-1616

Acta Electronica Universitatis Tamperensis 874
ISBN 978-951-44-7804-8 (pdf)
ISSN 1456-954X
http://acta.uta.fi

Tampereen Yliopistopaino Oy – Juvenes Print
Tampere 2009

To My Beloved **Ying** and **Toni**

CONTENTS

LIST OF ORIGINAL COMMUNICATIONS

The present dissertation is based on the following original publications referred to in the text by their Roman numerals (I-IV):

I. **Jin HT**, Lämsä T, Merentie M, Hyvönen MT, Sand J, Räty S, Herzig KH, Alhonen L, Nordback I. Polyamine levels in the pancreas and the blood change according to the severity of pancreatitis. Pancreatology. 2008;8:15-24.

II. **Jin HT**, Lämsä T, Hyvönen MT, Sand J, Räty S, Grigorenko N, Khomutov AR, Herzig KH, Alhonen L, Nordback I. A polyamine analog bismethylspermine ameliorates severe pancreatitis induced by intraductal infusion of taurodeoxycholate. Surgery. 2008;144:49-56.

III. **Jin HT**, Lämsä T, Nordback PH, Hyvönen MT, Räty S, Sand J, Herzig K-H, Alhonen L, Nordback I. Polyamine catabolism in relation to trypsin activation and apoptosis in experimental acute pancreatitis. Surgery, in revision.

IV. **Jin HT**, Räty S, Minkkinen M, Järvinen S, Sand J, Alhonen L, Nordback I. Changes of Blood Polyamine Levels in Human Acute Pancreatitis. Scand J Gastroenterol. 2009;44:1004-1011.

ABBREVIATIONS

AdoMet	Adenosyl-L-methionine
ALAT	Alanine aminotransferase
AP	Acute pancreatitis
APACHE II	Acute physiology and chronic health evaluation II
APP	Abdominal perfusion pressure
ATP	Adenosine-5'-triphosphate
BMI	Body mass index
CCK	Cholecystokinin
CDE	Choline-deficient ethionine
CDL	Closed duodenal loop
CRP	C-reactive protein
CT	Computed tomography
dcAdoMet	Decarboxylated AdoMet
DNA	Deoxyribonucleic acid
ERCP	Endoscopic retrograde cholangiopancreatography
FNA	Fine-needle aspiration
HCO_3^-	Bicarbonate
HEPES	N-2-hydroxyethylpiperazine-N^1-2-ethanesulfonic acid
HPLC	High performance liquid chromatography
IAP	Intra-abdominal pressure
IL	Interleukin
MeSpd	Methylspermidine
Me_2Spm	Bismethylspermine
MODS	Multiple organ dysfunction syndrome

MRCP	Magnetic resonance cholangiopancreatography
MRI	Magnetic resonance imaging
ODC	Ornithine decarboxylase
OF	Organ failure
PAO	Polyamine oxidase
PCT	Procalcitonin
PMN	Polymorphonuclear
PRSS1	Cationic trypsinogen
RBC	Red blood cell
RNA	Ribonucleic acid
Rpm	Revolutions per minute
SIRS	Systemic inflammatory response syndrome
SMO	Spermine oxidase
SOFA	Sequential organ failure assessment
SPINK1	Serine protease inhibitor Kazal type 1
SSAT	Spermidine/spermine N^1-acetyltransferase
TAP	Trypsinogen activation peptide
TNF	Tumour necrosis factor
TUNEL	Terminal deoxynucleotidyl transferase (TdT)-mediated dUTP-biotin nick-end labelling
US	Ultrasonography

ABSTRACT

Acute pancreatitis (AP) is a disease with various causes, signs, classifications and treatments in clinical practice. It is generally believed that the activation of exocrine pancreatic enzymes, especially trypsin activation, mostly contribute to the initiation of pancreatic injury. The pathogenesis of AP is still not clear. Polyamine catabolism is essential to cellular survival and functions and its association with experimental AP in transgenic model has been demonstrated. However, the relationship between polyamine catabolism and AP in wild-type experimental and clinical settings had never been studied. The aim of this dissertation was to explore the role of polyamine catabolism in the disease according to time-courses, severity, and aetiology by administrating polyamine analogue bismethylspermine (Me_2Spm) in several different experimental models and clinical patients with AP.

The changes of polyamine catabolism both in pancreatic and blood were associated with the extent of pancreatic necrosis in 2% and 6% taurodeoxycholate-induced AP. Especially, the blood spermidine and spermine levels decreased when the severity and necrotic extent of experimental AP were enhanced by higher concentration of taurodeoxycholate infusion. Any injury, such as sham operation to experimental animals could induce the decrease of polyamine levels in blood. The significant correlation between blood and pancreatic polyamine levels indicated that blood polyamine values may be representative of that in pancreas during AP.

After the observational study, the intervention study was introduced by administering polyamine analogue Me_2Spm in 2% taurodeoxycholate-induced AP. Me_2Spm ameliorated the pancreatic injury in the early stage, but not in the late progress of AP at 72 h. The drug accumulation in pancreas by treatment after induction of AP is more prominent than that by prophylaxis, and extra dose at 30 h

led to death of animals due to its toxicity. The beneficial effect of Me_2Spm depends on the timing and dose administered.

These findings showed that polyamine catabolism had a role in the mechanism of AP and introduced the hypothesis whether polyamine catabolism may mediate the trypsin activation in taurodeoxycholate-induced AP. In Study III, the changes of pancreatic trypsin activation, polyamine catabolism, necrosis and apoptosis were investigated and compared in taurodeoxycholate and transgenic models. It was found in taurodeoxycholate-induced AP that the trypsin activation took place earlier than spermidine/spermine N^1-acetyltransferase (SSAT) activation in severe form (6%), and concurred with SSAT activation in moderate form (2%) for 6 h. As a mild form, the SSAT activation was extremely highly induced in transgenic model at a very early time point and significant trypsin activation was only detected at 24 h. The protective effect of Me_2Spm may be mediated via other mechanisms, because Me_2Spm did not suppress the trypsin activation in 2% taurodeoxycholate-induced AP, and only the values of trypsinogen activation peptide, but not SSAT activity, had a significant association with the extent of pancreatic necrosis in taurodeoxycholate-induced AP models. Contrary to transgenic SSAT over-induced AP, polyamine may not be a mediator of trypsin activation in taurodeoxycholate-induced AP.

These experimental studies led to the clinical investigation that examined the changes of blood polyamine levels in patients with AP. The levels of blood polyamine showed no difference between AP (on admission) and control diseases (on admission)/healthy volunteers. There was also no difference found in patients with AP according to different aetiologies. However, on admission a few necrotic AP patients had significantly higher levels of putrescine than those without necrosis. Polyamine levels increased after clinical recovery and they correlated with inflammatory mediators.

In several respects, the observation in the clinical setting was consistent with that in experimental settings.

Based on the series of four studies, polyamine catabolism is associated with the early development of experimental AP according to severity, and it is not an early mediator of trypsin activation in taurodeoxycholate-induced AP. Me_2Spm can only ameliorate experimental AP with sufficient dose in early phase and its protective effect may not be mediated by suppressing trypsin activation. The changes of blood polyamine in human AP are consistent with that in experimental settings in several respects. A further experimental study will be introduced to monitor the changes of polyamine catabolism in remote organs during AP by administering polyamine analogue Me_2Spm. The effect of Me_2Spm on multiorgan injury and mortality needs to be studied during the development of the disease.

INTRODUCTION

Acute pancreatitis (AP) is acute inflammation of pancreas. The incidence of AP was 73 per 100,000 in Finland, including 70% of alcoholic and 20% of gallstone origin (Pelli et al. 2000, Pelli et al. 2008, Nordback et al. 2009). The diagnosis of the disease is based on the history of alcohol abuse and gallstone disease, the presence of symptoms with elevated amylase and lipase levels and confirmed by imaging investigations (Whitcomb 2006, Frossard et al. 2008). Currently there is no specific therapy for AP, except for supportive or preventive treatment (Frossard et al. 2008).

Generally the most accepted pathogenesis initiating AP is based on the discovery that the activation of trypsin in pancreatic acinar cells is the first step in the evolution of AP (Foitzik et al. 1994, Saluja et al. 1997, Hofbauer et al. 1998, Saluja et al. 1999). The associated genetic mutations are also found in several clinical investigations (Creighton et al. 2000, Felley et al. 2004, Frossard et al. 2008, Rebours et al. 2009). Besides trypsin activation, the following inflammatory cascades, such as release of cytokines, nitric oxide, reactive oxygen species and arachidonic acid metabolites, are believed to play important roles in the development of AP (Vollmar et al. 1989, Tsuji et al. 1994, Al-Mufti et al. 1998, Lundberg et al. 2000,).

Recently several studies have shown that experimental AP was induced by the over-activation of polyamine catabolism in transgenic rats (Alhonen et al. 2000, Hyvönen et al. 2006). Physiologically, polyamines are essential components for cellular survival and functions and very important in maintaining the integrity of pancreas (Alhonen et al. 2000). Furthermore, the use of polyamine analogues can ameliorate experimental AP in transgenic rats (Räsänen et al. 2002, Hyvönen et al. 2006).

The aim of the present dissertation is to investigate the role of polyamine catabolism in experimental taurodeoxycholate-induced and human AP by administering polyamine analogue bismethylspermine (Me_2Spm).

REVIEW OF THE LITERATURE

1 Acute pancreatitis

1.1 General

1.1.1 Anatomy

The pancreas was firstly described by the Greek anatomist and surgeon Herpophilus of Chalcedon in 300 B.C., and then named as an organ by Rufus of Ephesus in 100 B.C. (Busnardo et al. 1983, Howard and Hess 2002). The word "pancreas" originated from the Greek (*pan*: all; *kreas*: flesh) and referred to the organ that had no bone or cartilage present with uniform composition and consistency (Busnardo et al. 1983).

The adult pancreas is a transversely oriented organ extending from the "C" loop of the duodenum to the hilum of the spleen. Normally the pancreas is 20 cm in length and weighs 90 g in men and 85 g in women (Kumar et al. 2005).

The pancreas is a complex lobulated organ with distinct exocrine and endocrine components. The exocrine portion of the gland, which produces digestive enzymes, water and bicarbonate (HCO_3^-), constitutes 80% to 95% of the pancreas (Kumar et al. 2005, Lewis 2006). The endocrine portion is composed of about 1 million clusters of cells, the islets of Langerhans. The islet cells secrete insulin, glucagon, amylin, pancreatic polypeptide, somatostatin and ghrelin, and constitute only 1% to 2% of the organ (Kumar et al. 2005).

The pancreatic duct system varies greatly. The main pancreatic duct most commonly drains into the duodenum at the papilla of Vater, whereas the accessory pancreatic duct most often drains into the duodenum through a separate minor papilla approximately 2 cm proximal to the major papilla of

Vater (Kumar et al. 2005, Lerch et al. 2006). The main pancreatic duct merges with the common bile duct proximal to the papilla of Vater and creates the ampulla of Vater.

1.1.2 Physiology of exocrine pancreatic secretion

Pancreatic acinar cells produce the digestive enzymes, and a series of ductules and ducts to convey the secretions to the duodenum. The first discovery of the physiological function of the pancreas in digestion was made by Claude Bernard, who showed that pancreatic juice was important for the physiology of digestive system (Busnardo et al. 1983). Acinar cells form membrane-bound zymogen granules containing the digestive enzymes. The zymogen granules impart a distinctive eosinophilic granular appearance to the apices of the acinar cells. When the acinar cells are stimulated to secrete, the zymogen-containing granules migrate apically, fuse with the apical plasma membrane, and release their contents into the central acinar lumen. These secretions are then transported to the duodenum through a series of ducts. The epithelial cells lining the ducts are also active participants in pancreatic secretion and in producing a fluid rich in HCO_3^-. The epithelial cells lining the larger ducts are more columnar and can produce mucin (Kumar et al. 2005). Disorder in the pancreatic physiology may result in "autodigestion" of the gland associated with AP.

The pancreas is regulated by hormones in the blood and through the autonomic nervous system, involved in vagal nerves, secretin and cholecystokinin (CCK). It is evident that intake of chyme evokes pancreatic HCO_3^- secretion. In human secretin evokes a large volume of HCO_3^--rich fluid from the pancreatic ducts, however, CCK has no effect on secretion. Human pancreatic acini lack both CCK-A and CCK-B receptors. By contrast, in rats CCK evokes a great amount of electrolyte secretion which originates in the pancreatic acini (Case 2006).

1.2 Clinical acute pancreatitis

1.2.1 Epidemiology and Aetiology

The incidence of AP varies widely between countries. The incidence of the first attack of AP is 44 per 100,000 adults in California, USA (Frossard et al. 2008). In Finland, the incidence of in-hospital treated AP episodes was 73 per 100, 000 and kept increasing until the early 2000's (Pelli et al. 2008). During the past 6 years, the incidence of admissions for alcoholic AP decreased in Finland, although the average alcohol consumption still increased (Sand et al. 2009). Gallstones (38%) and abuse of alcohol (36%) are the leading risk factors for AP in adults (Whitcomb 2006, Frossard et al. 2008), which account for 20% and 70% of the causes of AP in Finland respectively (Pelli et al. 2000, Räty et al. 2003, Pelli et al. 2009). Co-factors including amounts of alcohol consumption, type of alcohol consumed, drinking patterns, dietary habits or smoking, also have effects on the occurrence of alcoholic AP (Sand et al. 2007). The amount of alcohol consumed had an association with the prevalence of AP in Finland and especially with the severity of the first attack of alcoholic AP (Räty et al. 2003). Alcohol is the leading risk factor of AP in central and northern Europe, whereas biliary cause seems more common in the United States (Beger et al. 2003). Other causes of AP are uncommon or controversial, including pancreas divisum or sphincter of Oddi dysfunction, pancreatic tumours, endoscopic retrograde cholangiopancreatography (ERCP), hypertriglyceridaemia, obesity, diabetes, pregnancy, hypercalcaemia, use of drugs and genetic mutation.

1.2.2 Diagnosis

AP is an acute inflammatory process of the pancreas, with variable involvement of peripancreatic tissues and remote organ systems (Mitchell et al. 2003). The clinical diagnosis of AP is based on the history of alcoholic abuse and gallstone diseases, the presence of clinical symptoms and signs such as nausea, abdominal pain, Cullen's sign or Grey-Turner's sign, companied by the elevation of

amylase and lipase levels. In the majority of patients with first alcoholic AP, the symptoms begin during the early withdrawal period (Nordback et al. 2005).

Of the laboratory tests blood amylase level more than 3-fold the upper limit of normal range supports the diagnosis of AP. Within 3-5 days after onset of symptoms its level returns to normal (Matull et al. 2006, Frossard et al. 2008). With this cut-off level, amylase as a diagnostic test has a specificity approaching 95% and a sensitivity as 61% (Matull et al. 2006). In comparison with amylase, serum lipase level remains high for a longer period (up to 8 – 14 days) (Matull et al. 2006, Frossard et al. 2008). Although lipase is also not specific to the pancreas, its diagnostic accuracy may be better than amylase with specificities above 95% and sensitivities ranging 55 – 100% with a cut-off value of 600 IU/l (Matull et al. 2006). Using urinary trypsinogen-2 test strip, a negative result excludes AP with high probability (Kylänpää-Bäck et al. 2000). Serum disialotransferrin is possibly an optimal marker to distinguish between alcoholic and non-alcoholic AP (Methuen et al. 2007).

Imaging investigations, such as plain computed tomography (CT), contrast-enhanced CT and magnetic resonance imaging (MRI), are useful to support or confirm the diagnosis of AP by detecting pancreatic oedema or necrosis and fluid collections (Whitcomb 2006, Frossard et al. 2008). Furthermore, the cause of the disease, like gallstones and sludge, can be identified or excluded by imaging studies. In a mild form of AP, CT findings may be false negative. MRI may identify necrosis and fluid collections even better than CT (Frossard et al. 2008).

1.2.3 Severity

According to Atlanta criteria, the severity of AP is defined by the presence or absence of organ failure (OF) (shock, pulmonary insufficiency, renal failure, gastrointestinal bleeding, disseminated

intravascular coagulation or severe hypocalemia) or local complications (pancreatic necrosis, abscess or pseudocyst) (Bradley 1993). In 80% of patients, AP is mild and self-limiting and resolves without serious morbidity, but in up to 20% the disease is severe and is complicated by substantial morbidity (Räty et al. 1998). Patients with severe AP have an expected mortality of 15-20% (Matull et al. 2006, Frossard et al. 2008).

The scoring systems for assessing organ injury include acute physiology and chronic health evaluation II (APACHE II), Ranson's score, modified Glasgow score and sequential organ failure assessment (SOFA) score. Their abilities vary in predicting severe AP in its early stages. The cut-off value for APACHE II ≥ 8 can identify patients with the severe form with 71% accuracy at 24 hours after admission to hospital with Ranson's score ≥ 3 with 58-63% accuracy and modified Glasgow score ≥ 3 with 59-84% accuracy at 48 hours (Whitcomb 2006). Daily maximum SOFA score has high discrimination values in predicting hospital mortality rates with 0.847 area under the curve value (Halonen et al. 2002). Multiple organ dysfunction syndrome (MODS) score (Marshall et al. 1995) is also used to define OF and it is very close to the definition of the Atlanta criteria. Recently, it was reported that the bedside index for severity in acute pancreatitis score could be used as a simple way to identify patients at risk of increased mortality and the development of intermediate markers of severity within 24 h of presentation (Singh et al. 2009).

Several laboratory markers for predicting the severity of AP have been developed. Serum amylase and lipase are poor predictors of severity. C-reactive protein (CRP) has been shown to be a severity marker with above 80% specificity and sensitivity at 48 hours after onset of symptoms if the cut-off level is set at 150 mg/l (Matull et al. 2006). On admission the sensitivity and specificity of the urinary trypsinogen-2 dipstick in predicting severe AP were 62% and 87% respectively (Lempinen et al. 2001). At 24 hours after admission, the sensitivity of pro-inflammatory cytokine Interleukin 6

(IL-6) for detecting severe AP is 100%, with specificity of 86% at a cut-off value of 2.7 ng/l (Matull et al. 2006). The anti-inflammatory cytokine IL-10 is increased throughout the course of severe AP and is higher than in mild AP within the first 3 days after the onset of symptoms (Mayer et al. 2000), and shows the prediction of severity with 62.5% sensitivity and 54.8% specificity by setting the cut-off value at 37 pg/ml (Stimac et al. 2006). The combination of IL-10 and calcium had a positive predictive value of 56% and negative predictive value of 99% for the prediction of OF (Mentula et al. 2005). However, serum concentration of IL decreases swiftly and its use in clinical practice is limited by the complexity of the assay. Amino acid propeptide procalcitonin (PCT) has 97% negative predictive value for identifying these patients who later developed OF (cut-off 0.5 pg/l) at 24 hours after admission and had 86% sensitivity and 89% specificity for predicting the death of patients with severe AP within 3-4 days after the onset of disease (Matull et al. 2006, Rau et al. 2007). In a Finnish study (Kylänpää-Bäck et al. 2001b), PCT assessment had a sensitivity of 94% and a specificity of 73% for the development of OF at 24 h after admission. Furthermore, PCT strip test showed better sensitivity and specificity in predicting severe AP than CRP, APACHE II or Ranson score (Kylänpää-Bäck et al. 2001a). A more than 10% decrease of haematocrit predicts severe AP (Whitcomb 2006). Body mass index (BMI) could be used as a predictor of outcome in AP while patients with obesity (BMI $\geq$ 30 kg/m^2) had a higher risk of complications and mortality (Martinez et al. 2006). Other markers, such as phospholipase A2, trypsinogen activation peptide (TAP), anionic trypsinogen 2, polymorphonuclear elastase (PMN elastase), urinary TAP have an association with the severity of the disease but are not used in routine clinical practice.

The severity of AP can also be classified by CT severity index. It is calculated by summing two scores: CT grade (CT scan grade for investigation of pancreas) and necrosis score (contrast enhanced-CT scan score for pancreatic necrosis) (Balthazar 2002, Frossard et al. 2008). There was a significant correlation, with a continuous increasing incidence of morbidity and mortality in patients

according to the CT severity index, with 76% sensitivity and 93% specificity by cut-off score 3 (Chatzicostas et al. 2003). The sensitivity and specificity of unenhanced CT grade for predicting mortality were 100% and 57% respectively within 72 hours after the onset of symptoms (Casas et al. 2004). For patients who are allergic to iodinated contrast materials or have renal insufficiency contrast-enhanced CT can not be performed and MRI is then a useful alternative measure to study the pancreas (Balthazar 2002). Recently the question about the safety of MRI with contrast agent has arisen and the reliance on Gadolinium contrast agents must be re-examined. Exposure to Gadolinium contrast agents has led to mild side effects like dizziness or nausea, or even severe complications including acute renal failure, AP or development of nephrogenic systemic fibrosis (Steen and Schwenger 2007).

1.2.4 Complications

Overall mortality from AP varies from 2% to 10% (Mitchell et al. 2003). Patients with severe disease are at risk of death. Half of early deaths occur within 14 days, whereas late deaths happen within 3 months, with multiple OF possibly originating from infection complications due to pancreatic necrosis (Frossard et al. 2008). Systemic inflammatory response syndrome (SIRS), which is induced by the activation of inflammatory cascade mediated by cytokines, implies the presence of non-specific inflammation in a clinical setting (Mofidi et al. 2006). The criteria for SIRS include body temperature > 38°C or < 36°C, heart rate > 90/min, hyperventilation evidenced by respiratory rate > 20/min or $PaCO_2$ < 32 mmHg, white blood cell count > 12,000 cells/ μl or < 4,000/ μl. The diagnosis of SIRS requires more than one of above findings (Levy et al. 2003). The presence of SIRS at 48 hours after the onset of symptoms and also persistent SIRS correlated strongly with mortality indicating that it may be useful in predicting the OF (Buter et al. 2002, Mofidi et al. 2006).

The deaths from AP are mostly attributed to an occurrence of MODS, especially respiratory failure followed by sequential failure of other organ systems. Single OF is often reversible and has low mortality (Buter et al. 2002, McKay and Buter 2003). The most often OF involved is respiratory insufficiency. The severity of pulmonary complications can vary from mild hypoxemia without clinical or radiological abnormalities to severe respiratory distress with two peaks: on admission and on day 5 after admission (McKay and Buter 2003, Frossard et al. 2008). Its incidence is highest in severe AP, ranging from 15% to 55%, and the involvement of additional organs is associated with substantially increased mortality (McKay and Buter 2003, Frossard et al. 2008). Hepatic injury is usually mild during AP but contributes to the systemic inflammatory response (Frossard et al. 2008). However, high risk of death is found in AP patients with a combination of hepatic and renal failure. In addition, early renal dysfunction in patients with a history of cardiovascular medication is a sign of poor prognosis (Halonen et al. 2002).

OF is a dynamic process that starts early and may improve, persist or deteriorate in the hours after onset (Buter et al. 2002). Among cases of severe AP, 44% are complicated by early MODS and 57% of early MODS occur already on admission (McKay and Buter 2003). Early and progressive OF is associated with high mortality, but patients with transient OF may have an uncomplicated course (Bollen et al. 2008). When transient OF resolves within 48 hours, there is a very low risk of death (Johnson and Abu-Hilal 2004). The presence of early OF in combination with necrosis is significantly associated with the development of infection of pancreatic necrosis. Persistent OF beyond 48 hours also carries a poor prognosis (Lytras et al. 2008). Patients with OF are susceptible to early refractory shock, bacterial translocation and later multiple OF due to sepsis (Lytras et al. 2008). However, the cause-effect relationship between the extension of necrosis and the persistent OF is still not clear.

Among patients with severe AP 10 – 20% is associated with identified pancreatic necrosis (Beger et al. 2003). This is classified into sterile and infected necrosis (Frossard et al. 2008). Necrosis is a potential nidus for secondary infection. Such may occur in 40 – 70% of patients with necrosis. The extent of necrosis is greater in infected necrosis than in sterile necrosis (Buchler et al. 2000, Beger et al. 2003). Patients with infected necrotizing AP more often have OF (Rau et al. 2000). Infected necrosis primarily leads to late death. Mortality in sterile and infected necrosis is 10% and 25% respectively (Bollen et al. 2008, Frossard et al. 2008). Systemic complications may also be present in patients with sterile necrosis, but the mortality still remains low (Beger et al. 2003).

According to site, necrosis may be peripancreatic necrosis and pancreatic parenchymal necrosis. Patients with only peripancreatic necrosis have better outcome (Acute Pancreatitis Classification Working Group 2008, Bollen et al. 2008). Both peripancreatic and pancreatic parenchymal necrosis may develop in the initial phase of severe AP (Beger et al. 2003). The accuracy of contrast-enhanced CT in diagnosing peripancreatic necrosis may not be good (Bollen et al. 2008). Although the sensitivity of contrast-enhanced CT for pancreatic necrosis is close to 100% in 4 – 10 days after onset, contrast-enhanced CT may still miss not only the peripancreatic but also the presence of parenchymal necrosis (Beger et al. 2003).

The organisms in pancreatic infection include 58% Gram-negative organisms, 55% Gram-positive organisms and 24% fungal organisms (Gloor et al. 2001). Gram-negative bacteria have been the predominant germs found in infected necrosis and the early use of antibiotics can change the characteristics of the organism spectrum in pancreatic infection and contribute to secondary fungal infection (Buchler et al. 2000, Beger et al. 2003, Berzin et al. 2007). Pancreatic infection may be indicated by the presence of a gas bubble in CT (Bollen et al. 2008), and it can be diagnosed by an image-guided fine-needle aspiration (FNA) with a positive Gram stain and culture (Acute

Pancreatitis Classification Working Group 2008). Pancreatic abscess is the consequence of liquefaction and infection of necrotic tissue, where a circumscribed intra-abdominal collection of pus and minimally necrotic debris is found more than 4 weeks after onset (Bradley 1993, Beger et al. 2003, Acute Pancreatitis Classification Working Group 2008).

Pancreatic pseudocysts are found in 1 – 10% of AP; 30 – 50% of them resolve spontaneously (Beger et al. 2003). Pancreatic pseudocyst is classified as acute, and occurs as a result of AP or trauma, or is chronic, arising as a consequence of chronic pancreatitis (Baillie 2004). Pseudocysts take 4 weeks or more to form from the onset of AP (Baillie 2004). The property of pseudocystic contents can be categorized by pure pancreatic juice or a mixture of necrotic debris and pancreatic secretions (Beger et al. 2003, Bollen et al. 2008).

Intra-abdominal pressure (IAP) and abdominal compartment syndrome are frequently found in patients with severe AP and are associated with development of OF (Al-Bahrani et al. 2008). IAP or abdominal perfusion pressure (APP) correlates significantly with the MODS, SOFA or APACHE II scores (De Waele et al. 2005, Pupelis et al. 2006, Al-Bahrani et al. 2008). The admission APP has a high sensitivity of 100% and specificity of 83% for predicting mortality (Al-Bahrani et al. 2008). Resolution of IAP is associated with significant decline in MODS and SOFA scores (Al-Bahrani et al. 2008).

1.2.5 Treatment

Many factors can cause AP. However, the specific therapy according to aetiology may only be possible in some cases of gallstone-induced AP. In most cases the therapies are only supportive or preventive. The early supportive therapy for AP includes fluid resuscitation, pain relief, oxygen administration and antiemetics. Severe cases need multidisciplinary management including

resuscitation, close monitoring and nutritional support (Frossard et al. 2008). Early enteral feeding seems to be safer and costs less than parenteral feeding, but the proportions of calories and type of nutrient mixtures are still under debate (Abou-Assi et al. 2002, Marik and Zaloga 2004, Frossard et al. 2008). ERCP and endoscopic sphincterotomy can be carried out within 72 hours for patients with severe gallstone AP, especially in the case of biliary obstruction, biliary sepsis and persistent OF. Systematic reviews have demonstrated that the beneficial effect of early ERCP is non-significant in severe AP without cholangitis, but the effect on complications remains controversial (Ayub et al. 2004, Petrov et al. 2008). Follow-up laparoscopic cholecystectomy is recommended for patients with gallstones to prevent recurrent AP, and it should preferably be done during the same admission after recovery from the first attack (Frossard et al. 2008). In mild biliary AP, laparoscopic cholecystectomy can be performed safely within 7 days (Uhl et al. 1999). In severe disease with extended pancreatic necrosis, at least 3 weeks should elapse because of an increased risk of pancreatic infection, but delaying cholecystectomy is associated with an increase of biliary complications in patients with non-necrotizing biliary AP (Uhl et al. 1999, Nebiker et al. 2009). Preoperatively the existence of choledochal stones can be diagnosed by magnetic resonance cholangiopancreatography (MRCP). When stones are present they can be removed by ERCP.

Even though pancreatic infection is an important cause of morbidity and mortality, the benefit of antibiotic prophylaxis is much debated since the findings are contradictory (Howard 2007). Two multicentre, prospective, double blind, randomized controlled studies have not shown that early antibiotic treatment could prevent (peri)pancreatic infection, mortality or requirement for surgical intervention (Mazaki et al. 2006, Dellinger et al. 2007). A single-centre randomized study has demonstrated that early antibiotics reduce the need for surgery and the occurrences of remote organ complications in necrotizing AP (Nordback et al. 2001). The final answer is far from clear. The current meta-analysis has shown that prophylactic antibiotics did not reduce the occurrence of

infected necrosis, the need for surgical intervention or mortality, but yielded limited benefits by reducing the rate of non-pancreatic infections and length of hospitalization (Hart et al. 2008). Another meta-analysis demonstrates that antibiotic prophylaxis for severe AP is not beneficial in avoiding infected necrosis, surgical intervention, or reducing mortality (Jafri et al. 2009). However, it is also known that once pancreatic necrosis develops, it is difficult for antibiotics to penetrate into the necrotic lesions, and that they can not halt the progression of necrotic infections (Foitzik et al. 1997). Thus, the effectiveness of prophylactic antibiotics may be shown when initiated before the development of pancreatic necrosis (Tsuji and Chiba 2008). When infection is suspected by FNA of the pancreas for bacteriology, antibiotic treatment is always required (Frossard et al. 2008); if the infection is not confirmed, there may still be an indication for antibiotics, since a part of patients in the control arm of randomized studies have also been given antibiotics.

Patients with infected pancreatic necrosis may require surgical intervention, including necrosectomy, debridement, drainage or lavage of the necrotic cavities (Working Party of the British Society of Gastroenterology et al. 2005, Jamdar and Siriwardena 2006). Minimally invasive techniques are currently used more often. They include CT-guided and ultrasonography (US)-guided percutaneous drainages, transgastric or transduodenal endoscopic drainage, or minimally invasive laparoscopy with retroperitoneal access (Bradley et al. 2008).

1.3 Experimental models of acute pancreatitis

1.3.1 Bile-salt induced acute pancreatitis models

Bile-induced experimental AP was first introduced by Opie according to the clinical observation that two patients with fatal AP had gallstone obstruction of the bile duct (Lankisch and Ihse 1987). From this came the proposal that gallstone migration into the common duct at the ampulla and subsequent bile reflux into the pancreatic duct led to the development of AP (Frossard et al. 2008).

A mixture of canine bile and pancreatic juice was found more readily to produce severe and fatal AP, compared with bile alone (Joyeuse et al. 1962).

The human pancreas is distinct and well defined, however, the rat pancreas is a diffuse organ enclosed in the dorsal mesentery (Case 2006). The major difference between human and rat pancreatic anatomy is that there is no separate pancreatic duct or common bile duct inside the pancreas of rat, but a combined biliopancreatic duct. The common bile duct continues as the pancreatic duct in rats (Hoogerwerf et al. 2004). The multiple pancreatic ducts drain into a common biliopancreatic duct which empties into the second part of the duodenum (Armstrong et al. 1985). Thus, the ligation of biliopancreatic duct at the hepatic and duodenal ends prevents entry of the injected substances into the liver or the duodenum and ensures perfusion into the pancreas (Hoogerwerf et al. 2004).

The mortality rate of bile-induced AP depends on the type or on the concentrations of bile salts. Bile salts can be divided into two types: unconjugated bile salts (dihydroxycholanic acids) and conjugated bile salts (trihydroxycholanic acid, taurocholate) (Lankisch and Ihse 1987). Taurocholate is a conjugate of cholic acid with taurine and can be converted into taurodeoxycholate by hydroxylation. Unconjugated bile salts are 8-fold more toxic than conjugated ones (Lankisch and Ihse 1987). The severity of AP can be modified from moderate to severe, or from moderate to lethal, according to different concentrations of bile salts (Lankisch and Ihse 1987, He et al. 1999a). By adding trypsin to bile salt infusion, mortality could increase (Nakae et al. 1995, Wang et al. 2001).

Necrosis takes place at a very early stage. Pancreatic necrosis of mice can be observed within 6 h after the infusion (Laukkarinen et al. 2007). The mechanism may include two steps: in the beginning, the acute detergent effect of bile salts leads to pancreatic necrosis, oedema and

haemorrhage. Later, trypsinogen is activated and autodigestion is involved in the further development of necrosis (Lankisch and Ihse 1987). Remote OF vary from study to study, some studies reporting different extents of lung or liver injury according to infusion pressure and bile salt concentration (He et al. 1999a, Lichtenstein et al. 2000, Wittel et al. 2008).

As an experimental model, bile-induced AP has both advantages and limitations. Bile salt infusion models are similar to clinical AP to some extent, and may mimic the aetiology of AP (Chan and Leung 2007). Moreover, this type of model is highly applicable in many species and can be combined with other stimulatory substances like trypsin or phospholipase A (Lankisch and Ihse 1987). Because it may be considered as one of the most relevant AP models from the clinical point of view, it has been used for investigating AP pathophysiology and potential therapies (Schwarz et al. 1994, Spicak et al. 1999, Hoyos et al. 2005, Chan and Leung 2007). Still, bile salt induced AP models have huge variations and the lack standardizations concerning the technique, especially the infusion pressure, volume, time, concentration and type of bile salt (Armstrong et al. 1985). Pancreatic damage with ductal extravasations through intercellular clefts can be induced by elevated hydrostatic pressure itself (Armstrong et al. 1985, Chan and Leung 2007). In such a situation, the mortality, onset of necrosis, release of TAP and development of MODS, differed from study to study (Lankisch and Ihse 1987, Fernandez-del Castillo et al. 1992, Schmidt et al. 1992b, Nakae et al. 1995, He et al. 1999a, Wang et al. 2001, Coelho et al. 2003).

1.3.2 Other acute pancreatitis models

Choline-deficient ethionine supplement (CDE) diet-induced AP was first reported in 1975 (Lombardi et al. 1975). Mice developed AP with 100% mortality when fed a CDE diet for 4 days. This model is suitable for studying the pathophysiology of AP and its therapy by assessing mortality ratios (Al-Mufti and Williamson 1999). Although CDE diet-induced AP is the most non-

invasive model and convenient in practice, slow progression of AP, direct side-effects on liver and brain and limited effect on small animals (only on young female mice) impair its relevance to clinical AP (Chan and Leung 2007).

Arginine-induced AP is a non-invasive AP model, whose severity is dependent on the dose of arginine (Tani et al. 1990, Tashiro et al. 2001, Ishiwata et al. 2006). However, this model has failed to achieve popularity due to lack of clinical relevance.

Secretagogue-induced AP models include those induced by various stimulatory substances, such as CCK analogue caerulein, muscarine receptor agonist carbachol and anticholinesterase agents. Caerulein-induced AP is most widely used in different animals. The main advantages of this model include rapid induction, highly reproducible course, non-invasive, AP-like histological changes, and *in vitro* research with similar stimulators (Al-Mufti and Williamson 1999). In addition to severity and MODS, sepsis can also be imitated by combining with lipopolysaccharide (He et al. 1999b, Mole et al. 2008); however, these efforts still can not strengthen the clinical relevance due to lack of hormonal over-stimulatory AP in clinical practice (Chan and Leung 2007).

AP may also be induced by the impairment of pancreatic blood inflow and outflow or disturbance of pancreatic microcirculation. This is not a standard tool to induce AP, but may be used for special purposes to investigate the consequences of circulatory changes in the disease (Waldner 1992). The limitation of this model is the extensive surgical trauma and non-specific damage to the pancreas, and the requirement for continuous analgesia yielding huge variation and low reproducibility (Chan and Leung 2007).

Closed duodenal loop (CDL)-induced AP is designed to establish a "blind loop" where duodenal contents with digestive enzymes are refluxed back to the pancreatic duct (Araki et al. 2002, Sawa et al. 2007). The CDL-induced model requires a complicated and invasive technique. Its relevance to clinical practice is very uncertain.

Duct obstruction-induced AP imitates the aetiology of gallstone or other obstruction-induced AP in a clinical setting (de Dios et al. 2002, Mooren et al. 2003). Ligation of the pancreatic duct in rodents can act as an established model of chronic pancreatic atrophy and is often used to study the regeneration of the exocrine pancreas (Al-Mufti and Williamson 1999). It also can be combined with the administration of stimulatory chemicals such as CCK, caerulein or secretin. The AP reverses when the duct obstruction is removed, allowing the study of treatment interventions (Toriumi et al. 1993, Chan and Leung 2007). Although this model may have some clinical relevance and requires less complicated surgical technique, its reproducibility has not been optimal, which is why it has not gained wide popularity.

Nowadays, with the development of various genetic techniques, experimental transgenic animals share the stage of AP models. Knock-in or knock-out technique can be introduced to animals to obtain certain gain-of-function or loss-of-function. Ribonucleic acid (RNA) silencing has yet been little applied in AP research (Lin et al. 2008). However, the possible interference of other genes might potentially cause unexpected side-effects in animal models.

1.4 Mechanisms of acute pancreatitis

1.4.1 Initiation of acute pancreatitis

The mechanism of initiating AP is still not clear. Although numerous controversies persist, it is generally accepted that inappropriate activation of trypsin in pancreatic acinar cells is the initial

common step in the development of AP (Foitzik et al. 1994, Saluja et al. 1997, Hofbauer et al. 1998, Saluja et al. 1999). In contrast to the activation of trypsin, the protective mechanisms as balancing factors are also widely studied, including autolysis of activated trypsin, enzyme compartmentalisation, production of trypsin inhibitors such as serine protease inhibitor Kazal type 1 (SPINK1) and trypsin-activated trypsin-like enzyme mesotrypsin (Mitchell et al. 2003, Frossard et al. 2008).

Genetic mutation may predispose to the development of AP. Frequent mutations of SPINK1, cationic trypsinogen (PRSS1) and cystic fibrosis transmembrane conductance receptor genes have been found in human immunodeficiency virus-positive patients with AP, recurrent AP and hereditary pancreatitis (Creighton et al. 2000, Felley et al. 2004, Rebours et al. 2009). The most often observed mutation of PRSS1 is associated with an increased function of trypsinogen, whereas the mutation of SPINK1 is always related to loss of its inhibitory function (Tukiainen et al. 2005, Frossard et al. 2008).

In summary, the initiation of AP can not be interpreted to be caused by any single risk factor alone, but by multiple factors including altered activation or inhibition of trypsin and mutations in counterpart genes, in association with environmental factors.

1.4.2 Activation of pancreatic trypsin

Kuhne firstly discovered trypsin as a result of pancreatic digestion of proteins in the 1800's (Busnardo et al. 1983). Trypsin originated from its zymogens by enterokinase. When activated, TAP is released. Enterokinase is activated at pH 6 – 9 and can be enhanced 2000 times in low concentrations of bile acids compared with the efficiency of trypsin. Biliary enterokinase may enter the pancreatic duct system to induce pancreatic autodigestion and to trigger enzyme cascade

(Rinderknecht 1986). In autoactivation theory, trypsinogen is activated by certain serine protease activity or independently of protease activity (Hirota et al. 2006). Such an event requires an acidic environment like the non-zymogen granule vesicular compartment, which contains vacuolar H^+-Adenosine-5'-triphosphate (ATPase) to maintain low pH (Pandol 2006). Recently, autophagy has been considered as a hypothetical mechanism of trypsinogen activation. Physiologically, autophagy would be a defensive mechanism of discharging trypsin. However, autophagosome containing trypsinogen may be actively formed fusing with lysosome to form autophagolysosome, and especially when pancreatic secretory trypsin inhibitor is not working normally (for example, when the gene is knock-out) trypsin is activated inside the cell instead of being discharged (Hirota et al. 2006).

Another hypothesis suggests that cathepsin B is highly involved in the activation of trypsinogen to trypsin. Cathepsin B could activate trypsinogen *in vitro*, transport from lysosomal to the zymogen-granule-enriched subcellular compartment and colocalize with digestive zymogens in AP models (Steer 1992, Halangk and Lerch 2005). Further, the specific disruption of cathepsin B gene in knock-out transgenic animals has been shown to reduce trypsinogen activation by 90% (Lerch and Halangk 2006). Nevertheless, the observation in human AP is a different issue. The cathepsin B gene carries a complex polymorphism and has uncertain roles in the development of AP (Halangk and Lerch 2005, Lerch and Halangk 2006).

1.4.3 Inflammatory cascades

After the activation of trypsinogen into active trypsin, inflammation is followed by the production of cytokines, nitric oxide, reactive oxygen species and arachidonic acid metabolites from pancreatic acinar cells, endothelial cells, neutrophils, macrophages and lymphocytes (Vollmar et al. 1989, Tsuji et al. 1994, Al-Mufti et al. 1998, Tsai et al. 1998, Lundberg et al. 2000, Ayub et al. 2001).

Immunocytes attracted by cytokines release more cytokines, free radicals and nitric oxide (Mitchell et al. 2003). The mediators involved in inflammatory responses can be divided into two categories: pro-inflammatory (including tumour necrosis factor [TNF] - α, IL-1, IL-2, IL-6, nitric oxide, reactive oxygen radicals, arachidonic acid metabolites, platelet-activating factor and substance P) and anti-inflammatory mediators (IL-1 receptor antagonist, IL-4 and IL-10) (Brivet et al. 1999, Granger and Remick 2005). The development of inflammatory responses between the pro- and anti-inflammatory mediators determines the course of AP. (Makhija and Kingsnorth 2002).

Cytokines are thought to be at the centre during inflammatory cascades. They are produced and secreted by immunocytes, and most of their expression is modulated by transcription factors such as nuclear factor kappa B (Frossard et al. 2001, Vaquero et al. 2001, Makhija and Kingsnorth 2002, Ramnath and Bhatia 2006). Several cytokines have been considered as potential mediators involved in SIRS or MODS. TNF-α is one of these which have important roles in the inflammatory response. In AP models, enhanced expression of TNF-α in forms of mRNA and protein can be found in acinar cells, or *in vitro*-cultured acinar cells; similarly, in human studies, patients with AP have higher concentrations of TNF-α and its receptors in peripheral blood (Pooran et al. 2003, Granger and Remick 2005, de-Madaria et al. 2008). IL-6 is secreted in the acute phase of AP and its levels are elevated in AP and serves as a marker of the severity of AP, in addition to parallelling the course of the disease (Leser et al. 1991, Viedma et al. 1992, Heath et al. 1993, Inagaki et al. 1997, Ohmoto and Yamamoto 2005). Anti-inflammatory IL-10 has been postulated as a treatment option for the inflammatory response that would decrease the production of pro-inflammatory cytokines (Simovic et al. 1999, Deviere et al. 2001, Ohmoto and Yamamoto 2005). However, in both experimental and human studies, IL-10 has been found to be already naturally increased during AP with possible prognostic significance (Granger and Remick 2005, Mentula et al. 2005).

1.4.4 Programmed cell death and necrosis of pancreas

Acinar cell death in AP is believed to have two patterns: apoptosis or necrosis (Gukovskaya et al. 1996). Morphologically, apoptosis is mainly characterized by cell shrinkage and chromatin condensation, whereas necrosis is characterized by swelling of the cell and its organelles and rupture of the plasma membrane (Mareninova et al. 2006). The severity of experimental AP correlates directly with the extent of necrosis and inversely with apoptosis (Gukovskaya et al. 2006, Odinokova et al. 2008). Most studies support the idea that pancreatic acinar cell apoptosis acts as a self-defence mechanism against the development of pancreatic necrosis (Takeyama 2005).

Apoptosis has two pathways: extrinsic and intrinsic. The former is initiated by the activation of caspase-8 followed by the activation of effector caspases (Mareninova et al. 2006). The family of caspases is typically activated in the early stage of apoptosis. Caspases can activate other degradative enzymes such as Dnases, which begin to cleave the Deoxyribonucleic acid (DNA) in the nucleus (Takeyama 2005). This pathway can be mediated by TNF-α *in vitro* or *in vivo* in experimental models of AP (Gukovskaya et al. 1997). In the intrinsic pathway, permeabilization of the mitochondrial outer membrane leads to the release of pro-apoptotic factors, and then cytochrome *c* induces the activation of caspase-9 and the other effectors caspases resulting in subsequent degradation of cellular constituents (Mareninova et al. 2006).

Necrosis also has two types: non-programmed and programmed necrosis (Mareninova et al. 2006, Criddle et al. 2007). The former is an accidental and irregular process triggered by severe cellular stress, which is always manifested as depletion of cellular ATP and explosion of the cell overwhelmed by ion fluxes (Mareninova et al. 2006). The programmed necrosis is mediated by a series of signalling events triggered via death receptors like receptor-interacting protein (Mareninova et al. 2006).

Current evidence suggests that the balance between apoptosis and necrosis may influence the severity of AP and decide the fate of acinar cells. Caspase activation is one major event in this balance, switching the cell-death response toward apoptosis and away from necrosis (Mareninova et al. 2006). Inhibition of caspase promotes necrosis and exacerbated AP, further, caspase activation not only stimulates apoptosis but also triggers the cleavage of receptor-interacting protein, the key mediator of programmed necrosis (Mareninova et al. 2006). Additionally, cytosolic calcium signalling has an influence on both apoptotic and necrotic cell death pathways (Criddle et al. 2007). The transient release of calcium induced by mild stimuli promotes apoptosis via the intrinsic pathway when reactive oxygen species are present (Odinokova et al. 2009). This action may be partially up to mitochondrial depolarization and transient opening of the mitochondrial permeability transition pore, which does not adversely influence ATP production (Criddle et al. 2007). Severe attacks cause depletion of calcium stores, dramatically reduce ATP production, paralyze energy-dependent processes and activate digestive enzymes (Criddle et al. 2007).

2 Physiology and metabolism of polyamines

2.1 Physiological function of polyamines

2.1.1 Cellular survival and growth

The natural polyamines (putrescine, spermidine and spermine) are aliphatic amines which are positively charged and expected to interact with negatively charged compounds and structures within the living cells (Jänne et al. 2005). Such negatively charged moieties include DNA, RNA, many proteins and phospholipids. One aim of this interaction is to stabilize their structure (Wallace et al. 2003). While polyamines stabilise DNA, they stimulate gene transcription and translation (Wallace et al. 2003). Their charges also play a role in the interaction of the polyamines with specific receptors and ion channels (Wallace 2000). By their stabilising effect, polyamines are able to interact with cell membrane receptors or modulate intracellular signalling (Moinard et al. 2005).

In all, polyamines are important molecules to maintain the survival of cells with several non-specific functions.

It is believed that the role of the polyamines is to work as intracellular growth factors, increasing the rate of cell growth and differentiation (Wallace et al. 2003, Gugliucci 2004). Normal cell growth is regulated in a cyclical manner by increases and decreases in specific proteins and protein kinases. The changes of both ornithine decarboxylase (ODC) and polyamine concentrations are also noted during the cell cycle (Wallace et al. 2003). It is found that the increases of polyamine levels go along with the increase of ODC activities during the cell cycle (Oredsson 2003). There are two peaks of ODC in the growth phases of cell cycle and depletion of polyamines in cell growth arrest (Wallace et al. 2003). Polyamines may affect cell growth via interacting with nuclear phosphoprotein p53, while depletion of polyamines produces an inhibition of cell growth (Moinard et al. 2005). Moreover, the requirement for polyamines during cell growth may be partly due to the need for spermidine as a substrate for eukaryotic initiation factor 5A, which is essential for eukaryotic cell proliferation (Moinard et al. 2005).

2.1.2 Cell death

Depletion of polyamines may be a general event in apoptosis activation (Seiler and Raul 2005). Exogenous spermine prevents apoptosis and depletion of polyamines induces apoptosis, implying a protective role for polyamines (Wallace et al. 2003). Despite the protective effects, exogenous polyamines can also be toxic in high concentrations. Massive accumulations of spermine and putrescine especially may cause direct toxicity and induce cell death (Wallace et al. 2003). The changes in intracellular polyamine content are transient during apoptosis, with polyamine levels mainly decreased in the later stages (Wallace et al. 2003). Abnormal polyamine concentrations are most probably not a primary cause of apoptosis, but the polyamines may promote apoptotic

mechanisms if they attain certain concentrations above or below physiological limits (Seiler and Raul 2005).

The key enzyme of polyamine catabolism, spermidine/spermine N^1-acetyltransferase (SSAT), is involved in cell death (Flamigni et al. 2007). Apoptosis is at least partially initiated via massive induction of catabolic key enzyme SSAT and related oxidative events and subsequently mediated by the mitochondrial apoptotic signalling pathway as indicated by cytochrome *c* release and caspase activation (Chen et al. 2001). TNF-α mediates the induction of SSAT, leading to lower levels of polyamines and the oxidization reactions in polyamine catabolism, and subsequently generating potential toxic hydrogen peroxide which causes DNA damage and cell death (Babbar et al. 2007).

Both induction and inhibition of biosynthesis and catabolic enzyme activities are associated with increased and decreased apoptosis (Wallace et al. 2003). Thus the response of the cell depends on multiple signals for survival or death, and one signal can produce either response, depending on the environment (Wallace et al. 2003).

2.2 Biosynthesis of polyamines

Polyamines are abundant in organ tissues like liver, pancreas, brain, heart and so on (Jänne et al. 2004). They also exist in circulation and 95% of blood polyamines (spermidine and spermine) are detected in red blood cells (RBC) (Moulinoux et al. 1991). In pancreas the polyamine levels are about 10^5-10^6 times the polyamine levels in RBC according to our experimental data. Exogenously, the sources of polyamines are dietary and human milk; endogenously, they come from intestinal and pancreatic secretions and catabolism products of intestinal cells and luminal bacteria (Larque et al. 2007). However, the contribution to the total polyamine pool from gut metabolism is less clear.

At present, it seems unlikely that microbially derived polyamines are a major contributor to the total body content (Wallace et al. 2003).

The primary sources of polyamines endogenously originate from the two amino acids, L-ornithine and L-methionine (Jänne et al. 2004). The former can be produced from arginine by the catalysis of arginase (Wallace et al. 2003). The amino acid arginine is a precursor of ornithine and can be exogenously obtained from dietary intake or endogenously synthesized in healthy adults (Larque et al. 2007).

The main pathway of polyamine biosynthesis includes the following procedures (Jänne et al. 2004): ornithine and S-adenosyl-L-methionine (AdoMet) are decarboxylated by two decarboxylases, ODC and AdoMet decarboxylase. The former reaction produces putrescine and the latter produces decarboxylated AdoMet (dcAdoMet). DcAdoMet contributes its aminopropyl group to the process yielding spermidine and spermine. Spermidine is yielded from putrescine in the reaction catalyzed by spermidine synthase, and spermine is yielded from spermidine by spermine synthase. Under normal circumstances polyamine concentrations regulate their own biosynthesis and prevent overproduction (Wallace et al. 2003).

ODC initiates the polyamine biosynthesis pathway and catalyzes the first step yielding putrescine (Pegg 2006). The activity of ODC is highly regulated and is a response to many stimuli. ODC has a short half-life, and its activity and quantity is induced by several growth factors or inhibited by inhibitors such as alpha-difluoromethylornithine (Pegg 2006, Larque et al. 2007). The induction of ODC is causally related to the activation of cellular growth and accumulation of putrescine, whereas the inhibition of the enzyme reverses the process of proliferation (Jänne et al. 2004).

2.3 Catabolism of polyamines

The main pathway of polyamine homeostasis is shown in Figure 1 (Jänne et al. 2004, Pegg 2008): Cellular spermidine and spermine are acetylated by the catalysis of SSAT, and subsequently the acetylated spermidine and spermine are converted respectively into spermine and putrescine by the action of polyamine oxidase (PAO) (Pegg 2008). In brief, the whole procedure contains two steps: spermine is converted into spermidine, and then spermidine is converted into putrescine. Both steps need the catalysis of SSAT and PAO. Additionally, spermine can be directly oxidized back to spermidine by spermine oxidase (SMO) (Jänne et al. 2004).

SSAT is the rate-controlling enzyme in the pathway of polyamine catabolism. It has a molecular mass of about 80 kilodaltons and is generally considered to be a cytosolic enzyme, sometimes also existing in mitochondria under particular pathophysiological conditions (Wallace et al. 2003, Pegg 2008). SSAT adds acetyl groups to spermidine and spermine. The acetylation reduces the charge on the polyamines and affects their function (Pegg 2008). Spermidine and spermine serve as the substrate of SSAT and spermidine is about 3 times more efficient than spermine (Wallace et al. 2003). SSAT, like ODC, has short half-life (20-40 min) and is highly regulated and easily inducible (Wallace et al. 2003). SSAT plays a key role in maintaining polyamine homeostasis and influencing cellular processes such as normal and neoplastic growth, which are related to polyamine content (Pegg 2008). Many factors, including polyamines, polyamine analogues, toxins, and stress pathways, lead to large increases in SSAT activity and proteins (Wallace et al. 2003, Pegg 2008).

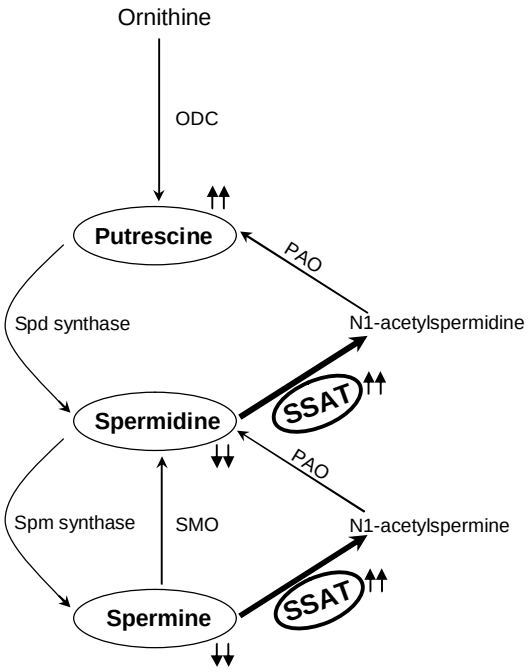

Figure 1. Polyamine catabolism of pancreas in acute pancreatitis model.

Polyamine biosynthesis includes yielding putrescine from ornithine by the catalysis of ODC, yielding spermidine from putrescine by spermidine (spd) synthase and yielding spermine from spermidine by spermine (spm) synthase. SSAT catabolizes spermine to N^1-acetylspermine, which is catabolized by PAO to spermidine. SSAT and PAO also catabolize spermidine to putrescine, which is again the precursor of spermidine and spermine. Spermine is also partially converted back to spermidine by the catalysis of SMO. SSAT is the rate-limiting enzyme in polyamine catabolism. Increased SSAT and putrescine, with depletion of spermidine and spermine in pancreas, can be observed in taurodeoxycholate-induced AP. "↑↑", increase; "↓↓", decrease.

Polyamine catabolism can be activated when SSAT is highly induced. Transgenic animals overexpressing SSAT have a greatly depleted polyamine pool so that spermidine or spermine content is dramatically reduced and the putrescine pool is greatly expanded (Jänne et al. 2004). In acute inflammation, the depletion of intracellular polyamine pools, following the induction of polyamine catabolism, can itself be growth inhibitory and help cells to adapt to inflammatory stress; long-term exposure to reactive oxygen species, caused by the activation of polyamine catabolism, results in genetic damage and mutation which are associated with the development of carcinoma (Babbar et al. 2007).

The inhibition of polyamine catabolism has not been extensively studied, because a specific inhibitor of SSAT has not yet been found. Various inhibitors tested also have a weak ability to enter cells and inhibit SSAT *in vivo* (Wallace and Fraser 2004, Pegg 2008). In SSAT knock-out mice, the targeted disruption of SSAT only resulted in a slight fluctuation of polyamine homeostasis (Niiranen et al. 2006).

3 Acute pancreatitis and polyamine catabolism

3.1 Transgenic acute pancreatitis model induced by over-activation of polyamine catabolism

Over-expression of SSAT gene, leading to extreme activation of polyamine catabolism in transgenic rodents, produces a new experimental model of AP and introduces a completely different insight into the mechanisms of the disease (Alhonen et al. 2000, Jänne et al. 2004, Jänne et al. 2005, Jänne et al. 2006).

In transgenic rats with over-expression of SSAT, pancreatic integrity is readily susceptible to the depletion of spermidine and spermine which are run out by the catalysis of SSAT (Räsänen et al. 2003, Herzig et al. 2005). In histological studies, pancreatic necrosis is simultaneously initiated. To a great extent, necrosis is exacerbated by the use of SSAT stimulatory agent gossypol or inhibitor of

PAO (Alhonen et al. 2000, Räsänen et al. 2003). Enhancing of pancreatic cathepsin B and trypsin activity, combined with elevated serum TNF-α and IL-6 levels, are found in the early stage of SSAT over-expression induced AP, followed by the presence of occasional pancreatic necrosis at 4 hours and extensive necrosis at 24 hours (Hyvönen et al. 2006, Hyvönen et al. 2007, Merentie et al. 2007). In isolated acini, intracellular trypsinogen activation is heavily dependent on the extent of SSAT activation (Hyvönen et al. 2006).

3.2 Use of polyamine analogues in preventing experimental acute pancreatitis

Methyl derivatives of spermidine and spermine have been studied as analogues for natural polyamines in a transgenic model. These analogues include methylspermidine (MeSpd) and Me_2Spm. They have more stable chemical characteristics than natural polyamines, which are easily acetylated and degraded (Järvinen et al. 2005). Both the polyamine analogues can reverse cytostasis and restore liver regeneration in transgenic rats after partial hepatectomy (Räsänen et al. 2002, Järvinen et al. 2005).

Either MeSpd or Me_2Spm can prevent the development of AP in transgenic models induced by over-expression of SSAT gene (Räsänen et al. 2002, Hyvönen et al. 2006). In mortality study, the death rate was reduced from 100% to 20% by the administration of MeSpd and to 0% by Me_2Spm at 72 hours (Hyvönen et al. 2006). The extent of pancreatic necrosis is ameliorated by the injection of MeSpd or Me_2Spm before the induction of AP (Räsänen et al. 2002, Hyvönen et al. 2006). In addition, the results of biochemical examination indicate that Me_2Spm ameliorates the activities of pancreatic cathepsin B and trypsin, and decreases the level of blood amylase, alanine aminotransferase (ALAT), haematocrit and cytokines (Räsänen et al. 2002, Hyvönen et al. 2006, Hyvönen et al. 2007, Merentie et al. 2007). Me_2Spm is more stable and more efficient than MeSpd at halting the development of this particular AP model.

In addition to the transgenic model, two AP models in wild-type animals (one induced by caerulein and the other by L-Arginine) have also been studied as to the effect of MeSpd. Especially in the L-Arginine induced model, MeSpd prophylaxis was able to reduce by 75% the amount of plasma amlyase and ameliorate histological injury (Hyvönen et al. 2006). The effect of MeSpd on caerulein-induced AP was not significant (Hyvönen et al. 2006). However, the clinically perhaps most relevant model (bile salt induced AP) has not been studied, either as to the changes of polyamine catabolism or the role of polyamine analogue. Furthermore, currently there has been a lack of clinical studies on polyamine changes in patients with AP.

AIMS OF THE STUDY

The aims of the study were to investigate polyamine catabolism in experimental and in clinical AP.

The following items were studied:

1. Polyamine catabolism in experimental AP according to time-course and severity of disease. (Studies I, III)
2. The relationship between polyamine catabolism and trypsin activation in experimental AP. (Study III)
3. The effect of substitution of polyamine depletion with a synthetic polyamine analogue on experimental AP. (Studies II, III)
4. The changes of RBC polyamine levels in AP patients according to aetiology and severity of the disease. (Study IV)

MATERIALS AND METHODS

1 Experiments (Studies I-III)

1.1 Animals

Both wild-type and transgenic rats were used in the experimental studies:

Wild-type adult male Sprague-Dawley rats (weighing 250–500 g) were fed with standard chow until 12 h before the experiment, after which the animals were fasted until the end of the experiment. There were 102, 78 and 106 wild-type rats used in Studies I, II and III respectively. The rats were anaesthetized by intraperitoneal injection of pentobarbital before the operation and again for later sampling. All wild-type rats were euthanized after the sampling procedures.

Thirty transgenic adult male Wistar rats (280-400 g) were used in Study III. The production of transgenic rats was as follows: the donor females were superovulated and mated with mature males, and the zygotes were collected and microinjected with the metallothionein-SSAT gene harbouring metallothionein I promoter; the zygotes were transferred into the oviducts of the recipient females immediately after microinjection (Alhonen et al. 2000). After birth, the transgenic Wistar rats were fed under the same conditions as the wild-type rats. All transgenic rats were euthanized after the sampling procedures.

1.2 Chemicals and materials

- Sodium taurodeoxycholate, used to induce experimental AP, was purchased from Sigma-Aldrich, Buchs, Switzerland
- The anaesthesia drug pentobarbital was from Orion, Espoo, Finland the dosage being 60 mg/kg
- The polyamine analogue Me_2Spm was synthesized as described before (Grigorenko et al. 2004) and dissolved in 0.9% NaCl solution at 25 mg/ml before use. Me_2Spm was used as

follows: the animals received Me_2Spm as a pre-treatment at 20 h and at 4 h before the induction of AP, intraperitoneally (25 mg/kg); the animals received Me_2Spm as treatment intraperitoneally (25 mg/kg) at 4 h and 8 h after the induction of AP

- Thin polyethylene tubes (inner diameter 0.28 mm, outer diameter 0.61 mm; Sims Portex, Hythe, UK) were used for cannulating the biliopancreatic duct through the duodenum while establishing the AP model in wild-type rats
- Pressuremeter 224, serial 72071, used to measure the pressure inside biliopancreatic duct, was made by Ollituote company, Finland
- Electric oven, used to dehydrate organ tissues, was TAMRO-APTA 90-544011 Memmert, Germany

1.3 Experimental procedures

1.3.1 Establishment of acute pancreatitis model

Wild-type rats: Taurodeoxycholate-induced AP was induced with intraductal administration of 0.2 ml phosphate buffered saline, pH 7.4 containing either 2 % or 6 % sodium taurodeoxycholate under 30 cm H_2O pressure, after cannulating the biliopancreatic duct through the duodenum with thin polyethene tubing and temporary clamping of the duct at the liver hilum (Fig 2).

Transgenic rats: In these rats, necrotizing AP was induced by activating SSAT transgene via metallothionein I promoter with an intraperitoneal injection of zinc sulphate. The zinc sulphate (10 mg/kg) was dissolved in distilled water and used to induce experimental AP in transgenic rats, as previously described (Alhonen et al. 2000, Hyvönen et al. 2006). The same dose of zinc sulphate did not affect polyamine levels or induce toxicity in wild-type animals (Alhonen et al. 2000).

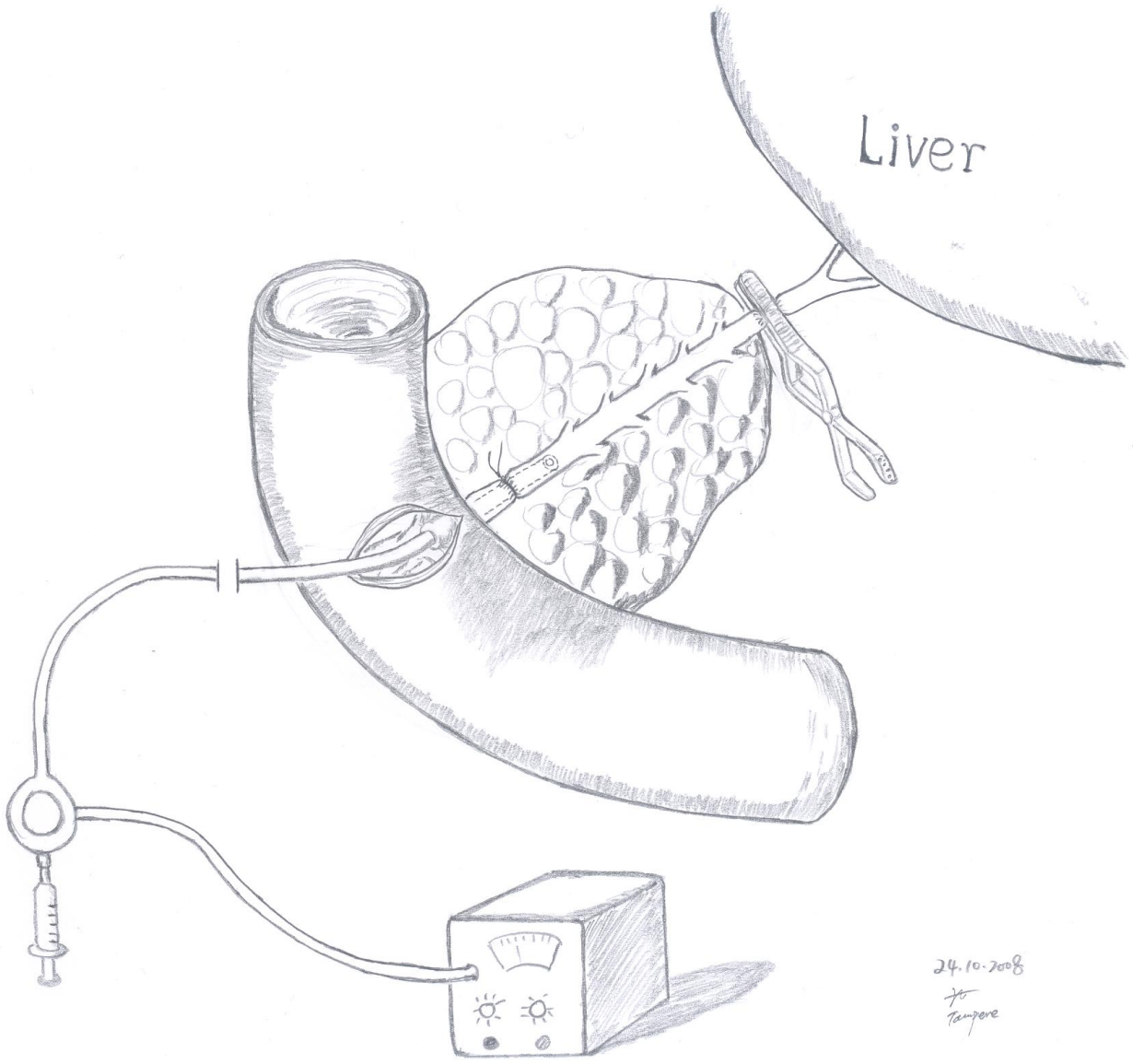

Figure 2. Model of experimental AP induced by intraductal infusion of sodium taurodeoxycholate.

1.3.2 Experimental design

- **Study I:** The aim of this study was to investigate whether pancreatic and blood polyamines change according to the severity of AP, whether polyamine levels in pancreas correlated with those in blood. The wild-type animals were randomly divided into the following 15 groups.

Controls (Group 1): Six rats served as 0 h references.

Sham operation (Group 2-6,): These animals only received laparotomy without induction of AP. They were sampled at 1.5 h (Group 2, n=6), 3 h (Group 3, n=6), 6 h (Group 4, n=6), 24 h (Group 5, n=6), or 48 h (Group 6, n=6).

2% taurodeoxycholate-induced AP (Group 7-11): These rats were infused with 2% sodium taurodeoxycholate intraductally, as described above, to induce AP. They were sampled at 1.5 h (Group 7, n=6), 3 h (Group 8, n=6), 6 h (Group 9, n=6), 24 h (Group 10, n=6), or 48 h (Group 11, n=6).

6% taurodeoxycholate-induced AP (Group 12-15): These animals were infused with 6% sodium taurodeoxycholate intraductally to induce more severe AP. They were sampled at 1.5 h (Group 12, n=6), 3 h (Group 13, n=6), 6 h (Group 14, n=6), and 24 h (Group 15, n=6) – originally 18 rats were needed to get 6 survivors at 24 h.

- **Study II:** The aim of this study was to investigate the mechanism of polyamine catabolism using a methylated polyamine analogue in taurodeoxycholate-induced AP model. The wild-type animals were divided randomly into 10 groups.

Control (Group 1): Six rats served as 0 h references.

Sham operation (Groups 2-3): Rats only received laparotomy without induction of AP. They were sampled at 24 h (Group 2, n=6) and at 72 h (Group 3, n=6) after the sham operation.

2% taurodeoxycholate-induced AP (Groups 4-5): Rats were infused with 2% sodium taurodeoxycholate intraductally as described above to induce AP. They were sampled at 24 h (Group 4, n=12) and at 72 h (Group 5, n=12) after induction of AP.

Me_2Spm + 2% taurodeoxycholate-induced AP (Groups 6-7): Rats were infused with 2% sodium taurodeoxycholate intraductally as above. The animals received Me_2Spm as a prophylaxis at 20 h and at 4 h before induction of AP as described above. They were sampled at 24 h (Group 6, n=6) and at 72 h (Group 7, n=6) after induction of AP.

2% taurodeoxycholate-induced AP + Me_2Spm (Groups 8-9): Rats were infused with 2% sodium taurodeoxycholate intraductally as in Groups 4-5 to induce AP. The animals received Me_2Spm as a treatment at 4 h and at 8 h after induction of AP as described above. They were sampled at 24 h (Group 8, n=12) and 72 h (Group 9, n=6) after induction of AP.

2% taurodeoxycholate-induced AP + extra Me_2Spm (Groups 10): Rats were infused to induce AP and they were administered Me_2Spm as a treatment at 4 h and at 8 h after induction of AP as in Group 9, however, with an extra dose of Me_2Spm (12.5 mg/kg) at 30 h. No tissue or serum samples were available due to 100% mortality.

- **Study III:** The aim of this study was to investigate whether polyamine catabolism is a mediator for trypsin activation in wild and transgenic models of AP. The total of 76 wild-type and 30 transgenic rats was divided into 18 groups:

Wild-type animals:

Control (Group 1): Six rats served as the 0 h references.

Sham operation (Groups 2-5): The rats only received laparotomy without induction of AP. They were sampled at 1.5 h (Group 2, n=6), 3 h (Group 3, n=6), 6 h (Group 4, n=6) and 24 h (Group 5, n=6) after the sham operation.

2% taurodeoxycholate-induced AP (Groups 6-9): AP was induced with intraductal infusion of 2% sodium taurodeoxycholate as described above. Rats were sampled at 1.5 h (Group 6, n=6), 3 h (Group 7, n=6), 6 h (Group 8, n=6) and 24 h (Group 9, n=6) after induction of AP.

2% taurodeoxycholate-induced AP + Me_2Spm (Group 10): AP was induced as in Group 9. The animals were injected with Me_2Spm at 4 h and at 8 h after the induction of AP, as described above. They were sampled at 24 h (Group 10, n=4) after induction of AP.

6% taurodeoxycholate-induced AP (Groups 11-13): AP was induced with intraductal infusion of 6% sodium taurodeoxycholate as described above. Rats were sampled at 1.5 h (Group 11, n=6), 3 h (Group 12, n=6) and 6 h (Group 13, n=6) after the infusion of 6% taurodeoxycholate. No samples were available after 6 h due to high mortality.

Transgenic animals:

Control (Group 14): Six transgenic rats without zinc sulphate injection served as 0 h references.

AP (Groups 15-18): AP was induced in these transgenic animals through SSAT induction by injection of zinc sulphate, as described above. The animals were sampled at 1.5 h (Group 15, n=6), 3 h (Group 16, n=6), 6 h (Group 17, n=6) and 24 h (Group 18, n=6).

1.4 Experimental measurements

1.4.1 Blood analysis

In order to obtain enough erythrocytes to measure polyamine levels from limited blood source, most blood samples were collected in tubes with sodium citrate solution (9NC Coagulation Sodium Citrate 3.2%) as an anticoagulant. After being centrifuged, separated erythrocytes were used to measure polyamine parameters. However, for assessing amylase activity, blood serum, not plasma, was collected by venous blood collection tubes (with spray dried clot activator inside).

Blood samples were collected and centrifuged at room temperature for 10 min at 2,500 (revolutions per minute) rpm, and the separated serum was stored at –20 °C for the assessment of amylase activity. The measurement was done according to the International Federation of Clinical Chemistry and Laboratory Medicine method for α-amylase (Lorentz 1998).

1.4.2 Tissue water contents

Tissue specimens were excised from pancreas and the tissues were weighed before and after dehydrating in an electric oven at 110 ^{o}C for 24 h. The water content of tissues was expressed by water weight ratio [(wet weight-dry weight)/dry weight].

1.4.3 Polyamines, Me_2Spm and SSAT analysis in blood or tissues

Blood samples were collected in tubes with sodium citrate solution and centrifuged at 1,200 *g* for 10 min at 4 °C. The red blood cells were washed three times in 9 g/l NaCl to remove white blood cells and stored at –70 °C (Quemener et al. 1996, Delzenne et al. 2000).

Excised pancreas samples were frozen in liquid nitrogen and stored at –70 °C for later SSAT and polyamine determinations (including Me_2Spm content in all excised pancreas samples).

The high performance liquid chromatography (HPLC) method for detecting polyamines and Me_2Spm was used. A 20 or 30 µl sample was applied to a reversed-phase column equilibrated for 5 min with buffer A. The column was then eluted with buffer A for 5 min, followed by a linear gradient to 75% buffer B over 15 min, then holding this mixture, 25% buffer A, 75% buffer B for 5 min, and back to the starting conditions over 3 min at a flow-rate of 0.5ml/min at 37^{o}C, using HP 1090 liquid chromatograph. The polyamines were detected by a fluorescence detector after post-column derivatization with *o*-phthalaldehyde. The *0*-phthalaldehyde solution was mixed under nitrogen with 1L of 1*M* borate buffer, pH 10.4. The mixture was allowed to stabilize under nitrogen for 1 h. The fluorescence reagent was pumped at a flow-rate of 0.4ml/min and the detection was carried out with a 340-nm excitation filter and a 425-nm emission filter (Hyvönen et al. 1992).

SSAT activities were measured as in an earlier study (Libby et al. 1989, Bernacki et al. 1995). Tissue cells were collected at a density of 2×10^7 per ml in 5 mM N-2-hydroxyethylpiperazine-N^1-2-ethanesulfonic acid (HEPES), pH 7.2, containing 1 mM dithiothreitol. Cellular cytosol was obtained at 35,000 rpm for one hour, and then incubated with 10 μmol HEPES buffer, pH 7.8, 0.15 nmol spermidine, and 0.5nmol [1-^{14}C]acetyl coenzyme A, in a final volume of 50 μl, for 5 minutes at 37 ^{o}C. The reaction was stopped by chilling, the addition of 20 μl of 0.5 M $NH_2OH.HCl$, and heating in a boiling water bath for 3 minutes. After centrifugation to remove precipitated protein, 50 μl of the reaction was spotted onto phosphocellulose disc and washed and counted.

SSAT activity is shown as pmol/mg protein/10 min. The observed reaction time is always kept at 10 minutes, so it also can be shortened to pmol/mg protein. The units of polyamine value are different according to the type of animal models: in wild-type animal models, polyamines were shown as nmol/mg protein; in the transgenic animal model, polyamines were shown as pmol/mg tissue. The protein concentration of each sample was assessed by Eppendorf BioPhotometer at A280.

1.4.4 Trypsinogen activation peptide in pancreas

Fresh pancreatic specimens were excised and placed in a boiling water bath for 15 minutes. The boiled samples were homogenised and centrifuged. The supernatant was transferred to 1.5 ml microcentrifuge tubes and then stored at –70 ^{o}C. Pancreatic TAP level was quantified in an aliquot of each supernatant using an enzyme immunoassay kit (Biotrin International Ltd, Dublin, Ireland). Typical calibration curve was obtained using Biotrin TAP enzyme immunoassay. Protein concentration of each sample was measured using bicinchoninic acid kit (Sigma-Aldrich, bicinchoninic acid -1, St. Louis, MO. USA). Pancreatic TAP contents are shown as pmol /mg protein.

1.4.5 Histology of pancreas

The pancreas were excised and fixed at room temperature in a pH-neutral, phosphate-buffered 10% formalin solution. The fixed tissue was embedded in paraffin, sectioned at 5 µm, stained with haematoxylin and eosin, and randomly coded for double blinded examination.

The histological changes in pancreas, including oedema, haemorrhage, leukocytes and necrosis, were graded using the previously validated scoring criteria at magnification ×400 (Schmidt et al. 1992b). The scores ranged from 0 to 4. The extent of PMN leukocyte infiltration was also scored in inter/intralobular blood vessels at the same magnification.

1.4.6 Detection of pancreatic apoptosis

Apoptosis, assessed as the presence of cells with active caspase-3 was determined by immunohistochemistry using cleaved caspase-3 (Asp175) as the primary antibody (Cell Signaling Technology, Inc., Danvers, MA). Detection and staining were performed using the Power Vision TM Poly-HRP anti-Rabbit IgG IHC Kit from ImmunoVision Technologies, Co., Brisbane, CA. The tissue was randomly coded for double blinded examination. The extent of apoptosis was scored as follows: 0 = no caspase-3 positive cells; 1 = 1-10 caspase-3 positive cells; 2 = 11-20 caspase-3 positive cells; 3 = 21-50 caspase-3 positive cells; 4 = 51-70 caspase-3 positive cells; 5 = 71-100 caspase-3 positive cells; 6 = more than 100 caspase-3 positive cells per field at magnification ×200.

2 Clinical investigation (Study IV)

2.1 Patients

During 2006-2007 thirty patients with AP were recruited for this study. We included 10 patients in each of the three aetiology subgroups (alcoholic, gallstone, unknown). From the initial 30 patients, one was excluded due to simultaneous prostate cancer, since the development of cancer may have

an effect on blood polyamine levels (Schipper et al. 2003, Stabellini et al. 2003, Gerner and Meyskens 2004). Another 3 patients were excluded because of unconfirmed clinical diagnosis of AP. Two patients were excluded because these patients were defined as having recurrent AP. Thus the final participants were 24 patients with first episode of AP (Table 1, Study IV).

Table 1. Descriptions of the patients and healthy volunteers.

	Acute pancreatitis			**Control†**	**Healthy**
Aetiology	Alcoholic	Gallstone	Unknown aetiology		
Number of patients	7*	10	7	18	6
Age (years, range)	52 (32 - 69)	75 (44 - 82)	67 (45 - 79)	63 (44 - 89)	57 (51 - 64)
Gender (male/female)	4/3	4/6	3/4	9/9	3/3
SOFA‡ on admission (range)	1 (0 - 5)	1.5 (0 - 3)	3 (0 - 10)		
No. of patients with SOFA ≥ 3 on admission	1	2	4		
No. of severe acute pancreatitis according to Atlanta Criteria	2	1	4		
• No. of patients with local complications	2	1	3		
No. of patients with pancreatic necrosis	0	0	3		
• No. of patients with OF§	0	0	3		
Hospital duration (days, range)	10 (5 - 19)	11.5 (5 - 48)	14 (3 - 54)	4 (3 - 11)	0 (0 - 0)
Mortality, n (%)	0 (0)	0 (0)	1 (14)	1 (6)	0 (0)

There was no significant difference in age and sex between the acute pancreatitis group and the acute abdominal disease group.

* One patient stayed at home for two weeks prior to admission.

† The patients with other acute abdominal diseases served as controls, including acute cholecystitis (n=2), cholecystolithiasis (n=2), gastric ulcer (n=2), diverticulitis (n=6), intestinal occlusion (n=2), intestinal necrosis (n=2), oesophageal ulcer (n=1) and reflux oesophagitis (n=1). None had increased amylase activity or AP in imaging.

‡ SOFA score

§ These patients with OF were also treated in the Intensive Care Unit

Twenty patients with other acute abdominal diseases were included as controls, manifesting symptoms, signs, and laboratory or imaging findings unrelated to AP. Two patients were excluded due to undergoing an operation within one month prior to hospital admission, since surgery may have an effect on the blood polyamine levels (Jänne 1967, Jänne and Raina 1968, Porciani et al. 2001, Stabellini et al. 2003). As references, 6 healthy adult volunteers were recruited for assessment of blood polyamine levels (Table 1, Study IV).

2.2 Study design

2.2.1 Diagnosis and classifications

The diagnosis of AP was based on the presentation of the first episode of AP with clinical symptoms and signs consistent with this disease, serum amylase activity over three-fold the upper limit of normal value or the imaging changes (US or CT). In the 24 patients studied, there were 3 with AP defined by US finding, one in each aetiology group (alcoholic, gallstone and unknown aetiology). One of the 3 patients, who were recruited in the alcoholic group, had elevated amylase level but less than three times the upper limit at the onset of symptoms.

The patients with AP were defined as alcoholic aetiology according to the history of excessive use of alcohol or Alcohol Use Disorders Identification Test > 8 points (Bohn et al. 1995, Nordback et al. 2007). For the patients with observed gallstones in US or increased ALAT or bilirubin level, their aetiology was defined as of gallstone origin when gallstones or biliary sludge were detected in the imaging studies (US, ERCP, MRCP) (Banerjee and Steele 1995, Whitcomb 2006). The aetiology was defined as unknown when alcoholic and gallstone origin, or trauma, post-ERCP, hereditary, hypercalcaemia and hyperlipaemia origin were excluded through the history.

Seventeen cases were defined as mild AP and 7 were defined as severe AP based on the manifestation of local complications (pancreatic necrosis or pseudocyst) or OF at any time during hospitalization, according to the Atlanta criteria (Bradley 1993).

2.2.2 Scoring system

SOFA score was calculated on admission for the AP patients as previously described (Vincent et al. 1996, Halonen et al. 2002).

2.3 Collection of blood specimens

For patients with AP or with other acute abdominal diseases, the samples were collected on 5 consecutive days after admission, then every second day during hospitalization. After recovery 14 AP patients visited the clinic within 41 days (median, range 30 days - 58 days) from the onset of symptoms. The blood samples were collected in tubes containing 9NC coagulation sodium citrate 3.2% as an anticoagulant. Plasma and RBC were separated and stored in aliquots at -70 ^{o}C until analyses.

2.4 Measurements of specimens

2.4.1 Polyamine analysis

RBC polyamine levels were determined with HPLC, as described in Section 1.4.3 Materials and Methods.

2.4.2 Inflammatory mediators and laboratory tests

Plasma was analysed for CRP concentration in accordance with hospital laboratory routine, IL-6 and IL-10 using ELISA kits (Quantikine, R&D Systems, Minneapolis, MN USA).

3 Statistics

The data are expressed as mean ± SE and were analysed using an independent Student's *t* test, when distributed normally (Study I). The data not distributed normally (Studies I, II, III and IV), are expressed as median [range] and compared with Mann-Whitney *U* test. Paired Wilcoxon signed ranks test was used when comparing data between different time points in the same patients (Study IV). Correlation was calculated by Pearson test (Studies I, III and IV). Multivariate analysis was performed using multinomial logistic regression for the cut-off values of spermine and putrescine respectively (Study IV). The statistical significance was set at $p < 0.05$ (two-tailed). The statistical analysis was performed using SPSS 11.0 version, Chicago, Illinois.

4 Ethical aspects

The experimental studies (Studies I, II and III) were approved by the Animal Committee of the University of Tampere and the Institutional Animal Care and Use Committee of the University of Kuopio and the respective provincial governments. The experiments were performed in accordance with the "Guidelines for the Care and Use of Laboratory Animals" (NIH publication No.86-23, revised 1985).

Study IV was approved by the ethical committee of Tampere University Hospital and was registered at ClinicalTrials.gov, number NCT00484042.

RESULTS

1 Changes in polyamine catabolism during experimental acute pancreatitis (Studies I - III)

1.1 Manifestation of experimental acute pancreatitis

Infusion of either 2% or 6% sodium taurodeoxycholate into the biliopancreatic duct induced AP in wild-type rats. Blood amylase activity increased significantly within 24 h after infusion of 2% taurodeoxycholate and returned to normal level at 72 h. There was also increased pancreatic oedema and increasing necrosis in histology characterizing the development of AP. After the injection of 2% taurodeoxycholate, acinar necrosis gradually increased over time until 72 h. In 6% taurodeoxycholate-induced model, necrosis already achieved its greatest extent at 6 h.

After over-induction of SSAT in transgenic rats, the signs of AP (oedema and necrosis) already started to develop at 3 h. The proportion of necrotic cells reached its highest levels at 24 h after the induction of AP induced by SSAT over-expression.

1.2 Changes in polyamine catabolism

SSAT activity in both 2% and 6% taurodeoxycholate-induced AP reached its highest level at 6 h and returned to normal level by 24 h (Study III).

Pancreatic spermidine diminished slightly in the sham operation group. Both 2% and 6% taurodeoxycholate-induced AP models were associated with a more pronounced decrease in spermidine at 24 h in comparison with the sham operation. Concentration of pancreatic putrescine, the catabolised product of spermidine and spermine, increased significantly in both 2% and 6% taurodeoxycholate-induced AP and reached its highest level at 24 h (Study I and III).

Especially in 2% taurodeoxycholate-induced AP, the sum of spermidine plus spermine decreased at 24 h indicating polyamine catabolism (Study II). Simultaneously, pancreatic putrescine increased. Ratios of putrescine/spermidine and putrescine/spermine, which indicate the extent of this catabolism, revealed polyamine catabolism to be high at 24 h but this returned to baseline level by 72 h (Study II).

This decrease of RBC spermidine was more prominent at most of the time points studied in the 2% or 6% AP group compared with the sham operation group (Fig 3 A, Study I). The sham operation likewise induced a minor decrease of 28% in RBC spermine levels (Fig 3 B, Study I). In the group with AP induced by 6% taurodeoxycholate, a significant decrease was found in the blood spermine levels (Fig 3 B, Study I). The sham operation induced a decrease in blood putrescine levels from the baseline level. There was a significant increase over the baseline level in blood putrescine 6 h and 24 h following exposure to 2% taurodeoxycholate to induce moderate AP (Fig 3 C, Study I). In the 6% AP group, the blood putrescine levels did not differ from those of the sham-operated group until 24 h, when the surviving rats showed a remarkable increase in blood putrescine concentration (Fig 3 C, Study I). Taking into account the data in both 2% and 6% AP, the concentrations of putrescine in blood correlated significantly with the putrescine concentrations in the pancreas ($r = 0.72$, $p < 0.01$). The best polyamine catabolism indicators, putrescine/spermidine ratio or putrescine/spermine ratio, both correlated well between pancreas and blood ($r = 0.75$ and $r = 0.72$ respectively, both $p < 0.01$).

In transgenic rats, SSAT activity increased significantly over time (Fig 4 A, Study III). Its value was 17- or 33-fold when compared with the SSAT activity found in either 2% or 6% taurodeoxycholate-induced AP at 6 h. Spermidine and spermine were depleted by 92% and 79% at 24 h respectively, which is more than what was found in either of the two taurodeoxycholate-induced AP models (Fig 4 B and C, Study III).

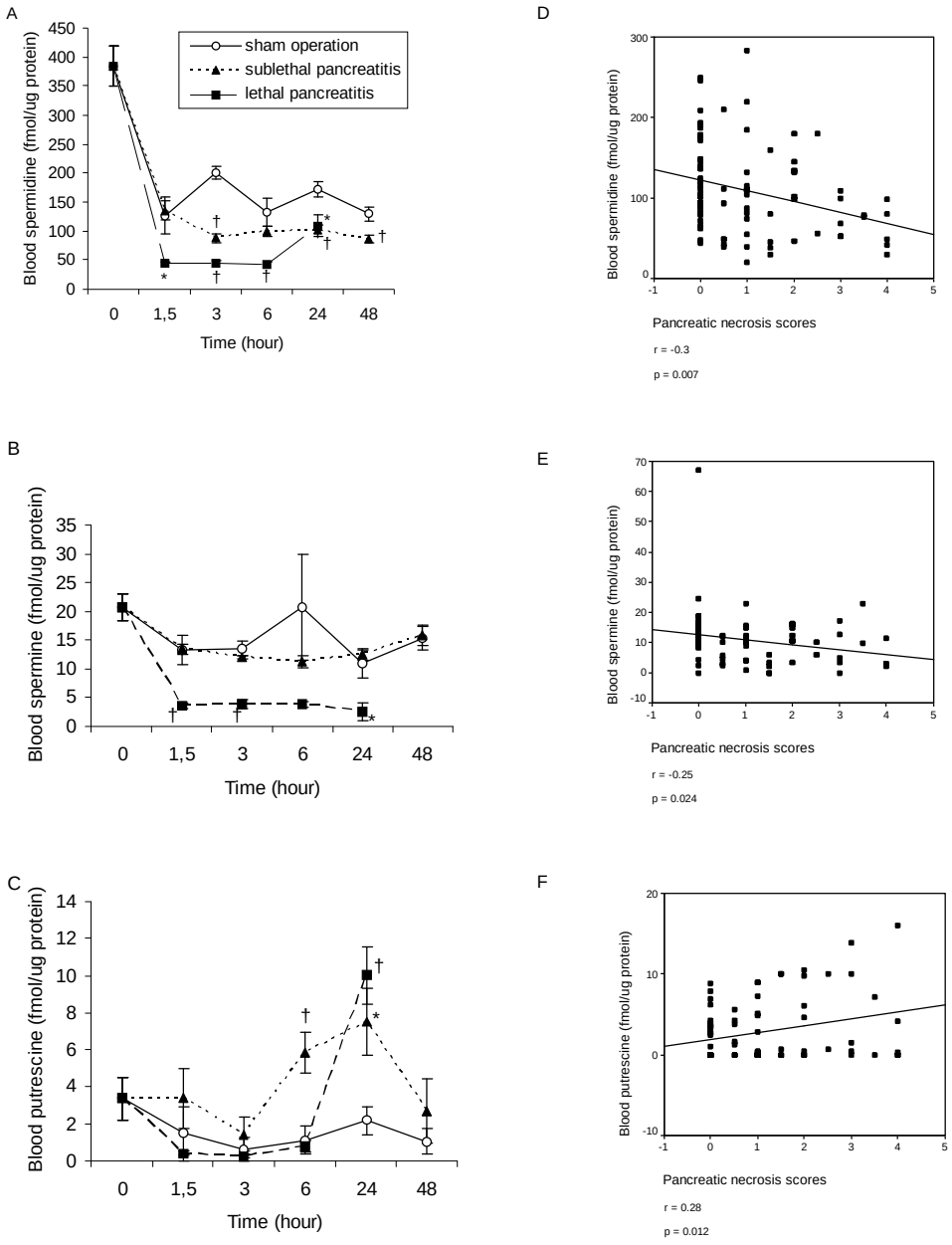

Figure 3. Blood polyamine levels in taurodeoxycholate-induced AP model and their correlation with the pancreatic necrosis.

Blood spermidine (A) decreased by sham operation only, but AP exacerbated this decrease, the more spermidine was depleted, the more severe was the disease. Blood spermine (B) decreased only in lethal AP. Blood putrescine (C) changed less logically. (In Figure 3, A-C, data are expressed as mean ± standard error, *$p < 0.05$, vs. sham operation; $^{\dagger}p < 0.01$, vs. sham operation). Blood spermidine (D), spermine (E) and putrescine (F) were significantly correlated with the necrosis of pancreas for 48 hours. In calculating the correlations, 2% and 6% taurodeoxycholate experiments were combined.

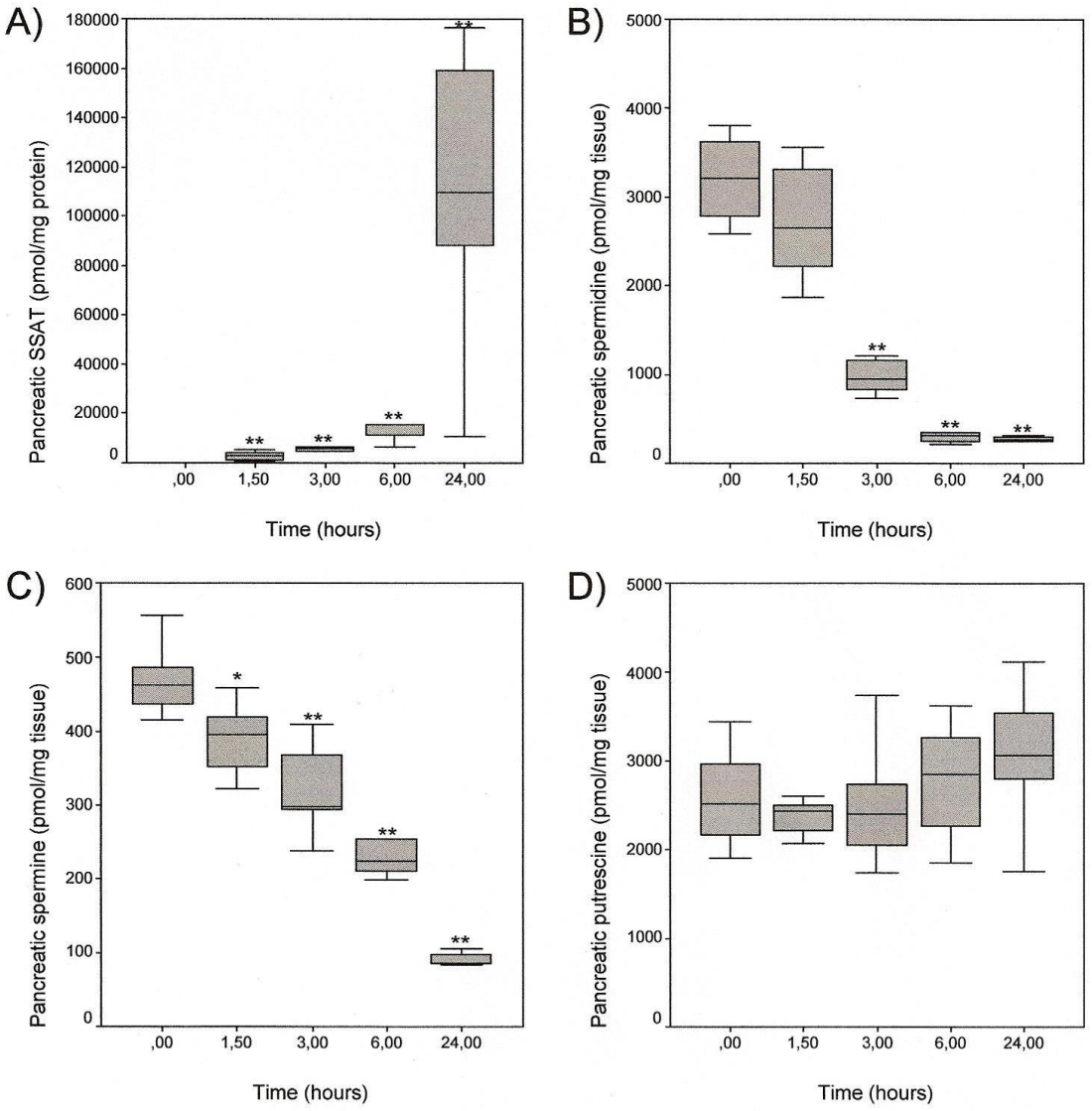

Figure 4. Changes of pancreatic polyamine catabolism in transgenic AP model.

In the SSAT transgenic Wistar rats, the AP was induced by intraperitoneal injection of zinc sulphate (10 mg/kg). Pancreatic SSAT increased significantly from 1.5 h to 24 h (A), meanwhile spermidine and spermine decreased remarkably (B,C). No significant change was observed in putrescine level (D). All data are expressed as medians [range]. $^{*}p < 0.05$, vs. 0h control group; $^{**}p < 0.01$, vs. 0h control group.

2 Polyamine catabolism and severity of acute pancreatitis (Studies I, III)

2.1 Severity of experimental acute pancreatitis

The mortality ratio of wild-type rats was different according to the concentrations of infused taurodeoxycholate: in 2% taurodeoxycholate-induced AP, the mortality ratio was 0% throughout the experiment; however, mortality was 66% at 24 h and 100% at 48 h in the 6% taurodeoxycholate-induced model (Study I). Mortality in transgenic rats was 0% during 24 h after the induction of AP by overexpressing SSAT (Study III).

AP was more severe in the 6% taurodeoxycholate-induced model, as revealed by amylase activity and histology, but the water content of the pancreas did not differ significantly between the two groups.

2.2 Polyamine catabolism

AP induced by 6% taurodeoxycholate was associated with higher SSAT levels than that induced by 2% taurodeoxycholate (Study III). Pancreatic spermidine decreased respectively by 64% and 44% at 24 h in the 2% and in 6% AP groups (Study I). In 6% AP, the pancreatic putrescine level started to increase later than in the moderate AP group and reached its highest level only 24 h after the induction of AP. However, activation of polyamine catabolism was not reflected in the spermine levels, as the changes of the pancreatic spermine concentrations were minimal at all time points studied.

In normal control rats, the blood polyamine concentrations were at a level of about 1: 1,000 of those in the pancreas. Blood spermidine already decreased from baseline by 60% in the sham-operated rats. This decrease was 74% in the 2% AP group (Fig 3 A, Study I). In the 6% AP group spermidine decreased by 84%, and more prominently than in the moderate group at most of the time points

studied (Fig 3 A, Study I). The sham operation likewise induced a minor decrease of 28% in the blood spermine levels (Fig 3 B, Study I). The blood spermine depletion (37%) did not change significantly in the 2% AP group during the follow- up period. In the group where AP was induced by 6% taurodeoxycholate, a significant decrease (83%) was found in the blood spermine levels (Fig 3 B, Study I).

3 Trypsin activation, polyamine catabolism and acinar cell death (Studies I, III)

3.1 TAP accumulation and SSAT activation

In the 6% taurodeoxycholate-induced AP model pancreatic TAP levels increased earlier from 1.5 h to 6 h compared to the respective sham groups (Fig 5 A, Study III). Compared with the 6% model, the 2% model showed slower accumulation of TAP, the maximum not being reached until 24 h (Fig 5 B, Study III). In transgenic rats, pancreatic TAP stayed low during the early stages of AP induced by SSAT over-induction, corresponding with the levels found after the sham operation in the wild-type rats (Fig 5 C, Study III). There was no significant increase in pancreatic TAP until 24 h, when pancreatic TAP reached a level similar to that observed much earlier in taurodeoxycholate-induced AP.

In the 6% taurodeoxycholate-induced model, the accumulation of pancreatic TAP occurred prior to the activation of pancreatic SSAT at the very beginning of AP (Fig 5 A, Study III); in 2% taurodeoxycholate-induced AP, TAP almost concurred with SSAT increase during the first 6 h (Fig 5 B, Study III); in the transgenic AP model, SSAT was highly activated during 1.5 h – 24 h, while TAP did not increase until 24 h (Fig 5 C, Study III).

3.2 Pancreatic TAP, SSAT and cell death

The presence of necrosis followed TAP accumulation, but preceded SSAT activation in 6% taurodeoxycholate-induced AP (Fig 5 A, Study III). The extent of necrosis correlated significantly with TAP level ($p = 0.012$).

In 2% taurodeoxycholate-induced AP, substantial necrosis (> 1 on a scale 0-4) developed by 6 h, concurring with the SSAT induction and the TAP accumulation (Fig 5 B, Study III). The extent of necrosis correlated significantly with the TAP level ($p = 0.001$). The correlation between the SSAT level and necrosis score did not quite reach statistical significance ($p = 0.063$).

No change was observed in the number of caspase-3 positive cells in either 2% or 6% taurodeoxycholate-induced AP.

In the transgenic AP model, substantial necrosis developed only after 6 h (Fig 5 C, Study III). Caspase-3 activity was already found to be increased before the necrosis developed, a few hours after SSAT induction (Fig 5 C, Study III). Both the necrosis and apoptosis scores correlated with the SSAT level ($p < 0.0001$).

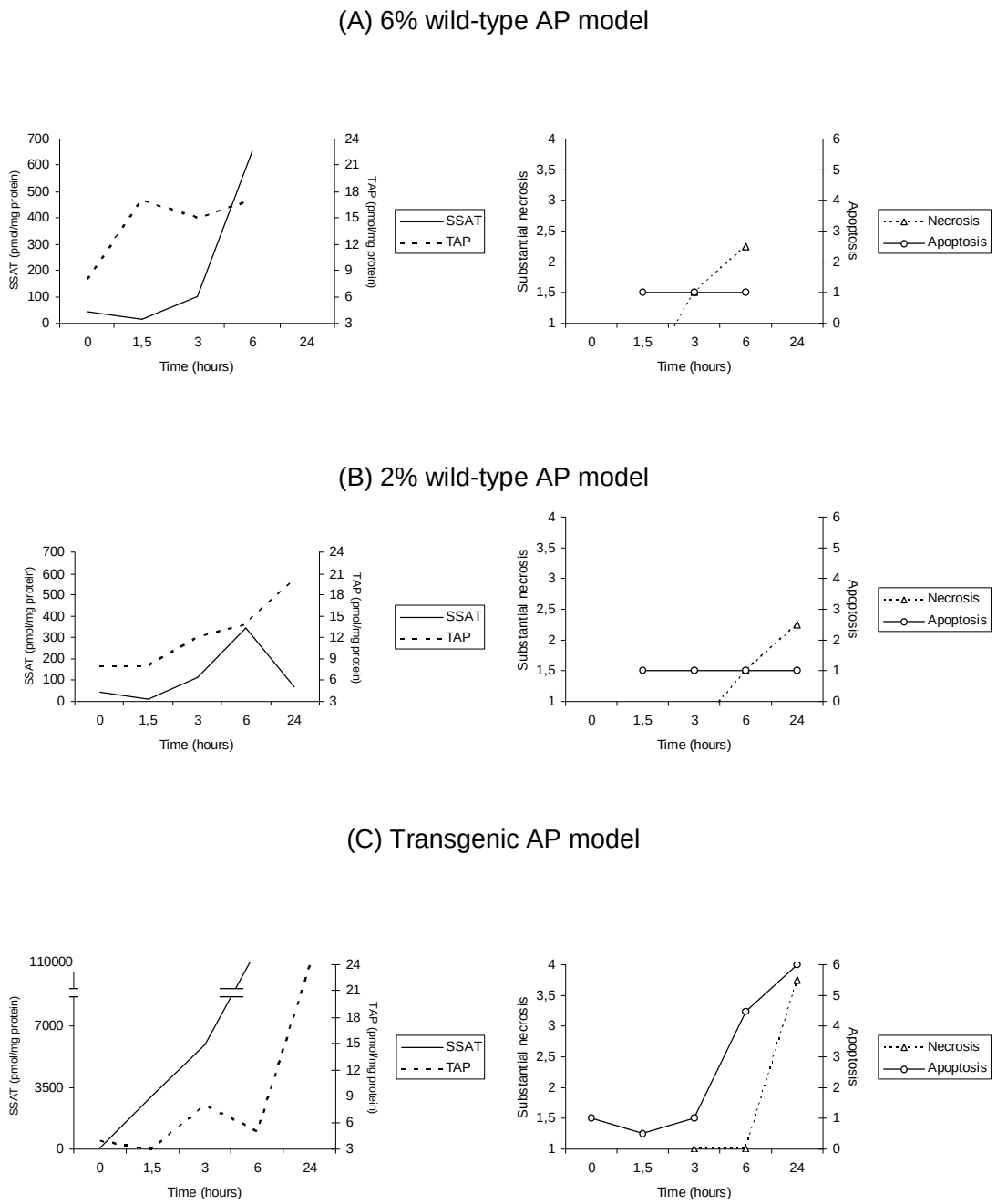

Figure 5. Changes of pancreatic SSAT, TAP, necrosis and apoptosis along with time-course in experimental AP models.

6% wild-type AP model, acute pancreatitis model in wild-type rats induced by 6% taurodeoxycholate; **2% wild-type AP model**, acute pancreatitis model in wild-type rats induced by 2% taurodeoxycholate; **Transgenic AP model**, acute pancreatitis model in SSAT over-expressed transgenic rats induced by zinc sulphate (10 mg/kg). The substantial necrosis is defined as pancreatic necrosis score > 1. All data are presented as medians.

3.3 Polyamine levels and pancreatic cell death

There was a significant correlation between the extent of pancreatic necrosis, as evaluated by histology, and the pancreatic putrescine levels ($r = 0.34$, $p < 0.01$), but not the spermine or spermidine levels, when taking into account the entire 48-hour time course in taurodeoxycholate-induced AP (Study I). The best polyamine catabolism indicators, the ratio of pancreatic putrescine/spermidine or putrescine/spermine, correlated well with the extent of pancreatic necrosis ($r = 0.29$ and $r = 0.32$, respectively, both $p < 0.01$). Blood spermidine ($r = –0.3$, $p < 0.01$), spermine ($r = –0.25$, $p < 0.05$), and putrescine ($r = 0.28$, $p < 0.05$) correlated with the extent of pancreatic necrosis, as evaluated by histology (Fig 3 D–F, Study I). Furthermore, the blood putrescine/spermidine and putrescine/spermine ratios correlated significantly with the pancreatic necrosis during the entire 48-hour evolution of AP ($r = 0.32$ and $r = 0.37$, respectively, both $p < 0.01$).

After induction of SSAT in transgenic rats, the extent of necrosis over 24 h was significantly and inversely associated with pancreatic spermidine and spermine levels (-0.84 and -0.89, both $p < 0.0001$). A slight increase in the number of pancreatic acinar cells with positive staining for cleaved caspase-3 was also already observed at an early time point (6 h), after which the number increased remarkably over time. The acinar cells started to lose their integrity from 6 h after induction, showing paler and more diffuse staining for cleaved caspase-3 than that seen in the acinar cells at earlier time points or in the taurodeoxycholate-induced model. The proportion of caspase-3 positive cells reached their highest levels at 24 h after the induction of AP induced by SSAT over-expression. The extent of apoptosis during the course correlated with pancreatic spermidine and spermine levels significantly and inversely (-0.78 and -0.88, both $p < 0.0001$).

4 Effect of polyamine analogue on experimental acute pancreatitis (Studies II, III)

4.1 Effect on mortality

In the 2% model, after the administration of Me_2Spm there were no deaths for 24 h, but the administration of an extra dose of Me_2Spm at 30 h resulted in 100% mortality by 72 h (Study II). Therefore, there were no samples available at 72 h to detect the effect on the pancreas. In the other groups there were only occasional deaths.

4.2 Effect on water content and necrosis

Me_2Spm was tested as pre-treatment and as treatment for the AP induced by 2% taurodeoxycholate infusion (Study II). The pre-treatment did not ameliorate the AP at 24 h as judged by amylase activity, pancreatic water content, or histology. By contrast, treatment with Me_2Spm after the induction of AP significantly reduced the pancreatic water content (Fig 6, Study II) and necrosis at 24 h but did not decrease the amylase activity.

This effect at 24 h was absent at 72 h, when neither the pre-treatment nor the treatment with Me_2Spm showed a significant effect on pancreatic histology or water content (Study II).

4.3 Effect on apoptosis and TAP

The administration of Me_2Spm had no effect on the extent of pancreatic apoptosis or the accumulation of TAP in the 2% taurodeoxycholate-induced models (Study III).

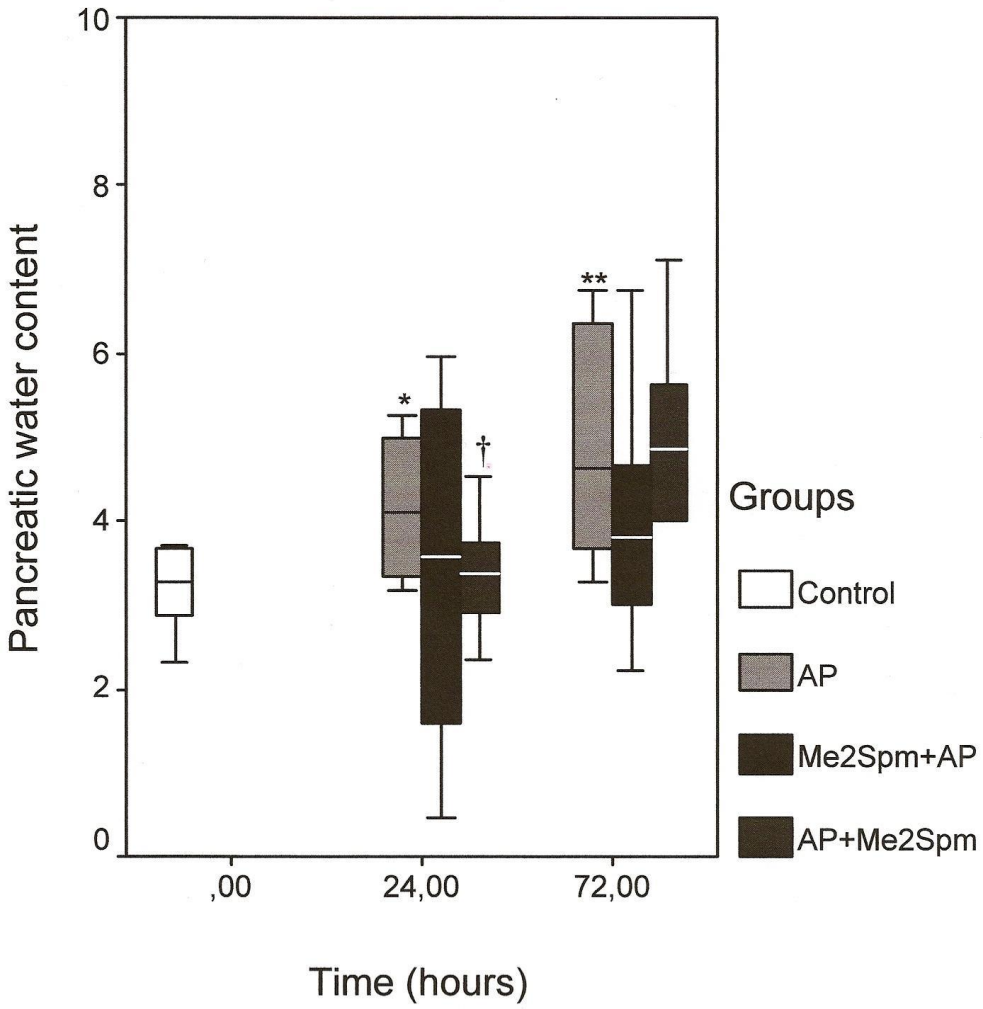

Figure 6. Effect of Me_2Spm on pancreatic water content during acute pancreatitis.

Water content = (wet weight-dry weight)/dry weight ratio. Only the treatment of Me_2Spm decreased the pancreatic water content at 24 h after the induction of 2% taurodeoxycholate-induced acute pancreatitis. The column at 0 h serves as a normal control. **AP**, Acute Pancreatitis; **Me_2Spm**, Bismethylspermine; **Me_2Spm + AP**, pre-treatment with Me_2Spm before acute pancreatitis induction; **AP + Me_2Spm**, treatment with Me_2Spm after acute pancreatitis induction. All data are medians [range]. * $p<0.05$ vs. control at 0 h; ** $p<0.01$ vs. control at 0 h; † $p<0.05$ vs. acute pancreatitis at 24 h

4.4 Effect on pancreatic polyamine catabolism

Me_2Spm accumulated in the pancreas as a result of both pre-treatment and treatment, with greater concentrations achieved during the later administration as treatment (Table 2, Study II). There was more Me_2Spm in pancreatic tissue at 72 h than at 24 h, especially when Me_2Spm was given after the induction of AP (Table 2, Study II).

Table 2. Accumulation of pancreatic Me_2Spm in 2% taurodeoxycholate-induced AP.

	Me_2Spm (nmol/mg protein) + AP	**AP + Me_2Spm (nmol/mg protein)**
24 h	2.52 [1.17-4.71]	4.15 [2.70-7.05]
72 h	4.99 [1.70-7.22]	10.00 [6.42-15.04]**

AP, Acute Pancreatitis; **Me_2Spm**, Bismethylspermine; **Me_2Spm + AP**, pre-treatment of Me_2Spm before acute pancreatitis induction; **AP + Me_2Spm**, treatment of Me_2Spm after acute pancreatitis induction. All data are medians [range]. $^{**}p<0.01$ vs AP+ Me_2Spm at 24 h

Me_2Spm did not increase SSAT activity in the AP model at 24 h (Study II). Both the pre-treatment and treatment with Me_2Spm led to a further accumulation of putrescine in the pancreas at 24 h, while spermidine and spermine levels decreased, suggesting increased catabolism of the natural polyamines in the presence of synthetic polyamine analogue. This observation was confirmed by calculating putrescine/spermidine and putrescine/spermine ratios (Study II). Previously it has been suggested that the decrease of spermidine and spermine is important in the deleterious effects of polyamine catabolism (Alhonen et al. 2000). We found that in AP the sum of spermidine plus spermine concentrations decreased to almost half from baseline by 24 h with return to baseline level by 72 h (Study II).

5 Changes of polyamines in human acute pancreatitis (Study IV)

5.1 Polyamine levels on admission

On admission to hospital, the polyamine levels were no different in the AP patients and in the control patients from those of the healthy volunteers. Spermidine and putrescine levels were highest among the patients with AP of unknown aetiology, while spermine level did not change according to the aetiology. None of the polyamines levels measured on admission were found vary according to the severity of AP as assessed by the Atlanta criteria or SOFA score. However, the patients who developed pancreatic necrosis had a higher level of putrescine in RBC on admission to hospital (Fig 7 A, Study IV), while spermidine and spermine levels remained unchanged when compared to patients without pancreatic necrosis. Furthermore, spermine level was significantly higher in the AP patients with CRP value > 250 mg/l on admission to hospital than the patients with lower CRP (Fig 7 B, Study IV). CRP, but not the duration of symptoms prior to admission, had an independent significant association with spermine level (with cut-off ≥ 60 pmol/mg protein, $p = 0.044$). Necrosis, but not aetiology, had an independent significant association with putrescine level (with the cut-off ≥ 3 pmol/mg protein, $p = 0.024$).

5.2 Polyamine levels during hospitalization

There was no change of RBC polyamine levels in AP patients during hospitalization compared to control patients with other acute abdominal diseases. However, spermidine and spermine levels appeared to stay significantly higher during hospitalization in patients with AP of unknown aetiology compared to patients with disease of alcoholic aetiology. Putrescine level was also significantly elevated in AP of unknown aetiology compared to alcoholic or gallstone aetiology.

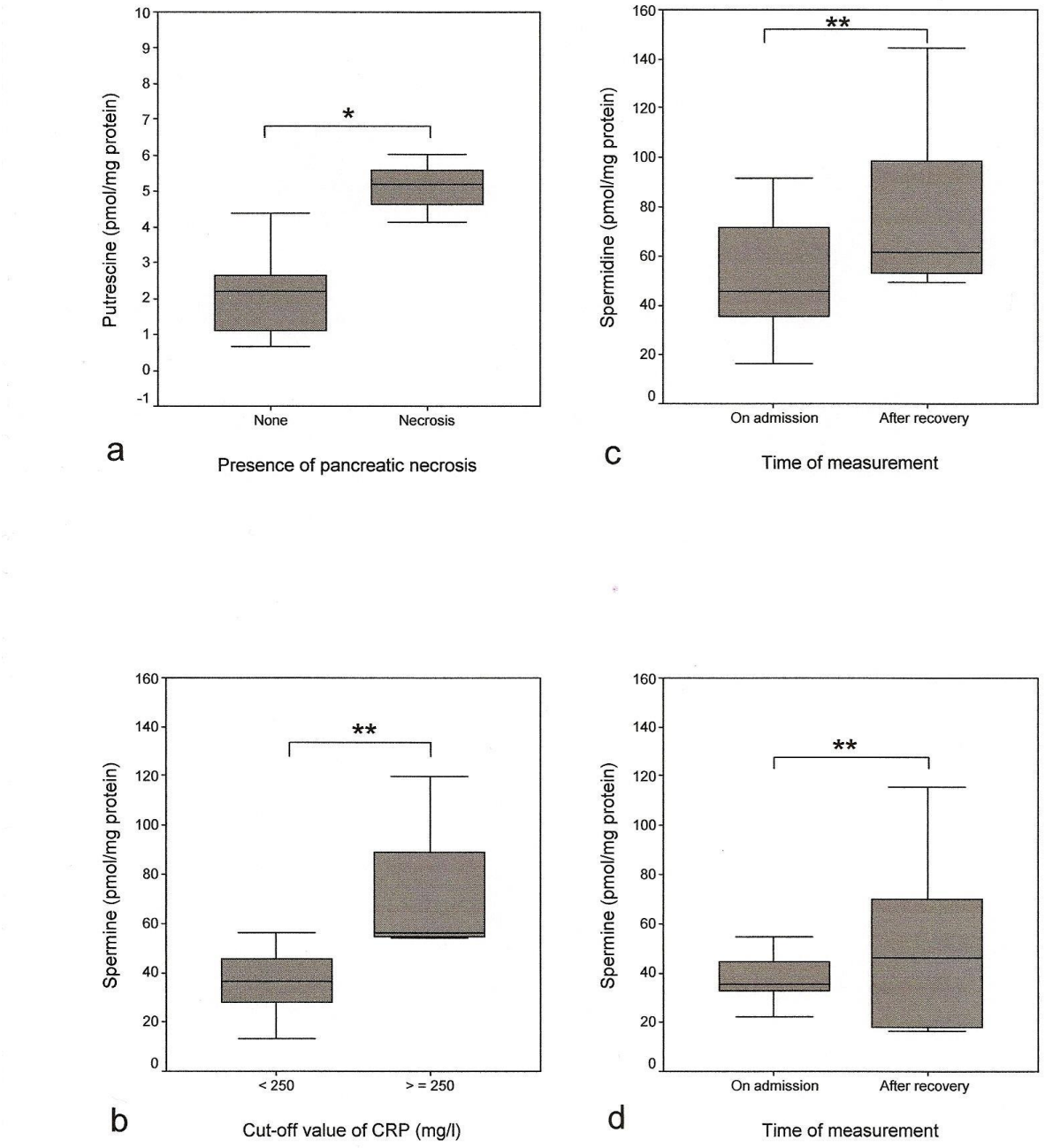

Figure 7. Change of blood polyamine levels on admission according to presence of pancreatic necrosis, CRP cut-off value and follow-up visit in patients with acute pancreatitis.

All data are expressed as medians [range]. $^{*}p < 0.05$; $^{**}p < 0.01$.

5.3 Polyamine levels after recovery

After recovery, the spermidine and spermine levels were significantly elevated from the levels found on admission to hospital, but with huge overlaps of ranges (Fig 7 C-D, Study IV). Putrescine level remained unchanged.

5.4 Inflammatory mediators and correlations with polyamines

On admission, IL-6 and CRP levels were significantly elevated in patients with AP as compared to the control and healthy groups. Both IL-6 and IL-10 returned to normal levels by the follow-up visit. The levels of IL-6 and CRP were increased during the first week of hospitalization in patients with AP compared to controls. Both IL-6 and IL-10 levels appeared to be highest in AP of unknown aetiology. They correlated significantly with the level of putrescine in the AP patients; CRP values correlated significantly with spermine levels.

DISCUSSION

1 Methods for investigating experimental acute pancreatitis and polyamine catabolism (Studies I - III)

In the present studies, wild-type male Sprague-Dawley rats were used to establish bile salt induced AP models. Sprague-Dawley rat is a readily available and economic study strain. The preference for using only male rats in our studies was based on the fact that the development of AP at least in some models may be affected by the action of estrogens (Rao et al. 1982).

Bile salt infusion model was used as the best model to simulate clinical AP. Furthermore, the severity of the disease may be adjustable when 7.5% taurocholate has been infused mixed with 5% trypsin, 50% mortality was found at 24 h (Nakae et al. 1995), approximating the 66% mortality at 24 h with 6% taurodeoxycholate in the present study. The infusion of 2% taurodeoxycholate induced 0% mortality. This is in agreement with earlier studies that different concentrations of bile salt are able to induce various severities of AP (Lankisch and Ihse 1987).

Besides the bile salt concentration, bile salt category, infusion volume, infusion pressure and infusion time may also have an influence. Several studies (Fernandez-del Castillo et al. 1992, Schmidt et al. 1992a, Nakae et al. 1995, Wang et al. 2001, Coelho et al. 2003) have reported that intraductal infusion of 0.5 ml of glycodeoxycholate or taurocholate with 0.48%-7.5% concentration led to trypsin increase of serum at 2 h, in urine at 3-4 h and in pancreas at 2 h. This is why two well standardized models were used, identical except for the bile salt concentration.

Currently the only strain which has been used as an established experimental transgenic AP model is the Wistar rat. In transgenic Wistar rat over-expressing SSAT gene, AP is induced by zinc sulphate 10 mg/kg and all animals die in 3 days. The occurrence of AP does not come from the

toxicity of zinc. The same zinc dose does not have an effect either on pancreatic polyamine levels or pancreatic histology or animal survival in wild-type Wistar rats (Alhonen et al. 2000). The toxicity to the pancreas requires an administration of high-dose zinc (50-300 mg/kg) which leads to pancreatic necrosis and fibrosis, and zinc-induced hyperamylasemia (Minami et al. 2001, Tetsuchikawahara et al. 2005).

SSAT activities were measured with highly excessive concentrations of the substrate in order to be able to measure the maximum performance of the enzyme. Thus small changes of substrate concentrations may be difficult to detect even if the enzyme is active. Furthermore, we measured the amount of acetylated spermidine in the reaction where (radioactively labelled) acetyl group from acetyl-coenzyme A is transferred to spermidine. As there is no labelled acetylspermidine at the beginning of the reaction, the quantitation of the product is reliable and the assay is relatively sensitive. The enzyme itself does not change during the process of catalysis but of course there is a limit to how long any protein will remain functional *in vitro* after disruption of the tissue. The proteins will eventually be inactivated or degraded by proteases in the samples. The time was minimized in the present experiments.

Previously the SSAT assay was found unspecific (Porter et al. 1991). It appeared to measure other enzyme activities capable of acetylating spermidine as well. Using SSAT-specific antiserum, it was found that immunoprecipitable SSAT only accounted for approximately 27% of the assay-detectable activity in MALME-3 cells. This suggests that the measured SSAT activity did not entirely represent SSAT at basal level. However, if the total activity is induced in response to some stimulus, the induced portion of the total activity is almost exclusively SSAT. Thus, the calculation of induction level using measured total activities may underestimate the extent of true SSAT induction. This limitation may partially explain the lack of correlation between SSAT activity and the severity of experimental AP in Study I.

Polyamines are mainly quantified by HPLC methods instead of more complicated gas chromatography (Larque et al. 2007). Putrescine values in control animals were generally rather low and very close to the limit of detection. In the taurodeoxycholate-induced AP model, the accumulation of putrescine was not as evident as in the transgenic model or clinical AP. The final result may also depend on the time (batch) of analysis that produces the technical variation (high background, dirty column, old *o*-phtalaldehyde reagent for detection and so on). Therefore all the current experiments in each study were measured simultaneously to allow comparison.

In the experimental settings, the method of detecting pancreatic apoptosis is activated caspase-3 staining. Aspartate-specific cysteine proteases, caspases, have been defined as key mediators of apoptotic cell death. The morphological phenotype of apoptotic cells depends on the activity of caspases (Stadelmann and Lassmann 2000). Caspase-3 is thought to be a key caspase that particularly prefers histological labelling of apoptosis (Hughes and Gobe 2007). Detection of caspase-3 is a valuable and specific method in identifying dying cells, and no activation of the caspase cascade has been found in necrotic cell death.

Terminal deoxynucleotidyl transferase (TdT)-mediated dUTP-biotin nick-end labelling (TUNEL) methods were not used in our studies. TUNEL is a sensitive method for detecting DNA fragmentation. However, DNA fragmentation is not always required for apoptosis (Hughes and Gobe 2007). Thus TUNEL may give false positive staining, which suggests that TUNEL staining is not specific for apoptosis. In contrast to TUNEL, the detection of activated caspase-3 is specific and accurate in accounting for apoptosis (Stadelmann and Lassmann 2000).

2 Role of polyamine catabolism in the actions of trypsin activation and cell death (Studies I - III)

The exact role of polyamine catabolism in the early phase of AP is not clear due to several limitations. Currently no chemical is available to specifically inhibit SSAT activity. In knock-out SSAT transgenic mice the data have shown, however, that the depletion of SSAT activity has only a slight effect on tissue polyamine levels (Wallace and Fraser 2004, Niiranen et al. 2006). It appears that SSAT induction is more important than SSAT decrease in AP. Because the induction is associated with a decrease in important polyamines, we investigated the polyamine catabolism in indirect manners by monitoring polyamine catabolism and the effect of a polyamine substitute.

In our studies, activation of polyamine catabolism was found in taurodeoxycholate-induced AP in wild-type rat: There was an increase of SSAT activity and depletion of polyamines in the pancreas. SSAT activity in the 6% model was higher than in the 2% model. Of the polyamines spermidine level declined, putrescine level rose temporarily, with the SSAT activation. For the transgenic model, over-activation of SSAT resulted in extreme polyamine depletion and the substantial necrosis of acinar cells did not start before 6 h. The activation of polyamine catabolism was over-induced so that the increased SSAT activity, putrescine level and decreased spermidine level at 24 h were about 1640-, 2.2- and 0.1-fold more than those found in 2% taurodeoxycholate-induced AP. These results demonstrate that polyamine catabolism is associated with the induction of AP.

However, trypsin activation in pancreas is generally thought to be an initial event in AP, that trypsin activity and TAP are first detectable in a heavy subcellular fraction (Hofbauer et al. 1998, Mithofer et al. 1998, Halangk et al. 2000, Chen et al. 2003a). Cleavage of TAP from the conversion of trypsinogen to active trypsin provides a quantitative index of active trypsin generation instead of trypsin (Mithofer et al. 1998). Hyvönen et al. reported that a dose-dependent activation of

trypsinogen was induced by treatment with different concentrations of zinc sulphate in isolated transgenic acini (Hyvönen et al. 2006). Furthermore, in the transgenic model activation of both cathepsin B and trypsin was observed at 4h after the induction of AP (Hyvönen et al. 2007).

To investigate the relationship between polyamine catabolism and trypsin activation in experimental AP, SSAT activation was compared with TAP accumulation. We found that pancreatic TAP is elevated earlier than SSAT in the severe form (6% taurodeoxycholate), however, in the moderate form (2% taurodeoxycholate), gradually enhanced SSAT activity concurred with TAP release. In the mildest form (transgenic AP), the accumulation of TAP is slowest for 24 h when SSAT activity was already extremely high at 1.5 h. These findings suggest that activation of polyamine catabolism may not be an early mediator of trypsin activation in the taurodeoxycholate-induced AP model. The timing of TAP and SSAT changes depends on the severity of the disease. The value of SSAT is not extremely high during earlier stage of taurodeoxycholate-induced AP, but it can be in transgenic AP. It is impossible to reach such an extreme extent of SSAT activation in the physiological condition of wild-type animals. The action of polyamine catabolism in early trypsin activation may be a phenomenon specific to particular animal model.

In pancreatic tissue, we found that the extents of pancreatic necrosis did not correlate with SSAT activities, but correlated significantly with TAP values in taurodeoxycholate-induced models. However, both necrosis and apoptosis scores significantly correlated with SSAT activities but not with TAP values. These observations led to the suggestion that both TAP and SSAT play certain roles in the mechanism of AP, but their contributions to pancreatic injury can be different depending on the mechanism of the respective animal model.

The activation of SSAT may mediate the early injury of pancreas through other mechanisms: (1) The pancreas is the richest source of the polyamine spermidine in mammalian animals and the depletion of polyamines is essential to destroy the integrity of the pancreas (Alhonen et al. 2000), the massive loss of cellular polyamines is actually a huge blow to the pancreas. (2) The stabilization of DNA requires a sufficient supply of polyamines in physiological circumstances (Jänne et al. 2004). Depletion of polyamines, initiated by SSAT can induce changes in chromatin and DNA structure (Moinard et al. 2005). The inhibition of anti-inflammatory response by spermine depletion with acute overexpression of the rate-limiting catabolic enzyme SSAT may contribute to the observed necrosis in the transgenic model (Alhonen et al. 2000).

Besides observational study, we also introduced interventional study by administering Me_2Spm to explore whether polyamine depletion has a role as a mediator in the pathophysiology. In our experimental studies, Me_2Spm was used as a therapeutic agent to ameliorate the pancreatic water content and acinar necrosis within 24 h after initiating disease in wild-type animals. Additionally, the protective effect of MeSpd was also observed in the L-arginine induced AP model (Hyvönen et al. 2006). Studies using transgenic AP models had shown that either MeSpd or Me_2Spm reduced the levels of plasma amylase and the extent of pancreatic necrosis (Räsänen et al. 2002, Hyvönen et al. 2006). The protective mechanism of polyamine analogue may include the following points: (1) Me_2Spm can prevent cell damage from regulating inflammatory mediators and oxidative stress (Merentie et al. 2007); (2) These analogues may fulfil cellular functions of polyamines in promoting the conversion of B-DNA to Z-DNA and restoring growth of polyamine-depleted cells (Hyvönen et al. 2006); (3) In the transgenic AP model, the activation of trypsinogen and cathepsin B can be inhibited by Me_2Spm in early stage of the disease (Hyvönen et al. 2007).

Me_2Spm also has an effect on polyamine catabolism in the pancreas. Polyamine depletion can be achieved by structural analogues which mimic spermidine and spermine in regulatory properties and cause the activation of catabolic pathways and release (Seiler and Raul 2005). Polyamine ratios may sometimes be used to make the extent of catabolism clearer. In polyamine metabolism, putrescine/spermidine or putrescine/spermine may not always be the most relevant way of describing catabolism. In some cases, activated acetylation is followed by enhanced excretion of acetylpolyamines from the cells causing no accumulation of putrescine despite extensive loss of spermidine and spermine. This may especially be the case if for some reason PAO is inhibited or unfunctional. In wild-type model, it is likely but not directly proven that PAO catalyzes the oxidation of acetylated polyamines. In all, it is the extent of polyamine depletion in molar terms that causes the consequences.

All together, activation of polyamine catabolism and trypsin are observed in both taurodeoxycholate-induced model and transgenic models, suggesting that they are both associated with the evolution of acinar necrosis. However, their proportions contributing to the early injury of pancreas depend on the individual mechanism involved in certain experimental AP models. In the taurodeoxycholate-induced AP model, the more severe the AP is, the earlier TAP increases and the later SSAT is activated. Contrary to the transgenic model, trypsin activation occurs prior to SSAT activation and polyamine catabolism is not a mediator of trypsin activation in wild-type models. The protective effect of Me_2Spm on necrosis without preventing trypsin activation is also against the hypothesis that polyamine catabolism mediates trypsin activation.

3 Severity and necrosis indicated by polyamine levels (Studies I, III, IV)

In both taurodeoxycholate and transgenic models, pancreatic polyamine levels correlated significantly with the extent of necrosis. The changes in polyamine catabolism were found not only

in pancreas but also in blood. Blood polyamine levels decreased and were associated with severity of taurodeoxycholate-induced AP. Polyamine ratios in blood correlated significantly with the ratios in pancreas. All the changes in blood took place within 24 h after AP induction, after that, the values of polyamines were generally ready to return to normal levels. These results suggest that blood polyamines are highly sensitive to pancreatic injury in the early stage. This is consistent with a previous finding that blood polyamines decrease after surgical or radiation injury (Porciani et al. 2001, Stabellini et al. 2003). The characteristics of polyamine changes may originate from the fact that polyamine pool in blood is much lower than that in pancreas. The mechanism for predicting severity may be due to the limitation of blood polyamine amounts and the connections between blood and pancreatic polyamines. However, abnormalities of blood polyamine homeostasis persist no longer than 48 h and gradually return to normal level.

As indicated above, blood polyamine levels were associated with severity of experimental AP and extent of pancreatic necrosis. Because erythrocytes lack the key enzyme of polyamine catabolism, SSAT, their polyamine content reflects changes taking place elsewhere in the body. Uptake of polyamine would not explain the changes because the rats were fasting during the experiment.

In taurodeoxycholate-induced AP, the decline of RBC spermidine and spermine levels was induced by either pancreatic injury or sham operation within 24 hours. Particularly, the extent of spermidine decline was proportionate to the severity of the injury. Spermidine decreased by 60% in sham operation, by 74% in moderate AP and by 84% in lethal AP respectively. Putrescine, as precursor of spermidine and spermine, had an opposite trend in that it increased over time. In clinical investigation, polyamine values did not differ between patients with AP and patients with other acute abdominal diseases on admission or throughout hospitalization. Interestingly, it was found on admission that patients with pancreatic necrosis had a significantly higher level of putrescine than

those without pancreatic necrosis. Furthermore, putrescine levels correlated statistically significantly with the values of IL-6 and IL-10 respectively.

There are several reasons for the depleted RBC polyamine levels associated with the severity of AP. (1) The way of depleting polyamines in erythrocytes in particular suggests that the process can operate without SSAT functioning, because erythrocytes contain no SSAT; (2) The pancreas is involved in circulation, if there is pancreatic injury that causes the pancreas to excrete less secretions to digestive lumen, polyamines uptake from lumen to circulation was reduced; (3) The pancreas needs polyamines (especially for a regeneration process after injury) and this would increase polyamine biosynthesis but also uptake from blood. The more severe damage occurs, the more the pancreas demands and the more polyamines are released from erythrocytes. Considering the whole body, polyamines excreted by some tissues may be utilized by other, at least to some extent.

In overview, the increase of putrescine levels is a common phenomenon in the development of AP. Putrescine elevation favours several pathophysiological situations such as the early phase of the disease, development of the severe form or presence of pancreatic necrosis. The fact that occurrence of AP is not predictable or controllable in the initial stage causes delayed sampling in clinical observation, and further makes investigators miss the best time to detect the fall in spermidine and spermine, although both the polyamine components show a promising performance in experimental settings. In detail, the interval between the onset of AP and first sampling on admission has an essential effect on the picture of polyamines in patients. Generally, polyamines are not a specific marker for referring diagnosis or predicting aetiology of AP in the clinical setting. The enhanced putrescine level found in patients with pancreatic necrosis is partially consistent with the

observation in experimental study. Under observation in well-controlled experimental settings, the action of polyamine catabolism could be regulated by an independent mechanism.

4 Polyamine catabolism and pancreatic apoptosis (Study III)

In taurodeoxycholate-induced AP, no extensive apoptosis was detected. In the transgenic model the apoptosis scores correlated positively with SSAT activity and correlated inversely with pancreatic spermidine and spermine levels. This suggests that polyamine catabolism is involved in the occurrence of apoptosis.

Damage to DNA or to other molecules causes a series of events that activate cellular apoptosis (Seiler and Raul 2005). Depletion of polyamines can induce changes in chromatin and DNA structure, and chromatin damage is a frequent primary cause of apoptosis induction, deficiency of polyamines being one of the plausible factors for the activation of apoptosis (Moinard et al. 2005, Seiler and Raul 2005). Polyamine depletion may be a common phenomenon in apoptosis induction that selective depletion of intracellular polyamines can induce apoptosis, however, selective polyamine depletion can also prevent cellular apoptosis (Seiler and Raul 2005). The possible mechanisms include binding to anionic structures, scavenging of radicals and formation of covalent bonds and cytotoxic products (Seiler and Raul 2005). Another cause of apoptosis may be associated with the release of oxidative stress produced by polyamine PAO (Moinard et al. 2005). Exogenous spermine can restore intracellular apoptosis and the polyamine pool in heart tissue (Zhao et al. 2007). Polyamine catabolism is involved in regulating molecules and genes linked to apoptosis (Jänne et al. 2004, Takeyama 2005). Tremendous acini apoptosis was found in the SSAT-overexpressed transgenic model. Activation of SSAT activity is associated with onset of apoptosis (Hegardt et al. 2000). It has been shown that selective interference of SSAT induction prevented development of apoptosis (Chen et al. 2003b).

The type of AP model can affect the extent of apoptotic activation. In the transgenic AP model, pancreatic acinar apoptosis is very extensive for 24 h and apoptosis develops over time. However, in wild-type models the apoptosis is not obvious and there was no significant difference in the extent of activated caspase-3 staining between AP groups. The reason may be associated with the degree of activation of polyamine catabolism. SSAT activity increased 1570-fold from baseline level at 24 h, meanwhile 92% spermidine and 80% spermine were depleted from pancreatic acinar in transgenic model. Compared with the transgenic model, the changes in SSAT and polyamines are mild in the wild-type model such that SSAT increased 3 folds and 55% spermidine was depleted at 24 h. Thus vast activation of pancreatic apoptosis may be associated with extreme activation of polyamine catabolism.

The treatment of Me_2Spm did not increase apoptosis in the 2% or 6% wild-type models but only decreased necrosis in 2% taurodeoxycholate-induced AP. The protective mechanism of polyamine analogue is unconnected with the regulation of apoptosis that plays a defensive role in the development of AP (Kaiser et al. 1995). Recent study has shown that the loss of mitochondrial integrity may initially lead to the induction of apoptosis signalling (Hyvönen et al. 2007). However, there was also extensive necrosis in the transgenic model. It is likely that caspases have also been implicated as mediators of necrosis elsewhere (Higuchi et al. 1998, Edelstein et al. 1999, Niquet et al. 2003, Dursun et al. 2006).

5 Effect of polyamine analogue Me_2Spm on experimental acute pancreatitis (Studies II, III)

Polyamine analogues, as derivatives of natural polyamines, are assorted to two categories, polyamine antimetabolite and polyamine mimetic, the latter decreasing growth without depleting polyamine pool (Wallace et al. 2003). Methyl derivatives of polyamines (spermidine and spermine) have been used as substitutes for the natural polyamines *in vivo* and *in vitro* and used to reverse

cytostasis (Järvinen et al. 2005). They are equally effective as natural polyamines, but more stable than natural polyamines, which would undergo rapid degradation due to activated acetylation (Järvinen et al. 2005, Hyvönen et al. 2006). The half-life of Me_2Spm is not known as it has still not been used extensively *in vivo*. However, reviewing the tissue concentrations of the drug at 24 h and 72 h, there is very little change, indicating that the drug is fairly stable in tissues.

In Studies II and III, it was found that the pancreatic injury was ameliorated within 24 h by Me_2Spm after initiating 2% taurodeoxycholate-induced AP in wild-type rats. This observation concurred with earlier studies that another polyamine analogue MeSpd played a protective role in the L-arginine induced AP model (Hyvönen et al. 2006). Furthermore, either MeSpd or Me_2Spm could diminish pancreatic damage in transgenic AP models (Räsänen et al. 2002, Hyvönen et al. 2006).

The TAP levels were not influenced by the intervention of Me_2Spm. The use of Me_2Spm does have a protective effect on pancreas in AP. The protective effect of Me_2Spm on 2% taurodeoxycholate-induced AP may come from the anti-oxidation and anti-inflammation mechanism. It was found in transgenic AP that Me_2Spm treatment increases pancreatic several anti-oxidants and decreases serum TNF-α and IL-6 (Merentie et al. 2007).

The protective effect of Me_2Spm needs certain conditions to take place, which, to some extent, depend on the time of administering and the dose. Not Me_2Spm prophylaxis, but Me_2Spm treatment after induction of 2% taurodeoxycholate-induced AP has a protective effect on the pancreas. This may be due to the observation that there was not enough Me_2Spm collected inside the pancreas by Me_2Spm prophylaxis compared with Me_2Spm treatment at 24 h or 72 h. However, the protection from Me_2Spm was not evident after 24 h, although more Me_2Spm accumulated in pancreas at 72 h.

The pharmacologically beneficial effect of Me_2Spm was overtaken by the toxicity of its over-dose so that additional Me_2Spm led to 100% mortality. Therefore polyamine analogue Me_2Spm exacerbates the later progress of AP, although it seems to act as a temporary therapeutic agent during the early stage of the disease in moderate form. Taking into consideration both the giving time and dose, Me_2Spm is helpful in ameliorating pancreatic injury only when sufficient Me_2Spm accumulates in pancreas within 24 h.

6 The value of polyamine measurements in human acute pancreatitis (Study IV)

It was found in the clinical investigation that patients with pancreatic necrosis were associated with high level of putrescine in RBC. The patients with necrotic AP had high levels of putrescine, which increased 156% compared to the patients with non-necrotic AP. The severe damage shown as necrosis could be associated with extensive activation of polyamine catabolism. Putrescine is an organic chemical compound related to cadaverine and is produced by the breakdown of amino acids in living and dead organisms (Lewis 1998). It is the terminal product in the chain of polyamine catabolism; necrotic tissue may have been catabolised to produce huge amounts of putrescine. In Study I it was found that pancreatic necrosis score correlated positively with blood putrescine level in wild-type experimental AP models. Conversely, the heavy accumulation of pancreatic putrescine with severe depletion of spermidine and spermine can induce pancreatic necrosis in transgenic AP model (Alhonen et al. 2000, Hyvönen et al. 2006). Thus, the accumulated putrescine may be gradually and persistently released from the pancreatic necrotic site into circulation when necrosis started to take place.

In our observation, the AP patients with CRP $\geq$ 250 mg/l had a significantly higher level of spermine than the AP patients with CRP < 250 mg/l did, so that the former had a 56% increase in blood spermine compared to the latter. According to the results, CRP, as an acute-phase protein

response to infection, inflammation, trauma or stress (Vermeire et al. 2005), may have an association with blood polyamine in AP patients. An earlier study showed that the levels of urinary polyamines in patients with rheumatoid arthritis correlated well with the concentrations of serum CRP (Furumitsu et al. 1993). This may partly explain the association between spermine and CRP, further, this also supports our current speculation that polyamines are non-specific markers for AP.

Polyamines are involved in the differentiation of immune cells and the regulation of the inflammatory response. Spermine can be released by damaged or killed cells and benefit cell migration and growth (Moinard et al. 2005). We found in AP patients that IL-6, IL-10, or CRP showed a positive correlation with blood putrescine levels or spermine levels. Additionally, in transgenic AP it was found that the levels of serum IL-6 and IL-10 increased along with course of the early stage of the disease (Merentie et al. 2007). Moreover, IL-6 level had a similar trend of fluctuation with the accumulation of pancreatic putrescine at 6 h and 24 h (Merentie et al. 2007). Therefore, the inflammatory mediators, like cytokines, CRP and polyamines concur in the development of AP.

Nowadays, the system and technique of HPLC have been improved and the determination is faster and more sensitive to obtain a better resolution (Teti et al. 2002). For detecting tissue polyamine levels, generally, it is difficult to collect pancreatic samples by biopsy in clinical practice. Instead, the fluid most frequently employed for polyamine analysis is blood. Taking blood samples and measuring the RBC polyamine levels would be a promising clinical routine. The HPLC assay of polyamines involves the following steps: collection of samples, concentration of samples, hydrochloric acid hydrolysis, filtration, extraction, evaporation, and solubilisation and finally HPLC chromatography. However, the method is not simple but complicated and time-consuming, and also not easily applicable to large numbers of samples. The lack of standardization of HPLC makes it

difficult to transfer it from the research field to the standard clinical laboratory (Gugliucci 2004). Modification might be needed to make the assay simpler and easier for emergency use in clinical setting.

SUMMARY AND CONCLUSIONS

In these studies infusion of taurodeoxycholate with two concentrations was used to induce AP of two severities in wild-type rats. SSAT over-induction was induced by injection of zinc sulphate using transgenic rats, resulting in AP. These different animal models were used to investigate polyamine catabolism during AP. The role of polyamine depletion was studied with an intervention of a polyamine analogue Me_2Spm. Furthermore, the changes in RBC polyamine levels were investigated in both experimental and in clinical settings.

The major findings and conclusions of the present dissertation are:

1. Polyamine catabolism is activated in the pancreas within the first 24 hours and RBC polyamine levels are associated with the severity in taurodeoxycholate-induced AP models. The extent of activation of polyamine catabolism, which is much less than in the SSAT over-induced transgenic model, is related to the extent of pancreatic necrosis.

2. Both polyamine catabolism and trypsin activation take place in the initial stage of experimental AP. Contrary to the transgenic AP model, polyamine catabolism is not a mediator of trypsin activation in taurodeoxycholate-induced AP. The timing of the two events may depend on the mechanisms of the different AP models.

3. Polyamine analogue Me_2Spm can ameliorate pancreatic injury in the early stage of experimental AP supporting the suggestion of the role of polyamine catabolism in the evolution of pancreatic damage. The protective effect of Me_2Spm is absent in the later phase and is not mediated by suppressing trypsin activation. This observation demonstrates that

Me_2Spm might ameliorate pancreatic injury via other mechanisms. Unfortunately, the drug may be toxic in large doses, suggesting that repeated doses are not beneficial to the pancreas.

4. RBC polyamine levels are not related to the aetiology of human AP. However, the few patients with pancreatic necrosis had the highest levels of RBC putrescine. The values of RBC polyamines are significantly correlated to those of inflammatory mediators during hospitalization. These findings were consistent with what was found in experimental AP, suggesting similar changes of polyamine catabolism in accordance with severity of human AP as evaluated by presence of pancreatic necrosis.

ACKNOWLEDGEMENTS

This study was carried out at the Department of Gastroenterology and Alimentary Tract Surgery in Tampere University Hospital and at the Faculty of Medicine, University of Tampere during the years 2004-2009.

I wish to express my sincere gratitude to Professor **Teuvo L.J. Tammela** M.D. and Professor Emeritus **Markku Järvinen** M.D. for offering me the opportunity to carry out this study, for supporting my work and for providing excellent working facilities in the faculty and the hospital.

My deepest gratitude is due to my supervisor, Docent **Isto Nordback** M.D., Director of Development, Pirkanmaa Hospital District, who suggested the present topic and introduced me to the challenging field of acute pancreatitis. I fully appreciate his sincere devotion and that he worked hard on every single detail of this study, ranging from opening a rough idea to setting the final publications, by donating huge amounts of time in his office, and in lab and group meetings. His tireless personal guidance, wide knowledge and constant support were extremely important and essential for me to finalize this dissertation.

I am deeply grateful to Docents **Juhani Sand** M.D. and **Sari Räty** M.D. as the official follow-up members of the present study. Their dedicated guidance and expert knowledge, including producing the protocol, commenting on the manuscripts and offering help to solve all kinds of problems in practice, made it possible for me to conduct this study and publish these articles.

It is an honour for me to have Docent **Marja-Leena Kylänpää** M.D. and Professor **Matti Eskelinen** M.D. as the reviewers of the dissertation. They both spent a lot of time and energy on reading the manuscript, reviewing literature and made valuable and constructive criticism which was vital to improve the quality of this dissertation. I express my warmest thanks for their contributions.

I am truly thankful for Professor **Leena Alhonen,** as she opened a totally new way of studying acute pancreatitis. Her broad understanding the world of polyamine, profound knowledge of molecular biology and practical laboratory assistance were crucial for me to set the experimental design and to process all publications of this study.

To **Teemu Lämsä** M.D. and **Panu H. Nordback** B.M., I would like to express my profound thanks as it would have been impossible to conduct this study without either of them. Teemu gave me the first impression of Finland by introducing me to the Finnish medical education system and national history. Panu took part as an active partner and never refused any of my requests for help. The whole laboratory work in Tampere was processed by our six hands, at the same time the partnership between us extended and became the friendship that was one of the best parts of my personal memories during the 5 years.

Professor **Karl-Heinz Herzig** M.D. is sincerely acknowledged for his great contribution to this study as co-authorship of the 3 of 4 original articles. His comments added a lot of valuable credit to the present study.

I had gained a lot of technical support from those nice and lovely people in the University of Tampere, including **Pekka Vilja**, **Leena Honkaniemi**, **Pirkko Rajala**, **Marja-Leena Koskinen** and **Sami Oikarinen**. I wish to thank all of them for the huge and constant help.

I am deeply grateful to these four members of the Medical School, **Tuija Hartin**, **Anne Hartin-Gathuo**, **Sanna Lehti** and **Tarja Tulonen**, who kindly provided me with all the help as I asked for. And they were always patient and kind to me. I could not forget that at several winter nights they even drove far away from home to give me a hand in the lab.

I am very thankful to **Marja-Leena Haukkavaara** and **Tarja Tuomisaari** for practical assistance at the Department of Gastroenterology and Alimentary Tract Surgery.

It would have been difficult for me to turn the whole idea into reality without the personnel in the A.I. Virtanen Institute for Molecular Sciences, University of Kuopio, including **Mervi T. Hyvönen**, **Mari Merentie**, **Riitta Sinervirta**, **Sisko Juutinen** and **Tuula Reponen**. I am truly thankful for their technical help and support.

My deep thanks go to **Minna Minkkinen** M.D., **Satu Järvinen**, **Annamari Aitolahti** and **Tuula Kuningas** for helping me in investigating patients, collecting clinical data and arranging the sampling.

I would like to express my truthful gratitude to **Teemu Honkanen**, Professor **Immo Rantala** and Professor **Heikki Kainulainen**, for guiding me in scoring histological slides and helping me to handle experimental models.

I wish to express my great thanks to **Alex R. Khomutov** and **Nikolay Grigorenko** of the Engelhardt Institute of Molecular Biology, Russian Academy of Sciences for supplying bismethylspermine.

I would like to express my warm thanks to **Eila Kaliste** of the Social and Health Care Department, the State Provincial Office of Southern Finland, for helping me to translate the application into Finnish to obtain the permission from the ethics committee.

I would like to express my sincere thankfulness to **Arja Vallivaara**, the Finnish Red Cross and **Heini Huhtala**, Tampere School of Public Health, for collecting samples and assisting statistics.

I express my true gratitude to **Maarit Mäki** for handling a lot of paperwork, arranging appointments and conference trips during my study. Great thanks go to **Elina Ranta** for her assistance at the later phase.

The special thanks belong to **Riitta Lappalainen-Lehto**, who gave me my first introduction to the working environment and helped me solve several problems in the clinical study.

I am very thankful to **Sisko Kammonen**, **Raila Melin**, **Minna Hämäläinen** and **Paula Nissilä,** who gave me a lot of help in literature searches in the library of the Medical School.

I am deeply grateful to **Virginia Mattila** for her wonderful linguistic assistance in this dissertation and several original publications.

This warm gratitude belongs to **Taina Ahlgren**, **Leena Nikkari**, **Sari Orhanen** and **Hanna Saressalo** who gave me a lot of helpful instructions about the registration of Ph.D. study, the clinical study application and regulations on Ph.D. dissertations.

I would like to thank all the members of Tampere Pancreas Team for your cooperation and support for my research.

I wish to express the great thanks to my sister **Hai-Ling Jin** and her husband **Peng-Cheng Lu** from the bottom of my heart, for their encouragement during the 5 years and for sharing a lot of the happiness of their family with me.

This profound gratitude belongs to my father **Yao-Zu Jin** and mother **Dai-Fen Yu** for their consideration and support for what I choose to do. I greatly respect my father for his attitude to suffering from the cancer and taking a positive view of every thing. I do appreciate the kindness of my mother as she always tries to comfort me in phone calls far away from Finland.

My son **Toni,** you are my most precious treasure. What you bring me every day is the endless happiness and the pride of being a father. For me, you are another dissertation which is more challenging, but I will write it by heart all my whole life.

Finally, I want to show the unique, faithful and truthful thanks to my lovely and beautiful wife **Ying**, for your emotional, intellectual and domestic support during the years of my research. Sometimes we had arguments when you rarely agreed with me, but I truly admire and respect your independent way of thinking. The agreement or disagreement between us just brings you and me closer to each other, and what you have never failed to give me during these years is one thing, your love. **Ying**, please accept this dissertation as a gift of my love.

This work was supported by a grant from Tampere University Hospital Research Fund and Competitive Research Funding of Pirkanmaa Hospital District. The copyright permissions of the 4 original full-text articles were kindly granted for this dissertation by publisher S. Karger AG, Elsevier and Taylor & Francis of Informa.

Tampere, September 2009

Hai-Tao Jin

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ORIGINAL COMMUNICATIONS

Original Paper

Pancreatology

Pancreatology 2008;8:15–24
DOI: 10.1159/000114851

Received: November 7, 2006
Accepted after revision: June 6, 2007
Published online: January 31, 2008

Polyamine Levels in the Pancreas and the Blood Change according to the Severity of Pancreatitis

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Key Words
Pancreatitis · Polyamines · Spermidine · Spermine · Putrescine · Spermidine/spermine N^1-acetyltransferase

Abstract
Background/Aims: Polyamines are essential to survival, growth, and proliferation of mammalian cells. Previous studies have suggested that the pancreatic polyamine levels may change in acute pancreatitis. In this study, the changes of polyamine levels in the pancreas have been studied with respect to the severity of pancreatitis. We investigated whether there is a relationship in polyamine levels between pancreas and blood, and whether pancreatic and blood polyamine levels change according to the severity of pancreatitis. ***Methods:*** In rats, sublethal pancreatitis was induced by intraductal infusion of 2% taurodeoxycholate, while lethal pancreatitis was induced with 6% taurodeoxycholate. ***Results:*** Infusion of 6% taurodeoxycholate as compared with 2% resulted in more severe pancreatitis, as revealed by mortality, histology, and serum amylase activity. Pancreatic spermidine/spermine N^1-acetyltransferase was induced early after pancreatitis and was associated with increased putrescine and decreased spermidine levels. The extent of pancreatic necrosis significantly correlated with the polyamine catabolism indicators pancreatic putrescine/spermidine ratio ($r = 0.29$, $p < 0.01$) and pancreatic putrescine/spermine ratio ($r = 0.32$, $p < 0.01$). The two pancreatic polyamine ratios correlated well also with the red blood cell polyamine ratios ($r = 0.75$ and $r = 0.72$, respectively, both $p < 0.01$). Furthermore, the extent of pancreatic necrosis correlated with red blood cell putrescine/spermidine ($r = 0.32$, $p < 0.01$) and putrescine/spermine ($r = 0.37$, $p < 0.01$) ratios. ***Conclusions:*** Acute experimental pancreatitis is associated with an early pancreatic spermidine/spermine N^1-acetyltransferase induction and consequent changes in polyamine levels in pancreas and red blood cells, depending on the severity of pancreatitis. Because changes in red blood cell spermidine, spermine, and putrescine levels evolve already early during the time course of pancreatitis, and correlate with the extent of pancreatic necrosis, their clinical value as early markers of the severity of acute pancreatitis needs to be further evaluated.

Introduction

Mammalian cell survival and growth are dependent on many substances, of which the importance of the polyamines spermidine, spermine, and putrescine has been recently emphasized [1, 2]. The intracellular levels of

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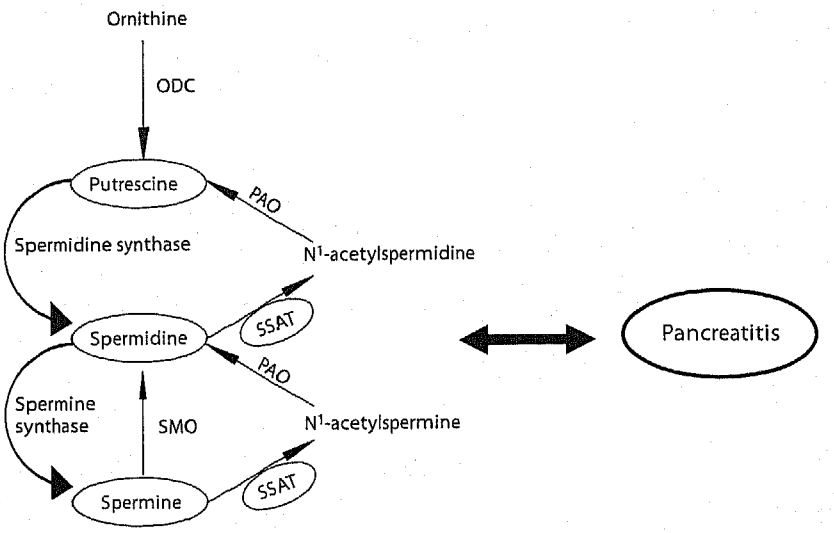

Fig. 1. Pancreatitis is associated with polyamine catabolism. Activation of the polyamine catabolism is known to induce pancreatitis in transgenic animals, and the induction of experimental pancreatitis by other means induces activation of SSAT and disturbs the balance of polyamine pools. ODC = Ornithine decarboxylase; PAO = polyamine oxidase; SMO = spermine oxidase.

these polyamines are tightly maintained by a homeostatic mechanism, by which the spermidine and spermine catabolism to putrescine is regulated by the cytosolic enzyme spermidine/spermine N^1-acetyltransferase (SSAT; fig. 1). Interestingly, the pancreas is the richest source of spermidine in the mammalian body [2, 3].

A transgenic model with SSAT overinduction has recently demonstrated that pancreatic cell survival is seriously threatened, when the spermidine level decreases to around 10% of normal [4]. Furthermore, this polyamine depletion achieved in the transgenic model is followed by acute pancreatitis [4–7]. Interestingly, it has also been shown that some other experimental models of acute pancreatitis may be associated with changes in the pancreatic polyamine levels. These models include acute pancreatitis induced by exogenous cerulein [8] or L-arginine [8] or infusion of 2% sodium taurodeoxycholate into the pancreatic duct [9]. The pancreatic polyamine changes have never been compared according to the severity of experimental pancreatitis.

It has been suggested previously [10, 11] that the blood polyamine levels decrease after injury such as surgery or radiation, but the blood polyamine levels have never been studied in acute pancreatitis. These findings led us to hypothesize that the blood polyamine levels may decrease in pancreatitis as well and might be an indicator for the severity of the injury.

The present study was designed to investigate whether there is a relationship between the polyamine levels in the pancreas and those in the blood and whether changes in polyamine levels in pancreas and blood are associated with the severity of pancreatitis using the well-established animal models of sublethal and lethal taurodeoxycholate-induced pancreatitis.

Materials and Methods

Animals and Surgical Procedures

The study was approved by the Institutional Animal Care and Use Committee of the Tampere University and the Provincial Government. The experiments were performed in accordance with the *Guidelines for the Care and Use of Laboratory Animals* [NIH publication No. 86-23, revised 1985]. Adult male Sprague-Dawley rats (weighing 300–370 g) were fed with standard chow until 12 h before the experiment, after which the animals were fasted until the end of the experiment. The rats were anesthetized by intraperitoneal injection of pentobarbital (60 mg/kg; Orion, Espoo, Finland). Sodium taurodeoxycholate (2%) has been previously used to induce sublethal pancreatitis to allow experiments exceeding 1 day [12]. We used two models of acute pancreatitis with different severity by intraductal administration of 0.2 ml phosphate-buffered saline (pH 7.4) containing either 2 or 6% sodium taurodeoxycholate (Sigma-Aldrich Chemie, Buchs, Switzerland). The drug was infused at a pressure of 30 cm H_2O, after cannulating the biliopancreatic duct through the duodenum with a thin polyethylene tubing (inner diameter 0.28 mm, outer diameter 0.61 mm; Sims Portex, UK) and temporary clamping of the duct at the liver hilum.

Experimental Design

The animals were randomly divided into the following four groups: (1) Normal controls (n = 6): These rats served as controls and were just anesthetized for sampling. (2) Sham operation (n = 30, 6 rats for each time point): These animals were managed to

undergo sham operation without any intraductal infusion of sodium taurodeoxycholate. They were anesthetized a second time for samplings at 1.5, 3, 6, 24, or 48 h and were euthanized after the sampling procedures. (3) Sublethal pancreatitis (n = 30, 6 rats for each time point): These rats were infused with 2% sodium taurodeoxycholate intraductally, as described above, to induce pancreatitis. They were anesthetized a second time for samplings at 1.5, 3, 6, 24, or 48 h and were euthanized after the sampling procedures. (4) Lethal pancreatitis (n = 24, 6 surviving rats for each time point): These animals were infused with 6% sodium taurodeoxycholate intraductally to induce more severe pancreatitis. They were anesthetized a second time for samplings at 1.5, 3, 6, and 24 h and were euthanized after the sampling procedures – originally 18 rats were needed to get 6 survivors at 24 h. After 48 h of follow-up, there were no survivors, that is why no results could be obtained for this time point.

Pancreatitis Assessment

Pancreas and blood specimens were taken under general anesthesia. For measuring pancreatic edema, part of the pancreas was excised and weighed before and after dehydrating at 110°C for 24 h. For measuring the serum amylase activity, blood samples were collected and centrifuged at room temperature for 10 min at 2,500 rpm, and the separated serum was stored at –20°C for later determinations. The serum amylase activity was measured according to the IFCC (International Federation of Clinical Chemistry and Laboratory Medicine) method for α-amylase [13].

Polyamine Analysis

Excised pancreatic tissue was frozen in liquid nitrogen and stored at –70°C for later SSAT and polyamine determinations. For assessing the blood polyamine concentrations, blood samples were collected in tubes with sodium citrate solution and centrifuged at 1,200 *g* for 10 min at 4°C. The red blood cells were washed three times in 9 g/l NaCl to remove white blood cells and stored at –70°C [14, 15]. SSAT activity and natural polyamines (spermidine, spermine, and putrescine) were determined using high-performance liquid chromatography according to the method of Hyvönen et al. [8, 16].

Histological Examination

For histology, the pancreatic specimens were fixed at room temperature in a pH-neutral, phosphate-buffered 10% formalin solution. The fixed tissue was embedded in paraffin, sectioned at 5 μm, stained with hematoxylin and eosin, and coded for blinded examination. The severity of pancreatitis was graded by using the scoring criteria described by Schmidt et al. [17]. Hemorrhages were scored from low-power fields and acinar necroses and inflammation from high-power fields. The scores ranged from 0 to 4.

Statistics

Data are expressed as mean ± SE and were analyzed by using an independent Student t test, when distributed normally. Histological data are expressed as median (minimum–maximum) and were compared using the Mann-Whitney U test. The correlations were assessed by SPSS version 11.0 software (SPSS, Chicago, Ill., USA). The level of significance was set at $p < 0.05$.

Results

Severity of Pancreatitis

Sham operation did not result in changes in histology or serum amylase activity (table 1, fig. 2). Infusion of sodium taurodeoxycholate into the pancreatic duct induced acute pancreatitis when administered at either 2 or 6% (table 1, fig. 2). The pancreatitis was more severe in the 6% group, as revealed by mortality, histology, and amylase activity, but the water content of the pancreas did not differ significantly between the two groups. All animals in the sublethal pancreatitis groups survived throughout the experiment, whereas in the lethal pancreatitis groups only one third of the rats survived for 24 h, and none survived to obtain 48-hour samples.

Pancreatic Polyamines

Both sublethal and lethal pancreatitides were associated with a six- to tenfold increase in pancreatic SSAT activity. The SSAT induction took place slightly earlier in the sublethal group than in the lethal group (fig. 3a).

The pancreatic spermidine concentration declined from the baseline level in both sublethal and lethal pancreatitis groups, without difference between the groups (fig. 3b). The pancreatic spermidine levels decreased by 64 and 44% at 24 h in the sublethal and in the lethal pancreatitis group, respectively, whereas activation of the polyamine catabolism was not reflected in the spermine levels, as the changes of the pancreatic spermine concentrations were minimal at all time points studied (data not shown). The pancreatic putrescine level in the sublethal pancreatitis group was significantly higher than that in the sham-operated group throughout the 24-hour observation period (fig. 3c). In the lethal pancreatitis group, the pancreatic putrescine level started to increase later than in the sublethal pancreatitis group and reached its highest level not before 24 h after the induction of pancreatitis (fig. 3c).

There was a significant correlation between the extent of pancreatic necrosis, as evaluated by histology, and the pancreatic putrescine levels ($r = 0.34$, $p < 0.01$), but not the spermine or spermidine levels, when taking into account the entire 48-hour time course (fig. 3d). The best polyamine catabolism indicators, the ratio of putrescine to spermidine or that of putrescine to spermine, correlated well with the extent of pancreatic necrosis ($r = 0.29$ and $r = 0.32$, respectively, $p < 0.01$; fig. 3e, f).

Blood Polyamines

There was no measurable SSAT activity in the red blood cells. In normal control rats, the blood polyamine

Table 1. Histological scoring for pancreas

	Time, h	Hemorrhage	Inflammation	Necrosis
Control	0	0 (0–0)	0 (0–0)	0 (0–0)
Sham operation	1.5	0 (0–2)	0.75 (0–1)	0 (0–1)
Sublethal pancreatitis	1.5	1 (0–2)	1 (1–2)[a]	1 (1–2)[b]
Lethal pancreatitis	1.5	4 (3–4)[b, d]	0.75 (0.5–1)[c]	1.25 (0–3)
Sham operation	3	0 (0–0)	0 (0–1)	0 (0–0.5)
Sublethal pancreatitis	3	0 (0–2)	0.5 (0–1)	0 (0–1)
Lethal pancreatitis	3	3.5 (2.5–4)[b, d]	1 (0.5–1.5)[a]	1.25 (0.5–2.5)[b, c]
Sham operation	6	0 (0–0)	1 (0–1)	0 (0–1)
Sublethal pancreatitis	6	0 (0–1)	1 (1–2)	1.5 (0.5–3.5)[b]
Lethal pancreatitis	6	4 (0–4)[b, c]	0.5 (0–1.5)	1.75 (0.5–4)[a]
Sham operation	24	0 (0–2)	0.75 (0–3)	0 (0–1)
Sublethal pancreatitis	24	2.5 (0–3)[a]	2 (1–4)	0.5 (0–3)
Lethal pancreatitis	24	2.5 (0–4)[a]	0.5 (0–3)	2 (0–4)[a]
Sham operation	48	0 (0–1)	0.25 (0–3)	0 (0–2)
Sublethal pancreatitis	48	2 (0–4)[a]	2.25 (0–3)	2.5 (0–4)
Lethal pancreatitis	48	0 survivors	0 survivors	0 survivors

All data are presented as median (minimum–maximum) and were analyzed by Mann-Whitney U test.
[a] $p < 0.05$ versus sham operation; [b] $p < 0.01$ versus sham operation; [c] $p < 0.05$ versus sublethal pancreatitis; [d] $p < 0.01$ versus sublethal pancreatitis.

concentrations were at a level of about 1:1,000 of those in the pancreas. Blood spermidine decreased from baseline by 60% already in the sham-operated rats. This decrease was 74% in the sublethal group (fig. 4a). In the lethal pancreatitis group spermidine decreased by 84%, and more prominently than in the sublethal group at most of the time points studied (fig. 4a).

The sham operation likewise induced a minor decrease of 28% in the blood spermine levels (fig. 4b). The blood spermine depletion (37%) was not changed significantly in the sublethal pancreatitis group during the follow-up period. In the group with lethal pancreatitis induced by 6% taurodeoxycholate, a significant decrease (83%) was found in the blood spermine levels (fig. 4b).

The sham operation induced a decrease in the blood putrescine levels from the baseline level. There was a significant increase over the baseline level in blood putrescine 6 and 24 h following exposure to 2% taurodeoxycholate to induce sublethal pancreatitis (fig. 4c). In the lethal pancreatitis group, the blood putrescine levels did not differ from those of the sham-operated group until 24 h, when the still surviving rats showed a remarkable increase in the blood putrescine concentration (fig. 4c).

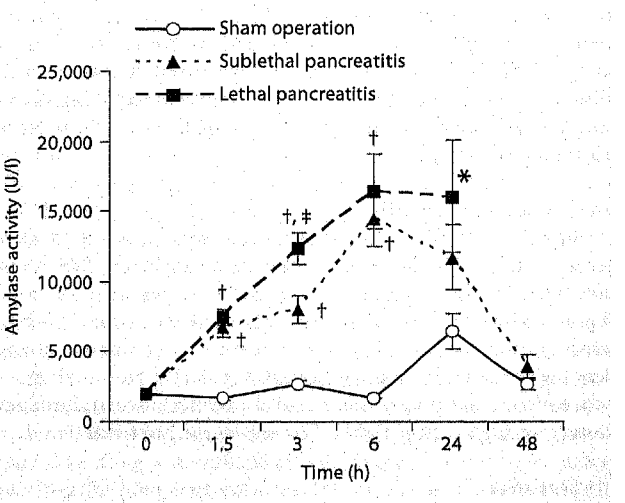

Fig. 2. Serum amylase activity in sublethal and lethal pancreatitis induced by sodium taurodeoxycholate. Data are expressed as mean ± SE. * $p < 0.05$ versus sham operation; † $p < 0.01$ versus sham operation; ‡ $p < 0.05$ versus sublethal pancreatitis.

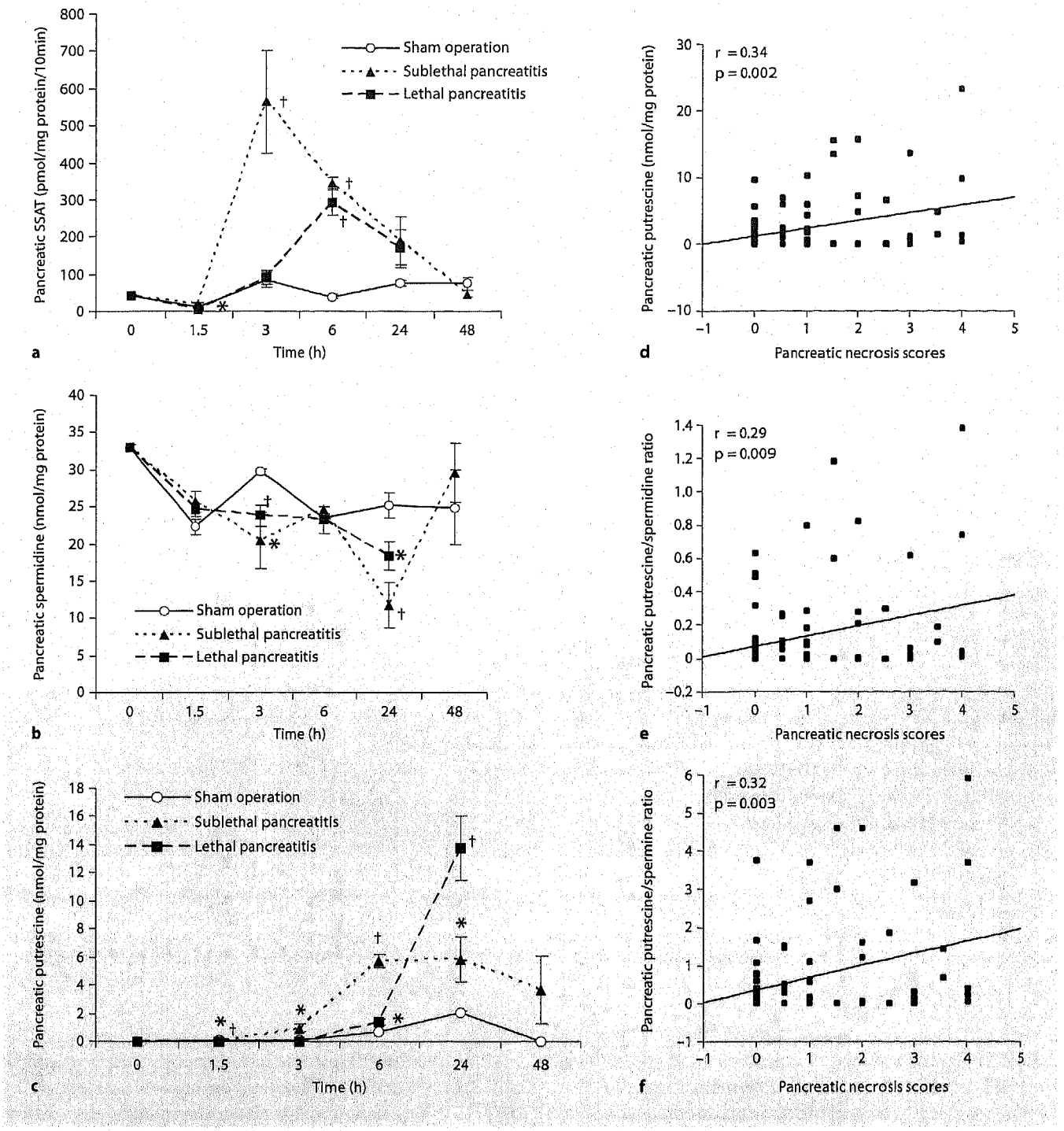

Fig. 3. Change of the pancreatic polyamine catabolism in the sodium taurodeoxycholate pancreatitis model and its correlation with pancreatic necrosis. The pancreatic SSAT activity (**a**) increased and was followed by a decrease of the spermidine level (**b**) and an increase of the putrescine level (**c**). **a–c** The data are expressed as mean ± SE. * $p < 0.05$ versus sham operation; † $p < 0.01$ versus sham operation. Pancreatic putrescine (**d**), putrescine/spermidine ratio (**e**), and putrescine/spermine ratio (**f**) significantly correlated with pancreatic necrosis during 48 h. In calculating the correlations, both 2 and 6% taurodeoxycholate experiments were combined.

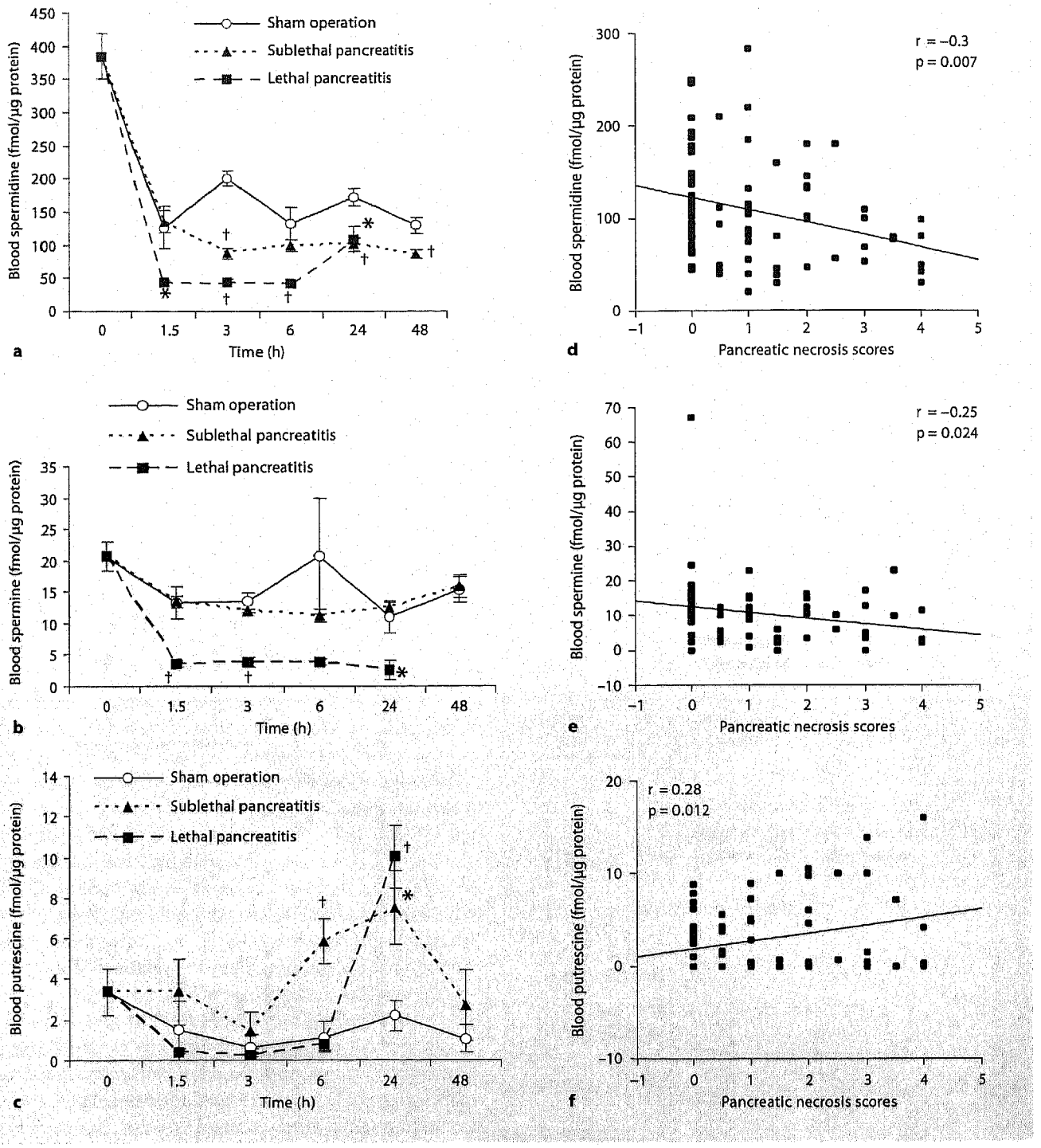

Fig. 4. Change of the blood polyamine catabolism in the sodium taurodeoxycholate pancreatitis models and correlation between blood polyamines and pancreatic necrosis. **a** Blood spermidine decreased by sham operation only, but pancreatitis enhanced this decrease; the more spermidine was depleted, the more severe was the disease. **b** Blood spermine decreased only in the lethal pancreatitis model. **c** Blood putrescine changed less logically. **a–c** Data are expressed as mean ± SE. * $p < 0.05$ versus sham operation; † $p < 0.01$ versus sham operation. Blood spermidine (**d**), spermine (**e**), and putrescine (**f**) significantly correlated with the necrosis of the pancreas during 48 h. In calculating the correlations, both 2 and 6% taurodeoxycholate experiments were combined.

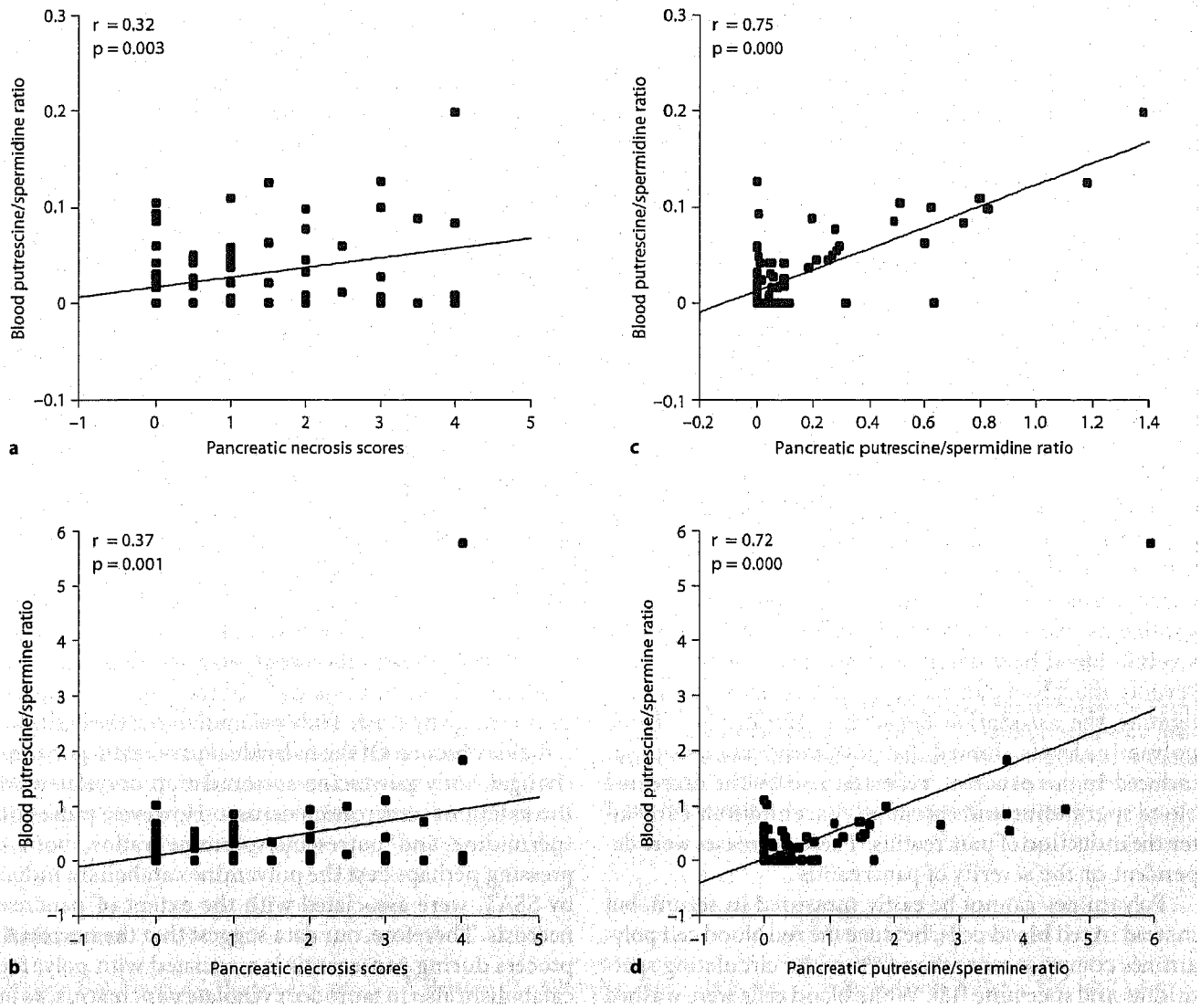

Fig. 5. The blood polyamine ratios correlated with pancreatic necrosis and pancreatic polyamine ratios. Both blood putrescine/spermidine ratio (**a**) and blood putrescine/spermine ratio (**b**) increased with the extent of necrosis of the pancreas; r = 0.32 ($p < 0.01$) and r = 0.37 ($p < 0.01$), respectively. Furthermore, they significantly positively correlated with pancreatic putrescine/spermidine and putrescine/spermine ratios; r = 0.75 ($p < 0.01$) and r = 0.72 ($p < 0.01$), respectively (**c**, **d**).

In the entire experimental setting, the concentrations of putrescine in blood correlated significantly with the putrescine concentrations in the pancreas (r = 0.72, $p < 0.01$). Blood spermidine (r = −0.3, $p < 0.01$), spermine (r = −0.25, $p < 0.05$), and putrescine (r = 0.28, $p < 0.05$) correlated with the extent of pancreatic necrosis, as evaluated by histology (fig. 4d–f). Furthermore, the putrescine/spermidine and the putrescine/spermine ratios correlated significantly with the pancreatic necrosis during the entire 48-hour evolution of pancreatitis (r = 0.32 and r = 0.37, respectively, both $p < 0.01$; fig. 5a, b). The best polyamine catabolism indicators, putrescine/spermidine ratio or putrescine/spermine ratio (fig. 5c, d), both correlated well between pancreas and blood (r = 0.75 and r = 0.72, respectively, both $p < 0.01$).

Discussion

The association between acute pancreatitis and pancreatic polyamine depletion has been recently described [4–9]. There are some data to support the hypothesis that polyamine depletion mediates, at least in part, the injury in some experimental settings [4–9]. These include the fact that the histological changes of injury may be ameliorated by substitution with polyamine analogues [4, 8]. The possible changes of the polyamine levels according to the severity of pancreatitis have not been previously studied in a single experimental model. It is difficult to compare pancreatitis models of various severities, because these models may be totally different referring to the induction mechanism which might have an influence on the results. Therefore, we chose a model with two severity modifications, one described earlier to be sublethal [12] and the other more severe, resulting in death between 12 and 48 h after pancreatitis induction. We needed 18 animals at 24 h to obtain data from 6 surviving rats, while 48 h after induction of lethal pancreatitis it was impossible to obtain specimens due to the lack of survivors. The polyamine levels in blood have never been studied before in various severity models of pancreatitis. The current study, investigating the association between pancreatic and blood polyamine levels, showed that polyamine catabolism was induced in the pancreas, reflected also by the decreased blood spermidine and spermine concentrations early after the induction of pancreatitis. These decreases were dependent on the severity of pancreatitis.

Polyamines cannot be easily measured in serum, but instead in red blood cells, because the red blood cell polyamines comprise more than 95% of the circulating spermidine and spermine [18]. White blood cells were washed out to rule out any changes according to white blood cell accumulation or activation, as described before [15, 18, 19].

In blood, both spermidine and spermine levels were the lowest in the lethal pancreatitis model. On the contrary, blood putrescine changes did not follow the sequence of the clinical severity of pancreatitis (mortality). The red blood cells did not contain measurable SSAT, which has been considered the rate-controlling enzyme, metabolizing spermine via N^1-acetylspermine to spermidine and spermidine via N^1-acetylspermidine to putrescine (fig. 1). However, remarkable decreases were found in both blood spermidine and spermine levels in our pancreatitis models. This suggests that the polyamine changes are not due to the metabolism in the blood, but rather are a reflection of what is happening elsewhere in the body. The blood polyamine pool is quite small. The concentrations were about 1,000-fold less than observed in the pancreas. Therefore, even minor changes might be reflected in the blood polyamines.

Another confounding effect might be the mortality in the lethal pancreatitis model. Only one third of the rats survived until 24 h, and none survived at 48 h, which is why the data are lacking at 48 h. The data at 24 h represent only the survivors that might, in fact, have even milder pancreatitis than those who already died. Therefore, this might be a selection bias after the first few hours, when the disease becomes lethal. The fact that the blood polyamine levels clearly differed between the two severity groups only at the very early time frame might be problematic in a clinical situation, because only few patients with pancreatitis are detected that early.

As previously described [9, 20], there was induction of SSAT and the following spermidine reduction in the pancreas early after the induction of pancreatitis. The spermidine decrease did not vary between the two models. SSAT induction and the increase in pancreatic putrescine levels were slightly delayed in the lethal pancreatitis model as compared with the sublethal model. Also, there was some inconsistency, especially in the pancreatic spermidine change by time. The explanation for these findings remains obscure. Of the individual pancreatic polyamine changes, only putrescine accumulation correlated with the extent of pancreatic necrosis. However, putrescine/spermidine and putrescine/spermine ratios, both expressing perhaps best the polyamine catabolism induced by SSAT, were associated with the extent of pancreatic necrosis. Therefore, our data suggest that the necrotizing process during pancreatitis is associated with polyamine catabolism also in taurodeoxycholate pancreatitis, as previously suggested for L-arginine pancreatitis and transgenic pancreatitis [5, 8].

During the entire experimental course, pancreatic putrescine correlated with the blood putrescine, even when the two accumulated with different kinetics after the induction of pancreatitis. Furthermore, the polyamine catabolism in the pancreas, as expressed by the putrescine/spermidine or putrescine/spermine ratios, correlated with the respective ratios in blood. Severe pancreatitis induces changes also in many other organs besides the pancreas [12, 21, 22]. The clinical severity of pancreatitis is dependent on remote organ failure. The functions of liver, kidneys, and the cardiovascular system are independent early prognostic indicators of pancreatitis [23–26]. In this study, we did not measure the polyamine concentrations elsewhere in the body. Even when the indica-

tors of the polyamine catabolism correlated between pancreas and blood, it might be suggested, however, that blood polyamines are not simply a reflection of only pancreatic polyamines, but rather the reflection of the pool of polyamines in all organs rich of these compounds. These include brain, liver, skin, and reproductive organs [2], of which at least the liver is associated with the clinical severity of pancreatitis.

Independent of the mechanisms involved, it was interesting to find early after the induction of pancreatitis the concentrations of red blood cell spermidine and spermine to be very low and the concentration of putrescine higher in the more severe form of pancreatitis than in the less severe form. During the entire experimental course, both pancreatic and blood polyamine catabolism indicators correlated with the extent of pancreatic necrosis. To apply any data for routine requires, however, the development of new and faster assay methods. Such requirements would not be urgent, if animal experiments and pilot clinical experiments were not encouraging. The current experimental findings suggest that polyamines in the blood need to be studied also in human pancreatitis.

The correlation between pancreatic necrosis and blood polyamine levels was significant, but not very strong. This might again be explained by the fact that most of necrosis and polyamine data came from the sublethal pancreatitis group, since few rats only survived for 24 h in the more severe pancreatitis group, which might result from some selection bias. Secondly, the necrosis difference between sublethal and lethal pancreatitis was not significant during the first 6 h, when the polyamine levels differed most. Therefore, necrosis might not be an ideal indicator to evaluate the severity of pancreatitis. This is in accordance with the recent understanding of clinical acute pancreatitis.

In conclusion, acute pancreatitis is associated with pancreatic SSAT induction and the consequent changes in polyamine levels in pancreas and blood. Significant correlations between pancreatic polyamines and blood polyamines show that blood polyamine levels may indicate changes of pancreatic polyamines during pancreatitis. Because the blood polyamine levels change early, especially spermidine and spermine levels, which are dependent on the severity of the acute experimental pancreatitis and correlate with the extent of pancreatic necrosis, their value as clinical markers of the severity of acute pancreatitis needs to be further evaluated.

Acknowledgments

This work was supported by a grant from the Tampere University Hospital. Equipment and drugs were provided by the Tampere University Hospital and the A.I. Virtanen Institute for Molecular Sciences, University of Kuopio. We are grateful to Panu Nordback, Pirkko Rajala, Riitta Sinervirta, Sisko Juutinen, and Tuula Reponen for their skillful technical assistance.

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A polyamine analog bismethylspermine ameliorates severe pancreatitis induced by intraductal infusion of taurodeoxycholate

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Background. *Stable polyamine homeostasis is important for cell survival and regeneration. Our experimental studies have shown that catabolism of spermidine and spermine to putrescine is associated with the development of pancreatitis. We investigated the pathogenetic role of polyamine catabolism by studying the effect of a methylated polyamine analog on taurodeoxycholate-induced acute experimental pancreatitis.*

Methods. *Acute pancreatitis was induced by infusion of sodium taurodeoxycholate (2%) into the pancreatic duct. Bismethylspermine (Me_2Spm) was administered as a pretreatment before the induction of pancreatitis or as a treatment after the induction of pancreatitis. The sham operation included laparotomy only. Pancreas tissue and blood were sampled at 24 h and 72 h after the infusion of taurodeoxycholate and studied for pancreatitis severity (serum amylase activity, pancreatic water content, and histology) and polyamine catabolism, which includes spermidine/spermine N^1-acetyltransferase (SSAT) activity as well as spermidine, spermine, and putrescine concentrations in the pancreas.*

Results. *Sodium taurodeoxycholate-induced acute pancreatitis manifests as increases in serum amylase and pancreatic water content, leukocytosis, and acinar cell necrosis in the pancreas. The activity of SSAT increased significantly together with an increase in the ratios of pancreatic putrescine/spermidine and putrescine/spermine at 24 h, which indicates SSAT-induced polyamine catabolism. Pancreatic water content and necrosis were reduced significantly by the treatment with Me_2Spm at 24 h but not at 72 h when the polyamine homeostasis had recovered, and the pancreatitis had progressed.*

Conclusions. *Taurodeoxycholate-induced acute pancreatitis was associated with activation of polyamine catabolism in the pancreas. The polyamine analog Me_2Spm ameliorated the injury in the early stage, but it did not ameliorate the late progression of the pancreatic necrosis at 72 h. Thus, besides proteolytic enzyme activation and the cascades of inflammation, polyamine catabolism may be an important pathogenetic mediator of the early stages of acute pancreatitis. (Surgery 2008;144:49-56.)*

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Supported by a grant from Tampere University Hospital, A.I. Virtanen Institute for Molecular Sciences, University of Kuopio, and Engelhardt Institute of Molecular Biology, Russian Academy of Sciences.

Accepted for publication March 30, 2008.

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0039-6060/$ - see front matter

doi:10.1016/j.surg.2008.03.029

NATURAL POLYAMINES PUTRESCINE, SPERMIDINE, AND SPERMINE exist in all mammalian cells. Although the precise molecular mechanism of polyamines is not well known, they are thought to play an important role in cellular growth and proliferation.[1,2] Polyamine homeostasis, as modulated by polyamine biosynthesis and catabolism, is crucial in cellular survival; function and disruption of polyamine homeostasis is deleterious. Severe depletion of spermidine and spermine by overexpression of polyamine-catabolizing enzyme spermidine/spermine N^1-acetyltransferase (SSAT) in transgenic rats can

initiate severe experimental acute pancreatitis.[3-5] Depletion of spermidine and spermine has been reported in L-arginine–induced and cerulein-induced experimental pancreatitis and in the pancreatitis induced by intraductal infusion of taurodeoxycholate.[6,7] In the latter diseases, pancreatic polyamine catabolism is also reflected in the polyamine levels of red blood cells depending on the severity of pancreatitis.[7] All these studies suggest strongly that the disturbances of pancreatic polyamine homeostasis is associated with the development of pancreatitis (Fig 1).

L-Arginine is 1 of the primary precursors of polyamines in eukaryotic cells.[1,2] The mechanism of L-arginine–induced pancreatitis is thought to be different from arginine functions in polyamine metabolism.[8-10] Because of this theoretic connection, L-arginine induction is perhaps not the best model to study the role of polyamines in severe pancreatitis. To avoid any interference with a precursor of polyamines, intraductal infusion of taurodeoxycholate was used as a well-established model of acute necrotizing pancreatitis.

Under the conditions of activated polyamine catabolism, the natural polyamines are acetylated and degraded so rapidly that they cannot serve as polyamine supplements if given exogenously.[6,11] Therefore, the methylated polyamine analogs bismethylspermine (Me_2Spm) and methylspermidine, which are more stable than the respective natural polyamines, offer an option.[6] These substances can ameliorate the histologic injury and the animal mortality in pancreatitis induced by activated polyamine catabolism in transgenic animals and histologic injury in L-arginine–induced pancreatitis in wild-type animals.[6,12] No previous study explores the effect of Me_2Spm on a more severe experimental pancreatitis in wild-type animals, in which no drugs that could possibly interfere directly with the polyamine metabolism are used for the induction of the pancreatitis. The aim of this study was to investigate the role of polyamine catabolism in mediating the injury in acute pancreatitis by studying the polyamine levels in the pancreas and the effects of a long-acting, polyamine analog on pancreatic injury.

MATERIAL AND METHODS

Animals. The current study was approved by the Institutional Animal Care and Use Committee of the University of Tampere and the respective provincial government. The experiments were performed in accordance with the "Guidelines for the Care and Use of Laboratory Animals"

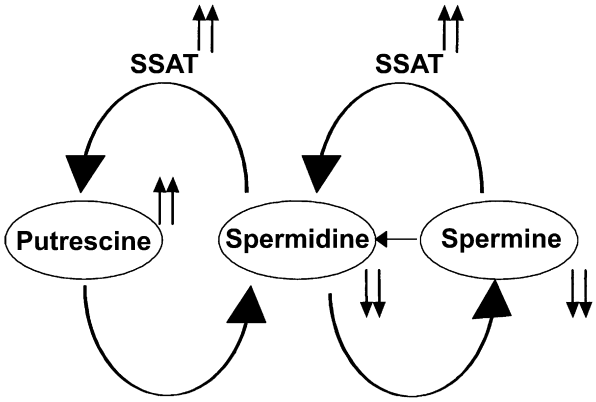

Fig 1. Overactivation of polyamine catabolism. Previous studies have shown that overinduction of SSAT in transgenic rats by overexpressing SSAT gene can induce acute pancreatitis in accompaniment with severe depletion of spermidine and spermine, and accumulation of putrescine. "↑↑," increase remarkably; "↓↓," decrease remarkably.

(NIH publication No.86-23, revised 1985). Adult male Sprague-Dawley rats (270–450 g) were fed with standard chow diet until 12 h before the experiment, and the animals were fasted overnight before the experiment. Rats were anesthetized with an intraperitoneal injection of pentobarbital (Orion, Espoo, Finland, 60 mg/kg) before the operation and for later tissue sampling.

Pancreatitis induction and administration of bismethylspermine. Sodium taurodeoxycholate (2%) (Sigma-Aldrich, Buchs, Switzerland) was used for the induction of acute pancreatitis. Sodium taurodeoxycholate was infused into the pancreatic duct with 0.2-mL phosphate-buffered saline, pH 7.4, as described previously to induce a sublethal, severe necrotizing pancreatitis to follow the evolution of the disease with time.[13] The infusion pressure was kept less than 30-cm H_2O. The common bile duct was clamped temporarily at the liver hilum during pancreatitis induction. Me_2Spm was synthesized as described before[14] and dissolved in 0.9% NaCl solution at 25 mg/mL before use. The administration of Me_2Spm was as follows: The animals received Me_2Spm as a pretreatment intraperitoneally (25 mg/kg) at 20 h and 4 h before the induction of pancreatitis, or the animals received Me_2Spm as a treatment intraperitoneally (25 mg/kg) at 4 h and 8 h after induction of pancreatitis. Additionally, 1 group of rats received an extra dose of Me_2Spm (12.5 mg/kg) as treatment at 30 h after induction of pancreatitis.

Experimental design. The animals were divided randomly into 10 groups:

- Control (group 1): Six rats without any manipulation served as the 0-h baseline group.
- Sham operation (groups 2 and 3): Rats received laparotomy only. They were anesthetized and killed for tissue sampling at 24 h (group 2, n = 6) and at 72 h (group 3, n = 6) after the sham operation.
- Acute pancreatitis (groups 4 and 5): Rats were infused with 2% sodium taurodeoxycholate intraductally as described above to induce pancreatitis. They were anesthetized and killed for tissue sampling at 24 h (group 4, n = 12) and at 72 h (group 5, n = 12) after the infusion of taurodeoxycholate.
- Acute pancreatitis, pretreatment with Me_2Spm (groups 6 and 7): Rats were infused with 2% sodium taurodeoxycholate intraductally as in groups 4 and 5 to induce pancreatitis. The animals received Me_2Spm as a pretreatment at 20 h and at 4 h before induction of pancreatitis as described above. They were anesthetized and killed for tissue sampling at 24 h (group 6, n = 6) and at 72 h (group 7, n = 6) after the infusion of taurodeoxycholate.
- Acute pancreatitis, treatment with Me_2Spm (groups 8 and 9): Rats were infused with 2% sodium taurodeoxycholate intraductally as in groups 4 and 5 to induce pancreatitis. The animals received Me_2Spm as a treatment at 4 h and at 8 h after induction of pancreatitis as described above. They were anesthetized and killed for tissue sampling at 24 h (group 8, n = 12) and 72 h (group 9, n = 6) after the infusion of taurodeoxycholate.
- Acute pancreatitis, treatment with extra Me_2Spm (group 10): Rats were infused to induce pancreatitis and administered Me_2Spm as in group 9; in addition, they received an extra dose of Me_2Spm (12.5 mg/kg) at 30 h. Because none of these rats survived to 72 h, no tissue or serum samples were available.

Evaluation of pancreatitis. *Blood amylase activity*: Blood samples were collected and centrifuged at room temperature for 10 min at 2500 rpm, and the separated serum was stored in –20°C for later determinations. The determination of serum amylase activity was performed by the International Federation of Clinical Chemistry and Laboratory Medicine method for α-amylase.[15]

Pancreatic water content: An aliquot of the pancreatic tissue was excised and weighed before and after dehydrating at 110°C for 24 h in an electric oven (TAMRO-APTA 90-544011, Memmert, Germany). Pancreatic water content was expressed by [(wet weight – dry weight) / dry weight].

Pancreatic histology: The pancreatic specimens were fixed at room temperature in a pH-neutral, phosphate-buffered, 10% formalin solution. The fixed tissue was embedded in paraffin, sectioned at 5 μm, stained with hematoxylin and eosin, and coded for blinded examination. The histologic injury of pancreatitis was graded by using modified scoring criteria.[16] The score ranged from 0 to 4.

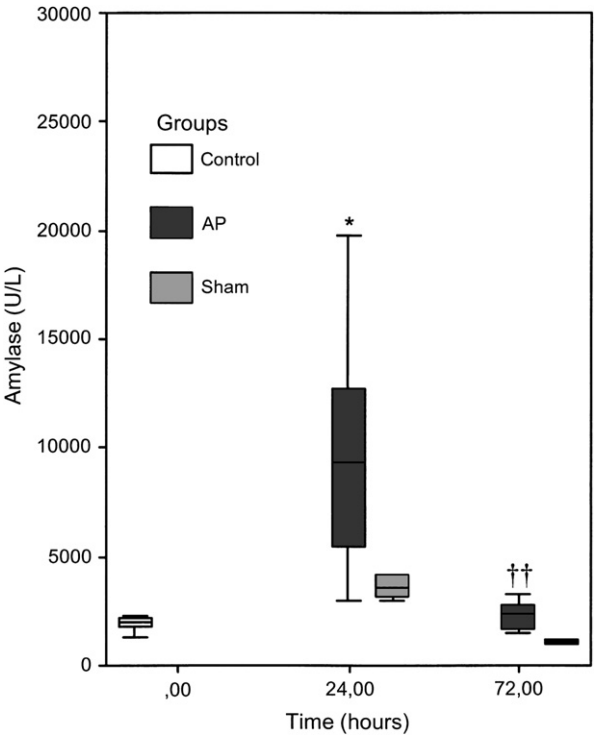

Fig 2. Blood amylase activity during acute pancreatitis. An increase in blood amylase activity is observed especially at 24 h after induction of acute pancreatitis. The column at 0 h serves as normal control. *AP*, acute pancreatitis; *Sham*, sham operation. All data are median [range]. *$P < .05$ vs sham operation at 24 h. ††$P < .01$ vs sham operation at 72 h.

Polyamine analysis. Pancreatic tissue was frozen in liquid nitrogen and stored at –70°C for later SSAT and polyamine determinations. SSAT activity was assayed according to Bernacki et al.[17] The natural polyamines (spermidine, spermine, and putrescine) and the polyamine analog (Me_2Spm) were determined by high-performance liquid chromatography according to the method of Hyvönen et al.[18]

Statistical analysis. All data are expressed as median [range] and compared with the Mann-Whitney *U* test between groups. The level of significance was set at *P* less than .05.

RESULTS

Induction of pancreatitis. Blood amylase activity increased significantly at 24 h and 72 h after induction of pancreatitis compared with the sham operation (Fig 2). Increased pancreatitis edema (Table I, Fig 3) and increasing necrosis

Table I. The effects of pretreatment and treatment with Me_2Spm on the histologic changes in sodium taurodeoxycholate-induced pancreatitis

Groups	*Edema*	*Hemorrhage*	*Inflammation*	*Necrosis*
AP, 24 h (n = 12)	3 [2–4] *	0 [0–4] *	1 [0–4]	3.5 [0.5–4] **
Me_2Spm+AP, 24 h (n = 6)	2.5 [1.5–3]	0 [0–0.5]	1 [0.5–1.5]	2 [0–4]
AP+Me_2Spm, 24 h (n = 8)	2.5 [1–4]	2 [0–4]	0.5 [0–0.5]	2 [0.5–3.5] ‡
Sham, 24 h (n = 6)	1.5 [1–3]	0 [0–0]	0.5 [0–1.5]	0 [0–1]
AP, 72 h (n = 12)	3 [3–4] ††	2 [0–4] †	0.5 [0–2]	4 [0–4] ††
Me_2Spm + AP, 72 h (n = 6)	3 [0.5–3.5]	0 [0–0]	0 [0–1.5]	3 [0–4]
AP + Me_2Spm, 72 h (n = 6)	3 [2–3]	0 [0–0]	1 [0.5–2]	3 [1–4]
Sham, 72 h (n = 6)	1.5 [1.5–3]	0 [0–0]	0.5 [0–2]	0 [0–3]

n, number of samples; *AP*, acute pancreatitis; *Me_2Spm + AP*, pretreatment of Me_2Spm before acute pancreatitis induction; *AP + Me_2Spm*, treatment of Me_2Spm after acute pancreatitis induction; *Sham*, sham operation. All data are median [range].
*$P < .05$ vs sham operation group at 24 h.
**$P < .01$ vs sham operation group at 24 h.
†$P < .05$ vs sham operation group at 72 h.
††$P < .01$ vs sham operation group at 72 h.
‡$P < .05$ vs acute pancreatitis group at 24 h.

were observed in histology, which characterizes the development of pancreatitis (Table I).

Pancreatic polyamine catabolism. SSAT activity in the pancreas was activated (Fig 4). Concentrations of both spermidine and spermine in the pancreas decreased at 24 h, which indicates polyamine catabolism (Fig 5). Simultaneously, pancreatic putrescine increased. Ratios of putrescine/spermidine and putrescine/spermine, which indicate the extent of this catabolism, revealed polyamine catabolism being high at 24 h but returned to baseline level by 72 h (Fig 6).

Effect of Me_2Spm on pancreatitis. Me_2Spm was tested as pretreatment and as treatment for the pancreatitis induced by taurodeoxycholate infusion. The pretreatment did not ameliorate the pancreatitis at 24 h as judged by amylase activity (not shown), pancreatic water content (Fig 3), or histology (Table I). In contrast, the treatment with Me_2Spm after the induction of pancreatitis significantly reduced the pancreatic water content and necrosis at 24 h (Table I and Fig 3) but did not decrease the amylase activity (not shown).

This effect at 24 h was absent at 72 h, when neither the pretreatment nor the treatment with Me_2Spm showed a significant effect on the pancreatic histology (Table I) or water content (Fig 3). Furthermore, the administration of an extra dose of Me_2Spm at 30 h resulted in 100% mortality by 72 h. Therefore, no samples were available in group 10 at 72 h to detect the effect on the pancreas. In the other groups, only occasional deaths had occurred.

Effect of Me_2Spm on pancreatic polyamine catabolism. Me_2Spm accumulated in the pancreas as a result of both pretreatment and treatment, with greater concentrations achieved during the later administration as treatment (Table II). More Me_2Spm was found in pancreatic tissue at 72 h

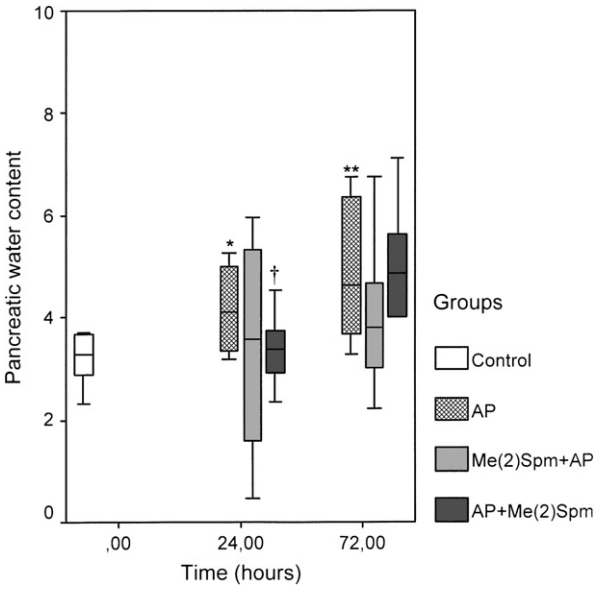

Fig 3. Change of pancreatic water content during acute pancreatitis with pretreatment or treatment of Me_2Spm. Water content = (wet weight − dry weight) / dry weight ratio. Only treatment of Me_2Spm decreased the pancreatic water content at 24 h after the induction of acute pancreatitis. The column at 0 h serves as normal control. Me(2)Spm = Me_2Spm; *AP*, acute pancreatitis; *Me_2Spm + AP*, pretreatment of Me_2Spm before acute pancreatitis induction; *AP + Me_2Spm*, treatment of Me_2Spm after acute pancreatitis induction. All data are median [range]. *$P < .05$ vs control at 0 h. **$P < .01$ vs control at 0 h. †$P < .05$ vs acute pancreatitis at 24 h.

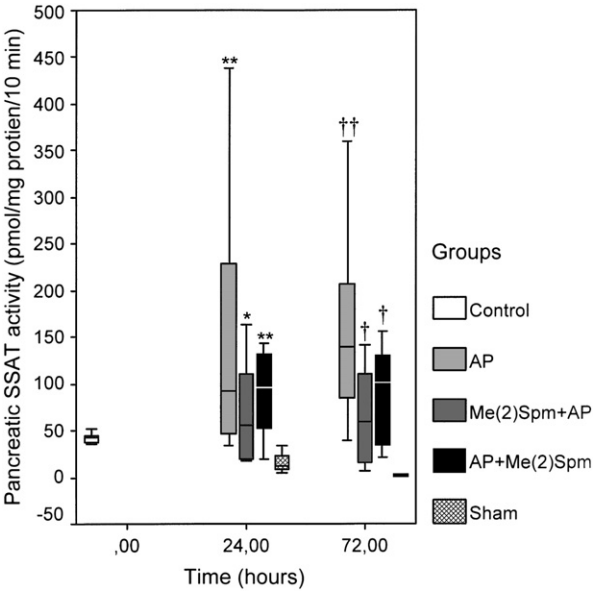

Fig 4. Pancreatic SSAT activity during acute pancreatitis with pretreatment or treatment of Me_2Spm. Pancreatic SSAT increased significantly along with time points after the induction of acute pancreatitis. The column at 0 h serves as normal control. Me(2)Spm = Me_2Spm; *SSAT*, spermidine/spermine N^1-acetyltransferase; *AP*, acute pancreatitis; *Me_2Spm + AP*, pretreatment of Me_2Spm before acute pancreatitis induction; *AP + Me_2Spm*, treatment of Me_2Spm after acute pancreatitis induction; *Sham*, sham operation. All data are median [range]. **P* < .05 vs sham operation at 24 h. ***P* < .01 vs sham operation at 24 h. †*P* < .05 vs sham operation at 72 h. ††*P* < .01 vs sham operation at 72 h.

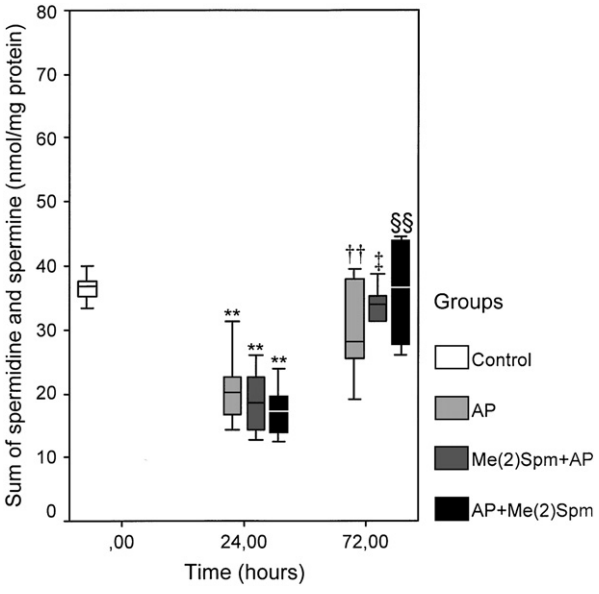

Fig 5. The sum of spermidine plus spermine concentration during acute pancreatitis with pretreatment or treatment of Me_2Spm. Acute pancreatitis led to decrease of spermidine and spermine concentration at 24 h, and it returned to baseline level at 72 h. With pretreatment and treatment of Me_2Spm at 24 h, but not at 72 h, the sum of spermidine plus spermine was significantly less than the sum at baseline. The sum significantly increased at 72 h. The column at 0 h serves as baseline control. Me(2)Spm = Me_2Spm. *AP*, acute pancreatitis; *Me_2Spm + AP*, pretreatment of Me_2Spm before acute pancreatitis induction; *AP + Me_2Spm*, treatment of Me_2Spm after acute pancreatitis induction; All data are median [range]. ***P* < .01 vs baseline, at 0 h. ††*P* < .01 vs AP, at 24 h. ‡*P* < .05 vs Me_2Spm +AP, at 24 h. §§*P* < .01 vs AP+ Me_2Spm, at 24 h.

than at 24 h; in particular, Me_2Spm was given after the induction of pancreatitis (Table II).

Me_2Spm did not increase SSAT activity in the pancreatitis model at 24 h. Both the pretreatment and treatment with Me_2Spm led to an accumulation of putrescine in the pancreas at 24 h, whereas spermidine and spermine levels decreased, which suggests increased catabolism of the natural polyamines in the presence of synthetic polyamine analog (Fig 5). This observation was confirmed by calculating putrescine/spermidine and putrescine/spermine ratios (Fig 6). Previously, it has been suggested that the decrease of spermidine and spermine is important in the deleterious effects of polyamine catabolism.[3] Therefore, the sum of these greater levels of polyamines (spermidine plus spermine) was calculated (Fig 5). We found that in acute pancreatitis, the sum of spermidine plus spermine concentrations decreased to almost half from baseline by 24 h with return to baseline level by 72 h (Fig 5).

DISCUSSION

Polyamines are necessary for normal cellular growth. Physiologically, polyamines are cationic molecules with positive charges that enable polyamines to interact electrostatically with polyanionic macromolecules in cells.[2] Natural polyamines include spermidine, spermine, and putrescine, and putrescine is a diamine precursor of spermidine and spermine.[1,3] Pancreas is the richest source of spermidine in the body.[19] It has been discussed previously that polyamines could prevent membrane structure damage by activated oxygen radicals.[20] In in vivo studies, activation of polyamine catabolism in transgenic animals leads to acute pancreatitis, and depletion of polyamines can be observed in some models of pancreatitis.[3-7,21] Nevertheless, very little is known of the pathogenetic role of polyamines in acute pancreatitis.

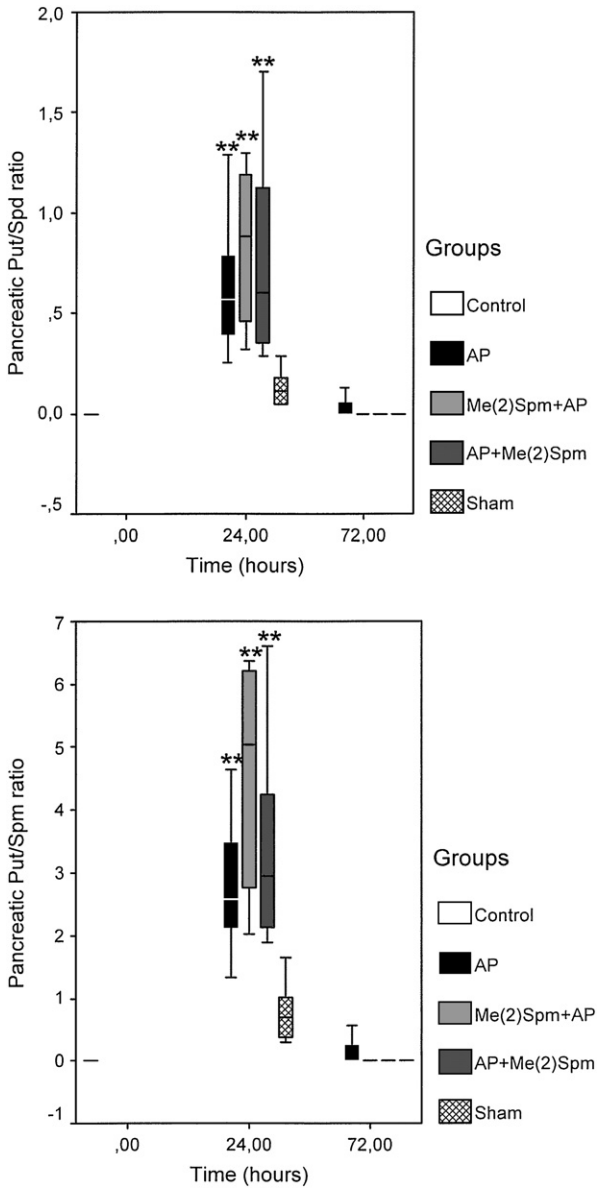

Fig 6. Change of pancreatic polyamine ratios during acute pancreatitis with pretreatment or treatment of Me_2Spm. Both pretreatment and treatment of Me_2Spm can induce the higher put/spd ratio and put/spm ratio in acute pancreatitis model at 24 h. The column at 0 h serves as normal control. Me(2)Spm = Me_2Spm; *AP*, acute pancreatitis; *Me_2Spm + AP*, pretreatment of Me_2Spm before acute pancreatitis induction; *AP + Me_2Spm*, treatment of Me_2Spm after acute pancreatitis induction; *Sham*, sham operation; *put/spd*, putrescine/spermidine; *put/spm*, putrescine/spermine. All data are median [range]. $^{**}P < .01$ vs sham operation at 24 h.

In the current study, we used a sublethal model of severe necrotizing pancreatitis induced by intraductal infusion of taurodeoxycholate.[13] Consistent with the previous findings,[7] we also observed in the current work an activation of SSAT in the pancreas during development of acute pancreatitis. This SSAT induction was accompanied by an increase in the polyamine catabolism, as measured by increased levels of putrescine and decreased levels of spermidine and spermine. These changes were prominent at 24 h but did not persist when measured at 72 h. Simultaneously, the acinar necrotizing process progressed from 24 h to 72 h. This observation suggests that the activation of polyamine catabolism is an early phenomenon during this model of acute pancreatitis.

Table II. Pancreatic concentration of Me_2Spm (nmol/mg protein) with pretreatment and treatment in sodium taurodeoxycholate-induced pancreatitis

	24 h	*72 h*
Me_2Spm + AP	2.52 [1.17–4.71]	4.99 [1.70–7.22]
AP + Me_2Spm	4.15 [2.70–7.05]	10.00 [6.42–15.04]**

AP, acute pancreatitis; *Me_2Spm + AP*, pretreatment of Me_2Spm before acute pancreatitis induction; *AP + Me_2Spm*, treatment of Me_2Spm after acute pancreatitis induction. All data are median [range].
$^{**}P < .01$ vs AP + Me_2Spm at 24 h.

Methyl derivatives of polyamines, such as Me_2Spm, act as substitutes for natural polyamines both in vitro and in vivo.[11] Me_2Spm is much more stable than the natural polyamines, which undergo rapid degradation because of acetylation.[6] Me_2Spm could ameliorate the injury to the pancreas, after early (24 h) induction of pancreatitis especially when Me_2Spm was given as a treatment after the induction of pancreatitis. This method of administration resulted in greater tissue concentrations of Me_2Spm than the pretreatment, which possibly explains the better result; however, although Me_2Spm was present in the pancreas throughout the experiment, the protective effect of Me_2Spm against pancreatic damage did not persist when evaluated at 72 h. This observation may be explained by the finding that at 72 h no more signs of high polyamine catabolism were observed, but the polyamine homeostasis had returned to baseline level. This observation suggests that polyamine depletion caused by increased catabolism may be important only early during the evolution of pancreatitis, whereas other mechanisms, such as locally and systematically released cytokines and other inflammatory mediators, in addition to protease activation, play a more important role than polyamine depletion after the first day.

Consequently, Me_2Spm may have a protective effect only on the early stages of the disease.

One explanation offered for the lack of effect of Me_2Spm at 72 h is that the dose of Me_2Spm was not sufficient. Therefore, 1 group of rats received an extra dose of Me_2Spm at 30 h. This treatment, however, resulted in lethal toxicity at 72 h, even when only half of the original dose was readministered. Furthermore, the accumulation of the synthetic Me_2Spm into the pancreas was more prominent without this additional dose at 72 h than at 24 h, which excludes the possibility of an insufficient dose. These findings exclude the possibility that repeated polyamine substitution might be of benefit.

Pancreatic polyamine catabolism was activated by the induction of acute pancreatitis. Me_2Spm was thought to be used as a substitute to compensate for the depletion of high polyamines in the pancreas. This function as a substitute is supported by the finding that although Me_2Spm could ameliorate the injury, it did not prohibit the catabolism of the natural polyamines spermidine and spermine.

The direct protective mechanism of Me_2Spm in ameliorating the injury in pancreatitis is not clear. Me_2Spm might mediate the partial protection of the cellular damage by affecting the attack of inflammatory mediators on their targets.[20] Multiple potential sites of action, however, may suggest that the mechanism may be at any point in the injury cascade of pancreatitis. Me_2Spm has been shown to convert right-hand B-DNA to left-hand Z-DNA, to interact with a variety of substances, and to restore the growth of polyamine-depleted cells.[6,11,22,23]

In summary, we have shown that the development of taurodeoxycholate-induced acute pancreatitis was associated with the activation of polyamine catabolism in the pancreas. Activated polyamine catabolism may play a role in mediating the pancreatic injury during the first day of pancreatitis. Furthermore, a long-acting polyamine analog given after the induction of acute taurodeoxycholate-induced pancreatitis ameliorated the injury during the first day. This protective effect, however, did not persist at 72 h, and when given again at 30 h, it led to toxicity and death. More studies are needed to explore the mechanisms of the effect of Me_2Spm, the toxicity of the substance, and the best timings and doses of administration. Thus, besides proteolytic enzyme activation and the cascades of inflammation, also polyamine catabolism might be important pathogenetic mediators of acute pancreatitis.

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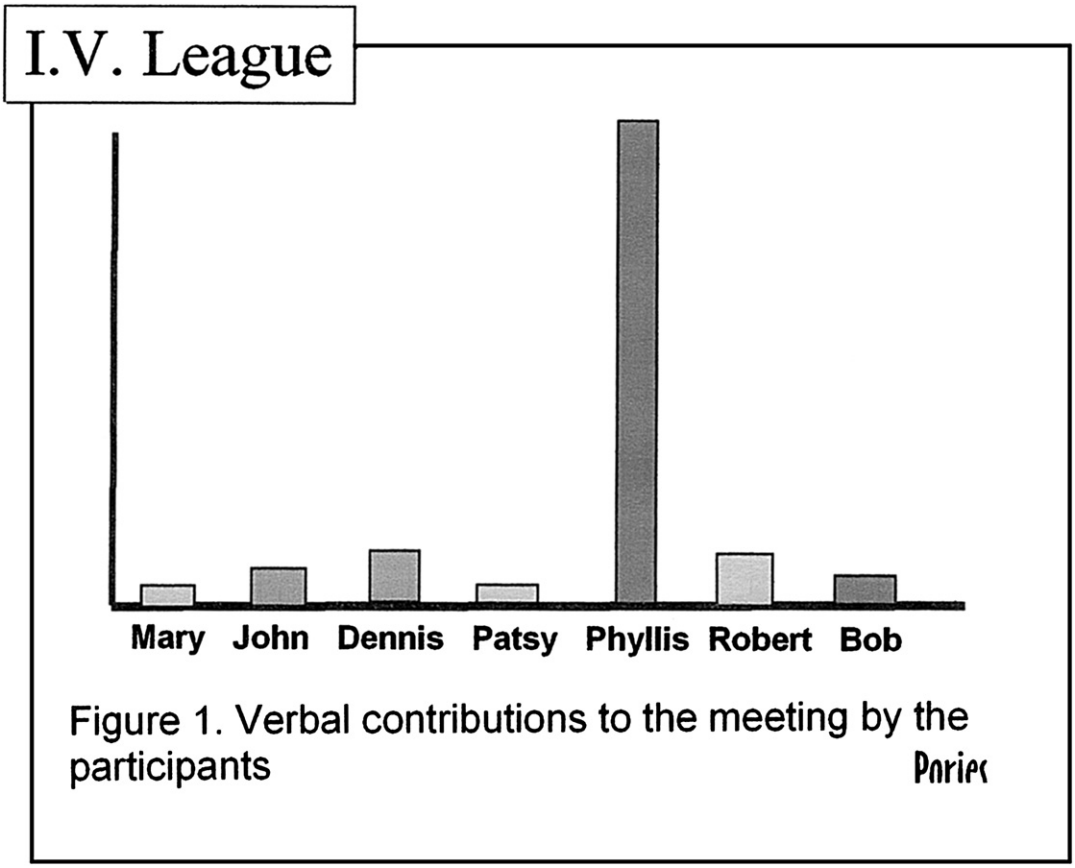

Figure 1. Verbal contributions to the meeting by the participants

Polyamine catabolism in relation to trypsin activation and apoptosis in experimental acute pancreatitis

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This work was supported by grant from Tampere University Hospital, equipment and drugs were supplied by Tampere University Hospital, A.I. Virtanen Institute for Molecular Sciences (University of Kuopio) and Engelhardt Institute of Molecular Biology (Russian Academy of Sciences).

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Structured abstract

Background. Over-induced polyamine catabolism (PC) in a transgenic rat model has been suggested to be a mediator of trypsin activation which is important in acinar cell necrosis. PC has been also observed also in experimental taurodeoxycholate pancreatitis. We hypothesized that PC may be a mediator of trypsin activation in taurodeoxycholate pancreatitis.

Methods. Pancreatitis was induced in wild-type rats by 2% or 6% taurodeoxycholate infusion or in transgenic rats by overexpressing spermidine/spermine N^1-acetyltransferase (SSAT). The time-courses of necrosis, caspase-3 immunostaining, SSAT, polyamine levels and trypsinogen activation peptide (TAP) were monitored. The effect of a polyamine analogue bismethylspermine (Me_2Spm) was investigated.

Results. In transgenic pancreatitis model, TAP and acinar necrosis increased simultaneously after the activation of SSAT, depletion of spermidine and development of apoptosis. In taurodeoxycholate pancreatitis, necrosis developed along with the accumulation of TAP. SSAT was activated simultaneously or after TAP accumulation and less than that in the transgenic model, with less depletion of spermidine than that in transgenic model. Supplementation with Me_2Spm ameliorated the extent of acinar necrosis at 24h, but contrary to the previous findings in transgenic model, in the taurodeoxycholate model it did not affect the trypsin activation. Compared with transgenic model, no extensive apoptosis was found in taurodeoxycholate pancreatitis.

Conclusions. Contrary to the transgneic SSAT over-induced pancreatitis, PC may not be a mediator of trypsin activation in the taurodeoxycholate pancreatitis. The beneficial effect of polyamine supplementation on the necrosis in the taurodeoxycholate pancreatitis may be rather mediated via other mechanisms than via amelioration of the trypsin activation.

Standard abbreviations

Me_2Spm, bismethylspermine;

PAO, polyamine oxidase;

PC, polyamine catabolism;

PMN leukocytes, polymorphonuclear leukocytes;

SSAT, spermidine/spermine N^1-acetyltransferase;

TAP, trypsinogen activation peptide;

TUNEL, Terminal deoxynucleotidyl transferase (TdT)-mediated dUTP-biotin nick-end labeling

INTRODUCTION

The polyamines spermidine, spermine and putrescine are essential to cell growth and many functions important for cell survival. The catabolism of polyamines is controlled by the rate-controlling enzyme spermidine/spermine N^1-acetyltransferase (SSAT) (Fig 1).[1, 2] There is irrefutable evidence showing that remarkably enhanced polyamine catabolism (PC), that can be induced in a transgenic rat model, can lead to pancreatitis with acinar necrosis.[3-5] The mechanism is probably mediated by the depletion of spermidine and spermine,[6, 7] since a polyamine analogue bismethylspermine (Me_2Spm) can ameliorate the acinar cell necrosis in these transgenic rats.[5] Also in wild-type rats the pancreatitis induced by taurodeoxycholate-infusion, a model perhaps best simulating clinical biliary pancreatitis, seems to be associated with SSAT activation and polyamine depletion which is followed by acinar necrosis that can be ameliorated with polyamine substitutes.[8, 9]

Trypsin has been shown to act as the trigger enzyme for the activation of other pancreatic digestive zymogens, as well as its own precursor trypsinogen.[10, 11] Physiologically this cascade is initiated by enteropeptidase of the duodenum cleaving the trypsinogen activation peptide (TAP) from trypsinogen, releasing active trypsin to digest nutrients.[12] Intrapancreatic trypsin activation is an early event in acute pancreatitis, and is associated with a release of TAP already in 15 minutes after pancreatitis induction in some models.[13]

More recent studies with the SSAT overexpressing rats showed that pancreatic trypsin activity was increased 10-fold at 4 h after the induction of pancreatitis.[14] It has also been reported that trypsinogen activation takes place in the acini isolated from SSAT overexpressing rats, and *in vitro* trypsinogen activation was blocked by pre-treatment of the animals *in vivo* with a polyamine

analogue.[5] These results let us hypothesize that the activation of PC may also be a mediator of trypsin activation in taurodeoxycholate-induced pancreatitis models.

Besides acinar cell necrosis, apoptosis is also induced in the pancreas during polyamine depletion.[3] Apoptosis has been associated with tissue defence rather than the injury in pancreatitis.[15-17] Apoptotic signalling pathway includes mitochondrial depolarization, cytochrome *c* release and activation of caspase cascade. Caspases are considered to be activated in the very early stages of apoptosis.[15, 17] In a melanoma cell line, induction of SSAT with diethylnorspermine resulted in apoptosis where the oxidation products of PC served as mediators for cytochrome *c* release and caspase activation.[18] The association of PC with apoptosis has not been studied in taurodeoxycholate-induced pancreatitis. Furthermore, to exclude a possibility to mix up necrosis and apoptosis, we also studied the development of apoptosis by caspase-3 immunohistochemistry.

MATERIALS AND METHODS

Animals

Both wild-type and transgenic animals were used in the experimental setting. The present study was approved by the Animal Committee of University of Tampere and the Institutional Animal Care and Use Committee of University of Kuopio and the respective provincial governments. The experiments were performed in accordance with the "Guidelines for the Care and Use of Laboratory Animals" (NIH publication No.86-23, revised 1985).

Wild-type rats: Adult male Sprague-Dawley rats (250-420 g) were fed with standard chow diet until 12 h before the experiment, after which the animals fasted until the end of the experiment.

Transgenic rats: Adult male transgenic Wistar rats (280-400 g) harboring a methallothionein-promoter driven SSAT transgene construct were used.[3] They were fed under the same conditions as the wild-type rats.

Experimental pancreatitis models in wild-type and transgenic animals

Wild-type rats: Acute taurodeoxycholate pancreatitis was induced with intraductal administration of 0.2 ml phosphate buffered saline, pH 7.4 containing either 2 % or 6 % sodium taurodeoxycholate (Sigma-Aldrich, Buchs, Switzerland) under 30 cm H_2O pressure, after cannulating the biliopancreatic duct through the duodenum with thin polyethene tubing and temporary clamping of the duct at the liver hilum. These two concentrations of sodium taurodeoxycholate have been shown to induce respectively moderate or severe necrotizing pancreatitis.[8]

Transgenic rats: In these rats, activation of the SSAT transgene was induced by an intraperitoneal injection of zinc sulphate (10 mg/kg) dissolved in distilled water, as previously described.[3-5, 19]

Intervention with Me_2Spm - a polyamine analogue

Me_2Spm was synthesized as previously described [20] and used in 0.9% NaCl solution at 25 mg/ml. The wild-type animals were injected with Me_2Spm as intraperitoneal treatment (25 mg/kg) at 4 h and at 8 h after the induction of pancreatitis by the 2 % taurodeoxycholate.

Experimental design

The totals of 106 animals were divided into 18 groups:

Wild-type animals:

Control (Group 1): Six rats served as the 0 h baseline group.

Sham operation (Groups 2-5): 24 rats were used, 6 in each group, rats underwent laparotomy only and were sampled at 1.5 h (Group 2), 3 h (Group 3), 6 h (Group 4) and 24 h (Group 5) after this sham operation.

2% taurodeoxycholate (Groups 6-9): 24 rats were used, 6 in each group. Acute pancreatitis was induced with intraductal infusion of 2% sodium taurodeoxycholate as described above. They were

sampled at 1.5 h (Group 6), 3 h (Group 7), 6 h (Group 8) and 24 h (Group 9) after the infusion of taurodeoxycholate.

2% taurodeoxycholate + Me_2Spm (Group 10): Acute pancreatitis was induced as in Group 9. The animals were injected with Me_2Spm at 4 h and at 8 h after the induction of pancreatitis, as described above. They were sampled at 24 h (Group 10, n=4) after the infusion of 2% taurodeoxycholate.

6% taurodeoxycholate (Groups 11-13): 18 rats were used, 6 in each group. Acute pancreatitis was induced with intraductal infusion of 6% sodium taurodeoxycholate as described above. They were sampled at 1.5 h (Group 11), 3 h (Group 12) and 6 h (Group 13) after the infusion of taurodeoxycholate. After 6 h there was considerable mortality. Experiments were not continued beyond this time point to exclude selection bias of sampling only the living animals.

Transgenic animals:

Control (Group 14): These transgenic rats (n=6) served as 0 h baseline controls. They were sampled without the induction of SSAT.

Acute pancreatitis (Groups 15-18): 24 rats were used, 6 in each group. Acute pancreatitis was induced in these transgenic animals through SSAT induction by injection of zinc sulphate, as described above. The animals were euthanized for samplings at 1.5 h (Group 15), 3 h (Group 16), 6 h (Group 17) and 24 h (Group 18).

Histology

For histology, the pieces of pancreas were fixed at room temperature in a pH-neutral, phosphate-buffered, 10% formalin solution. The fixed tissue was embedded in paraffin, sectioned at 5 µm, stained with hematoxylin and eosin, and coded for blinded examination. The histological changes of pancreas, including edema, leukocyte infiltration and necrosis, were graded using the previous validated scoring criteria at magnification ×400.[21]

Detection of cells with activated caspase-3

Apoptosis, assessed as the presence of cells with active caspase-3 was determined by immunohistochemistry using cleaved caspase-3 (Asp175) as the primary antibody (Cell Signaling Technology, Inc., Danvers, MA). The detection and staining were performed using the Power Vision TM Poly-HRP anti-Rabbit IgG IHC Kit from ImmunoVision Technologies, Co., Brisbane, CA. The extent of apoptosis was scored as follows: 0 = no caspase-3 positive cells; 1 = 1-10 caspase-3 positive cells; 2 = 11-20 caspase-3 positive cells; 3 = 21-50 caspase-3 positive cells; 4 = 51-70 caspase-3 positive cells; 5 = 71-100 caspase-3 positive cells; 6 = more than 100 caspase-3 positive cells per field at magnification ×200.

Pancreatic SSAT and polyamines

Pancreatic samples were frozen in liquid nitrogen and stored at -70 ^{0}C until determination of SSAT activity and polyamine levels. Pancreatic SSAT activity was assessed according to Bernacki et al., [22] and the natural polyamines (spermidine, spermine and putrescine) and the polyamine analogue (Me_2Spm) were determined with high-performance liquid chromatography as previously described by Hyvönen.[23]

Pancreatic TAP

Pancreatic trypsin activation was measured by assaying the TAP accumulation. Fresh pancreatic specimens were excised and placed in a boiling water bath for 15 minutes to inactivate further TAP generation. The boiled samples were homogenised and centrifuged. The supernatant was transferred to 1.5 ml microcentrifuge tubes and then stored at –70 ^{0}C. Pancreatic TAP level was quantified in an aliquot of each supernatant using an enzyme immunoassay kit (Biotrin International Ltd, Dublin, Ireland). Typical calibration curve was obtained using Biotrin TAP enzymeimmunoassay. Protein

concentration of each sample was measured using BCA (Bicinchoninic Acid) kit (Sigma-Aldrich, BCA-1, St. Louis, MO. USA). Pancreatic TAP contents are shown as pmol /mg protein.

Statistical analysis

All data are expressed as medians [range], and the groups were compared by Mann-Whitney *U* test using SPSS 11.00 software. The correlation analysis was performed by calculating the Pearson correlation coefficient. The level of significance was set at $p < 0.05$.

RESULTS

Histology

Pancreatitis manifested as edema, leukocyte infiltrations and necrosis in all these models of pancreatitis. After infusion of 2% taurodeoxycholate, acinar necrosis gradually increased over time until 24 h. In 6% taurodeoxycholate model, necrosis developed more rapidly (Table I). Few caspase-3 positive cells were observed in 2% and 6% taurodeoxycholate pancreatitis without changes during the observation periods.

In transgenic model the necrosis developed by 3 h, extending up to 24 h (Table I). Extensive caspase-3 positive staining was not obvious before 6 h (Table I).

SSAT and polyamines

SSAT activity in both 2% and 6% taurodeoxycholate pancreatitis reached its highest level at 6 h, being about double in the 6% compared with the 2% model (Table II). Pancreatic spermidine diminished respectively (Fig 2 A). The changes in spermine level were minimal. Concentration of pancreatic putrescine, the product of spermidine and spermine catabolism, increased significantly (Fig 2 B).

In the transgenic model SSAT activity increased enormously during the study period (Table II), when spermidine and spermine were depleted by 92% and 79% at 24 h respectively (Fig 3). The SSAT activity at 24 h was 1640-fold of that found in the 2% taurodeoxycholate pancreatitis model; moreover, spermidine, spermine and putrescine changes were remarkable: their values were roughly 10%, 20% and 220% of those found in the taurodeoxycholate pancreatitis, respectively.

TAP

In the 6% pancreatitis model pancreatic TAP levels were highly elevated already at 1.5 h, staying at the same level till 6 h. Compared with the 6% model, the 2% model showed slower accumulation of TAP, the maximum not being reached until 24 h (Table II).

In transgenic model there was no significant increase in pancreatic TAP until 24 h (Table II), when pancreatic TAP reached a level similar to that observed much earlier in taurodeoxycholate pancreatitis (Table II).

The effect of Me_2Spm

The effect of Me_2Spm has been previously studied in the transgenic model with SSAT overexpression.[5] In the 6% taurodeoxycholate pancreatitis, mortality increased after 6 h, before the completion of such Me_2Spm administration protocol that has been shown most effective in ameliorating necrosis in 2% taurodeoxycholate pancreatitis.[9] This protocol did ameliorate the necrosis at 24 h in the 2% taurodeoxycholate pancreatitis also with present experiment (Table I). In the 2% taurodeoxycholate pancreatitis models the polyamine analogue Me_2Spm did not decrease the pancreatic TAP levels (20 [16-34] pmol/mg protein vs. 28 [17-38] pmol/mg protein; NS). Neither did SSAT change significantly after the Me_2Spm administration (68 [51-167] pmol/mg protein vs. 110 [18-367] pmol/mg protein; NS).

Combined timings of changes in SSAT, TAP, necrosis and apoptosis

In the severe pancreatitis induced by 6% taurodeoxycholate, TAP started to accumulate very early, prior to SSAT induction (Fig 4 A). The necrosis followed the TAP accumulation, being found before the SSAT induction (Fig 4 A). No change was observed in the number of caspase-3 positive cells. In the correlation analysis, only the necrosis score significantly correlated with the TAP level ($p = 0.012$).

In the moderate pancreatitis induced by 2% taurodeoxycholate, SSAT was activated with the same time-course as TAP was found to accumulate into the pancreas (Fig 4 B). Substantial necrosis (> 1 in scale 0-4) developed by 6 h, concurring with the SSAT induction and the TAP accumulation (Fig 4 B). No change was observed in the number of caspase-3 positive cells. The necrosis score correlated significantly with the TAP level ($p = 0.001$). The correlation between the SSAT level and necrosis score did not quite reach statistical significance ($p = 0.063$).

In the transgenic model SSAT was induced first, already extremely highly at 1.5 h (Fig 4 C). TAP was not elevated until 24 h (Fig 4 C). Substantial necrosis (> 1 in scale 0-4) developed not until 24 h (Fig 4 C). Caspase-3 activity was already found increased before the necrosis developed, in a few hours after the SSAT induction (Fig 4 C). Both the necrosis and apoptosis scores correlated with the SSAT level ($p < 0.0001$).

DISCUSSION

PC,[3] trypsin activation [12, 24] and apoptosis [15] have all been studied during pancreatitis, but these three have not been compared in the same models. In transgenic pancreatitis model we found that

pancreatic TAP, the marker of trypsin activation, and acinar necrosis increased simultaneously. They were preceded with the activation of SSAT, depletion of spermidine and occurrence of apoptosis. In wild-type animals with pancreatitis induced with either 2% or 6% taurodeoxycholate, necrosis developed along with the accumulation of TAP. SSAT was also induced in the both taurodeoxycholate pancreatitis models, but not before TAP accumulation and much less than that in the transgenic model, resulting in less extent of spermidine depletion than that found in the transgenic model. Supplementation with a methylated polyamine analogue Me_2Spm ameliorated the extent of acinar necrosis at 24h, as also previously observed both in the transgenic and taurodeoxycholate models,[5, 9] but contrary to transgenic model, in the taurodeoxycholate model it did not affect the trypsin activation. The increase in the number of cells positive for cleaved caspase-3 was only seen in the transgenic model.

The present study is consistent with the previous studies confirming that the development of necrosis is associated with the trypsin activation.[10, 12, 25-28] It also supports the earlier suggestion that the early trypsin activation might be an important mediator of necrosis in the SSAT pancreatitis model as well.[14]

When the time-courses of TAP and SSAT changes were compared in both wild-type and transgenic pancreatitis models, the TAP accumulation seemed to precede the SSAT activation in the severe pancreatitis model induced by 6% taurodeoxycholate, it was concurrent in the moderate pancreatitis induced by 2% taurodeoxycholate, whereas it followed the SSAT activation in the mild transgenic model. These time-courses exclude the possibility of PC being the mediator of trypsin activation in 6% taurodeoxycholate pancreatitis and may make it questionable in the 2% taurodeoxycholate model. Therefore, the SSAT over-induced PC in the early trypsin activation may be a model specific phenomenon.

The supplementation with Me_2Spm did ameliorate the necrosis at 24 h in the moderate 2% taurodeoxycholate pancreatitis model. This effect was not extensive, although it has been repeatedly statistically significant. The protective mechanism may be, however, other than the effect on trypsin activation in this 2% taurodeoxycholate pancreatitis, since Me_2Spm did not diminish the TAP accumulation. There is also another possible mechanism, by which Me_2Spm could ameliorate necrosis. Besides the early trypsin activation, previous studies have demonstrated that inflammatory and oxidative stress may contribute to the cell injury.[29-31] Me_2Spm has been found to ameliorate the inflammatory response,[32] giving a possible explanation for the delayed ameliorative effect on the necrosis. Thus the present and previous studies suggest that while PC may play a role in the activation of trypsin in transgenic SSAT over-induction pancreatitis, it may rather have an effect on the secondary hit induced by cytokines and oxidative stress than on the initial hit induced by trypsin activation in the 2% taurodeoxycholate-induced pancreatitis. In more general, it may be hypothesized that the enormous depletion of the polyamine pool by the huge over-expression of SSAT in the transgenic model may serve as such a general disturbance of many essential functions of acinar cell that mediates the early trypsin activation in this particular model, whereas the minor changes in polyamine pools in taurodeoxycholate pancreatitis are insufficient for such induction via this mechanism.

Extensive apoptosis was clearly seen only in the transgenic model but not in the taurodeoxycholate models. In this study we used activated caspase-3 staining to detect apoptosis. Caspase-3 is thought to be the key caspase that has particularly been used for the histological labeling of apoptosis.[33, 34] Terminal deoxynucleotidyl transferase (TdT)-mediated dUTP-biotin nick-end labeling (TUNEL) method was not used in our studies, because although the TUNEL method may be sensitive to detect DNA fragmentation, such DNA fragmentation is not specific for apoptosis.[33]

Previous studies reported that apoptosis has been connected with the activation of SSAT and PC in melanoma cells.[18, 35] In transgenic pancreatitis model huge SSAT activation, 1640-fold of that in wild-type pancreatitis model, took place with remarkable drop of spermidine and spermine already well before the accumulation of TAP or the detection of necrosis, giving a time window for the apoptosis to develop. The transgenic model was milder than the taurodeoxycholate-induced models based on the presences of the trypsin activation and substantial necrosis during 6 h. Previously apoptosis has been defined as rather protective than injury promoting in the course of pancreatitis and has been found being associated with the mildest forms of pancreatitis.[36] Recent study has shown that acinar cells exhibited mitochondrial damage in transgenic pancreatitis.[14] Thus the loss of mitochondrial integrity may initially lead to the induction of apoptosis signaling. As the final outcome, however, is extensive necrosis also in the transgenic model, we may suggest that caspase-3 activation is mediated by some of the same mechanisms as necrosis. Caspases have also been implicated as necrosis mediators elsewhere.[37-40]

We conclude that the PC is associated with the taurodeoxycholate-induced pancreatitis. Contrary to the transgneic SSAT over-induced pancreatitis, PC may not be a mediator of trypsin activation in the taurodeoxycholate pancreatitis. The beneficial effect of polyamine supplementation on the necrosis, observed in 2% taurodeoxycholate pancreatitis at 24 h, may be rather mediated via other mechanisms than via amelioration of the trypsin activation. Future studies are needed to explore various mechanisms, including the effect on inflammatory mediators and oxidative stress. Extensive apoptosis is detected by the caspase-3 immunostaining only in the transgenic SSAT over-induced pancreatitis model, in which the necrosis develops slowest among the three studied pancreatitis models and where the SSAT activation is overwhelmingly highest.

Acknowledgements

We wish to thank Alex R Khomutov and Nikolay Grigorenko from Engelhardt Institute of Molecular Biology, Russian Academy of Sciences, as participating investigators, for supplying bismethylspermine to this study.

Great thanks to the following colleagues for their skillful technical assistance in this study:

Tampere University Hospital: Prof. Immo Rantala, Department of Pathology, Centre for Laboratory Medicine

University of Tampere: Tuija Hartin, Anne Hartin-Gathuo, Sanna Lehti and Tarja Tulonen; Leena Honkaniemi and Pirkko Rajala, Clinical Medicine, Medical School; Pekka Vilja and Marja-Leena Koskinen, Medical School; Sami Oikarinen, Department of Virology, Medical School

University of Jyväskylä: Prof. Heikki Kainulainen

Conflict of interest

Financial support: Grant of Tampere University Hospital.

There is no commercial association with or financial involvement that might pose a conflict of interest in connection with the study.

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Figure 1

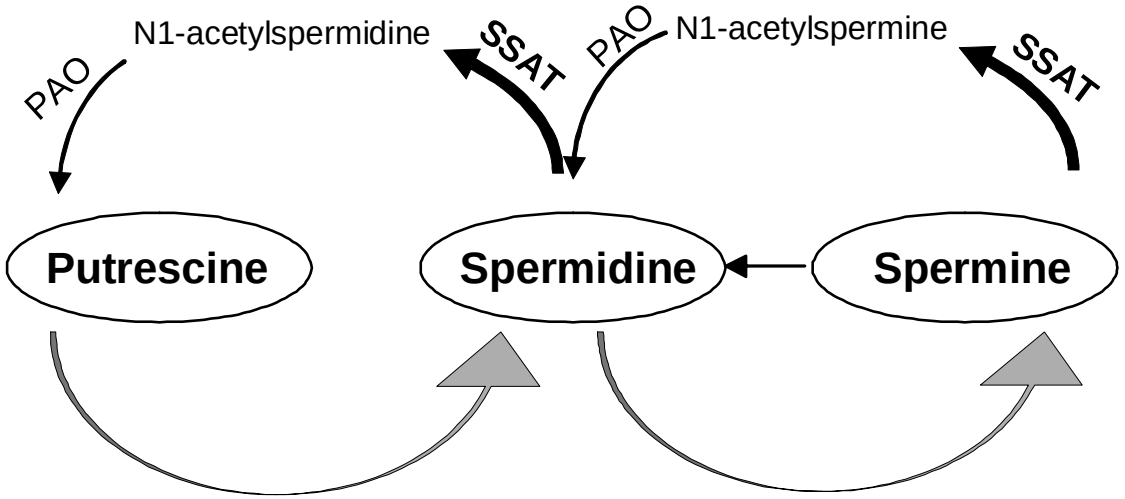
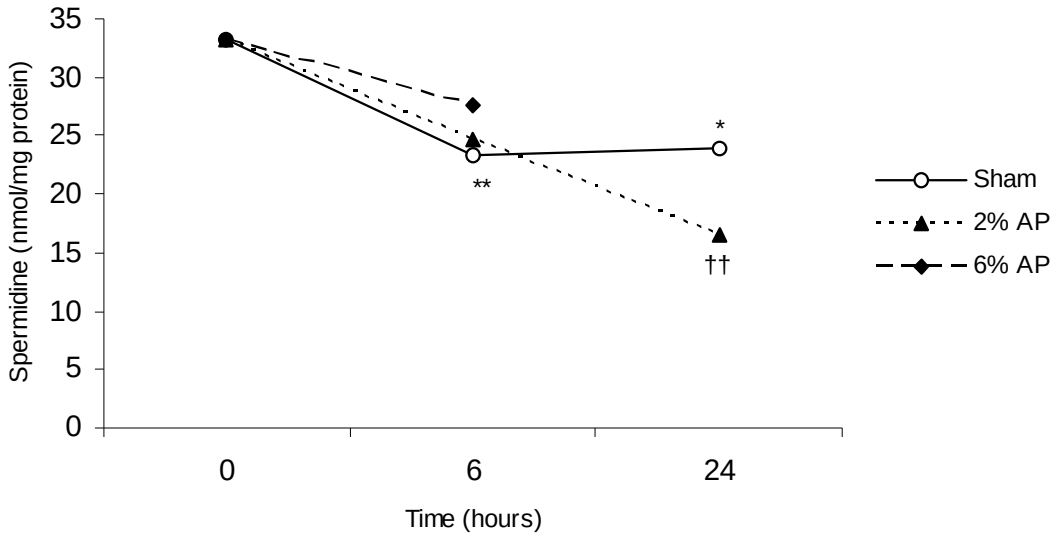


Fig 1. Polyamine catabolism.

Polyamine homeostasis includes synthesizing of putrescine to spermidine, which is used to synthesize spermine. Spermidine/spermine N^1-acetyltransferase (SSAT) catabolizes spermine to N^1-acetylspermine, which is catabolized by polyamine oxidase (PAO) to spermidine. SSAT and PAO also catabolize spermidine to putrescine, which is again the precursor of spermidine and spermine.

A

Figure 2



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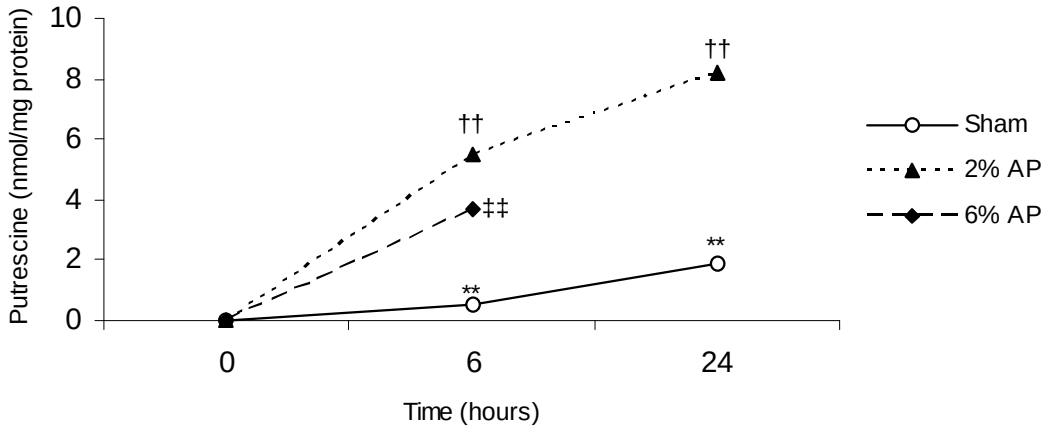

Fig 2. Changes of pancreatic polyamines in 2% and 6% taurodeoxycholate-induced pancreatitis models.

Control, served as 0 h baseline in wild-type rats; **Sham**, sham operation in wild-type rats; **2% AP**, 2% taurodeoxycholate-induced acute pancreatitis; **6% AP**, 6% taurodeoxycholate-induced acute pancreatitis. A) The decrease of spermidine was induced by sham operation. Compared with the sham operation group, the level of pancreatic spermidine in both 2% and 6% taurodeoxycholate pancreatitis models decreased further. B) The increase of putrescine was induced by sham operation. Compared with the sham operation group, the level of pancreatic putrescine in both 2% and 6% taurodeoxycholate pancreatitis models increased further. All data are expressed as medians. $^{*}p < 0.05$, sham operation vs. 0h control group; $^{**}p < 0.01$, sham operation vs. 0h control group; $^{\dagger\dagger}p < 0.01$, 2% AP vs. sham operation; $^{\ddagger\ddagger}p < 0.01$, 6% AP vs. sham operation.

Figure 3

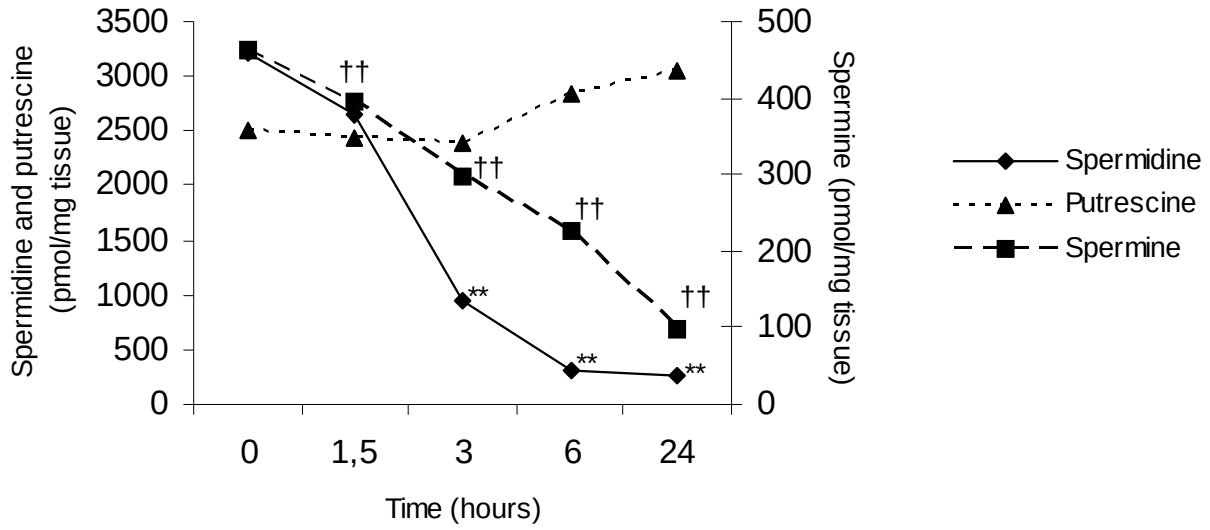

Fig 3. Changes of pancreatic polyamines in transgenic pancreatitis model.

In the spermidine/spermine N^1-acetyltransferase (SSAT) transgenic Wistar rats, the pancreatitis was induced by intraperitoneal injection of zinc sulphate (10 mg/kg). Pancreatic spermidine and spermine decreased remarkably. No significant change was observed in putrescine level. All data are expressed as medians. $^{**}p < 0.01$, vs. spermidine level of transgenic control at 0h; $^{††}p < 0.01$, vs. spermine level of transgenic control at 0h.

Figure 4

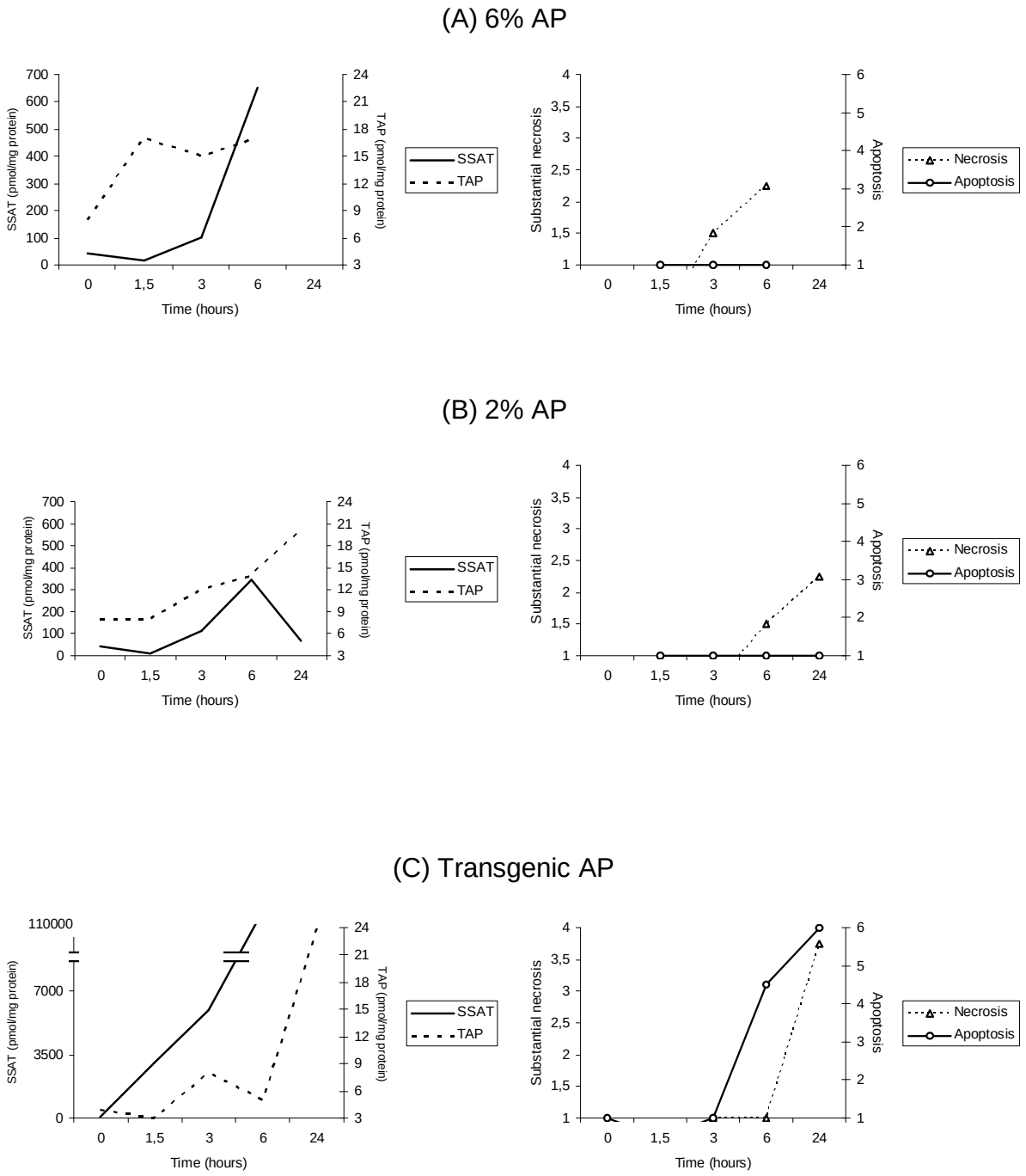

Fig 4. Combined timings of changes in pancreatic SSAT, TAP, necrosis and apoptosis.

6% AP, 6% taurodeoxycholate-induced acute pancreatitis; **2% AP**, 2% taurodeoxycholate-induced acute pancreatitis; **Transgenic AP**, acute pancreatitis model in SSAT over-expressed transgenic rats induced by intraperitoneal injection of zinc sulphate (10 mg/kg). **SSAT**, Spermidine/spermine N^1-acetyltransferase; **TAP**, trypsinogen activation peptide. The substantial necrosis is defined as pancreatic necrosis score > 1. All data are presented as medians.

Table I. Pancreatic histology and activated caspase-3 in both taurodeoxycholate-induced pancreatitis and transgenic pancreatitis with the treatment of Me_2Spm

Group, follow-up		Edema	PMN leukocytes	Necrosis	Activated caspase-3
Control	0h	0[0-0]	0[0-0]	0[0-0]	
Sham	1.5h	1[0.5-1]	0[0-1]	0[0-1]	
	3h	0[0-1]	0[0-0]	0[0-0.5]	
	6h	1[0.5-2]	0[0-0]	0[0-1]	
	24h	0.5[0-2]	0[0-1]	0[0-1]	
2% AP	1.5h	2.25[1-3]**	1[0-2]	0.75[0-2.5]*	1[1-1]
	3h	2[1.5-3]**	1[0-2]*	0.5[0.5-1]**	1[1-1]
	6h	2[1-3]	0.5[0-1]	1.5[0.5-3.5]*	1[1-1]
	24h	2.5[1.5-3]**	0.5[0-80]	2.25[1-3.5]**	1[1-2]
6% AP	1.5h	2.25[2-3]**	1[0-2]	0[0-4]	1[0-1]
	3h	2.5[2-3.5]**	2[1-3]**	1.5[1-4]**,††	1[1-1]
	6h	2.75[2-3]**	2[1-3]**,†	2.25[0.5-3]**	1[1-3]
2% AP+Me_2Spm	24h	2[2-3]	0[0-1]	0.5[0-1.5]‡	1[1-1]
Transgenic control	0h	0[0-0]	0[0-0]	0[0-0]	1[0-1]
Transgenic AP	1.5h	1[0.5-2]§§	0[0-0]	0[0-0]	0.5[0-1]
	3h	1.5[1-2]§§	0[0-0]	1[0.5-1]§§	1[1-2]
	6h	1.5[1-2]§§	0[0-0]	1[0-1]§§	4.5[1-6]§§
	24h	3[3-4]§§	2[0-3]§§	3.75[2.5-4]§§	6[6-6]§§

Control, served as 0 h baseline in wild-type rats; **Sham**, sham operation in wild-type rats; **2% AP**, 2% taurodeoxycholate-induced acute pancreatitis; **6% AP**, 6% taurodeoxycholate-induced acute pancreatitis; **Me_2Spm**, bismethylspermine; **2% AP + Me_2Spm**, treatment of Me_2Spm after induction of 2% taurodeoxycholate-induced acute pancreatitis; **Transgenic AP,** transgenic acute pancreatitis; **PMN leukocytes**, polymorphonuclear leukocytes (which is graded by counting cell numbers). In the SSAT transgenic Wistar rats, the pancreatitis was induced by intraperitoneal injection of zinc sulphate (10 mg/kg) that activated methallothionein-promoter dependent SSAT. All data are expressed as medians [range].

$^{*}p<0.05$, 2% or 6% AP (1.5h-24h) vs. sham operation (1.5h-24h) respectively;

$^{**}p<0.01$, 2% or 6% AP (1.5h-24h) vs. sham operation (1.5h-24h) respectively;

$^{\dagger}p<0.05$, 6% AP (1.5h-6h) vs. 2% AP groups (1.5h-6h) respectively;

$^{\dagger\dagger}p<0.01$, 6% AP (1.5h-6h) vs. 2% AP groups (1.5h-6h) respectively;

$^{\ddagger}p<0.05$, 2% AP+Me_2Spm (24h) vs. 2% AP (24h);

$^{\S\S}p<0.01$ vs. transgenic control at 0h.

Table II. Pancreatic SSAT activity and TAP content in taurodeoxycholate-induced pancreatitis and transgenic acute pancreatitis with Me_2Spm treatment

Group, follow-up		SSAT (pmol/mg protein)	TAP (pmol/mg protein)
Control	0h	42.01[36.33-51.92]	8.13[2.17-10.02]
Sham	1.5h	7.97[4.57-21.39]	7.88[4.60-20.31]
	3h	61.94[40.63-171.40]	6.24[4.56-26.33]
	6h	39.04[17.75-54.38]	7.54[4.01-12.61]
	24h	79.19[44.77-92.62]	9.99[3.73-16.13]
2% AP	1.5h	10.92[5.59-48.63]	7.5[3.10-23.66]
	3h	113.00[48.10-167.00]	11.69[6.69-20.77]
	6h	342.82[295.80-397.17]**	13.62[4.81-28.45]
	24h	67.70[51.40-167.40]	19.61[16.27-34.69]**
6% AP	1.5h	16.50[15.00-254.00]†	17.39[8.56-53.31]†
	3h	103.00[66.00-262.00]	15.37[11.00-52.67]†
	6h	654.00[447.00-1043.00]††,‡‡	17.06[12.98-37.38]††
2% AP+Me_2Spm	24h	109.65[18.3-366.7]	27.885[16.75-38.56]
Transgenic control	0h	69.50[17.00-144.00]	4.46[2.50-12.70]
Transgenic AP	1.5h	3059.00[711.00-5398.00]§§	3.36[1.36-10.40]
	3h	5939.00[4505.00-12991.00]§§	8.26[1.75-98.93]
	6h	11440.00[6588.00-23817.00]§§	5.03[1.35-445.28]
	24h	109950.00[10639.00-176260.00]§§	24.48[19.76-30.41]§§

Control, served as 0 h baseline in wild-type rats; **Sham**, sham operation in wild-type rats; **2% AP**, 2% taurodeoxycholate-induced acute pancreatitis; **6% AP**, 6% taurodeoxycholate-induced acute pancreatitis. **Transgenic AP,** transgenic acute pancreatitis. Pancreatic spermidine/spermine N^1-acetyltransferase (SSAT) increased to its highest level at 6h in both severities, where SSAT in the 6% taurodeoxycholate model was higher than in the 2% model; in the SSAT transgenic Wistar rats, the pancreatitis was induced by intraperitoneal injection of zinc sulphate (10 mg/kg), pancreatic SSAT increased significantly during 1.5h-24h. Pancreatic trypsinogen activation peptide (TAP) increased during 1.5h-6h in 6% taurodeoxycholate pancreatitis, whereas it raised at 24h in 2% taurodeoxycholate pancreatitis; in the SSAT transgenic Wistar rats, pancreatic TAP did not significantly increase until 24h. All data are expressed as medians [range].

$^{*}p < 0.05$, 2% taurodeoxycholate pancreatitits vs. sham operation;
$^{**}p < 0.01$, 2% taurodeoxycholate pancreatitits vs. sham operation;
$^{\dagger}p < 0.05$, 6% taurodeoxycholate pancreatitits vs. sham operation;
$^{\dagger\dagger}p < 0.01$, 6% taurodeoxycholate pancreatitits vs. sham operation;
$^{\ddagger\ddagger}p < 0.01$, 6% taurodeoxycholate pancreatitits vs. 2% taurodeoxycholate pancreatitits;
$^{\S}p < 0.05$, vs. transgenic control at 0h;
$^{\S\S}p < 0.01$, vs. transgenic control at 0h.

Scandinavian Journal of Gastroenterology, 2009; 44: 1004–1011

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ORIGINAL ARTICLE

Changes in blood polyamine levels in human acute pancreatitis

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Abstract

Objective. Experimental studies have shown that pancreatic activation of polyamine catabolism occurs during the early stage of acute pancreatitis. Changes in pancreatic polyamines are reflected in the red blood cell (RBC) polyamine contents, correlating with the extent of pancreatic necrosis. The aim of this human study was to examine the changes in polyamine levels in the RBCs of patients with acute pancreatitis. ***Material and methods.*** Twenty-four patients with acute pancreatitis (7 alcoholic, 10 gallstone and 7 of unknown etiology) were recruited in the study. Eighteen patients with non-pancreatic acute abdominal diseases were included as controls, and 6 volunteers were studied as references. Blood samples were collected on admission and during hospitalization to assess polyamine levels. After clinical recovery, the patients revisited the clinic, and RBC polyamine levels were measured again. For comparison, plasma interleukin-6 (IL-6), IL-10 and C-reactive protein (CRP) were measured. ***Results.*** In acute pancreatitis patients, there was no difference in RBC polyamine levels on admission compared with those in controls or in volunteers. Putrescine levels on admission were higher in patients with pancreatic necrosis than in patients without necrosis, but there was no difference in spermidine and spermine levels. Patients with pancreatitis of unknown etiology had significantly higher levels of polyamines on admission and throughout hospitalization, but they also had more necrosis, which explained the difference in multivariate analysis. Spermidine and spermine levels increased after clinical recovery. RBC putrescine correlated with IL-6 and IL-10, and spermine correlated with CRP. ***Conclusions.*** The results of this study suggest that RBC polyamines change in human acute pancreatitis in several respects, as has been previously observed in experimental pancreatitis.

Key Words: *Acute pancreatitis, etiology, necrosis, polyamine, putrescine, severity*

Introduction

In mammalian cells, spermidine and spermine together with their diamine precursor putrescine are considered as the natural polyamines. They are important physiological components for cellular survival, growth and proliferation, and are essential in maintaining the integrity of the tissues. Their concentrations are high in liver, the nervous system, reproductive organs and also in the pancreas [1–5].

The primary amino acid source of polyamines is L-ornithine, which yields putrescine in a reaction catalyzed by ornithine decarboxylase – the key regulatory enzyme of polyamine biosynthesis [5]. Putrescine is converted into spermidine by spermidine synthase and spermidine is converted into spermine by spermine synthase. Spermine is converted back to spermidine and spermidine again is converted back to putrescine by the concerted action of two polyamine catabolizing enzymes spermidine/spermine N^1-acetyltransferase (SSAT) and polyamine oxidase (PAO) [5]. SSAT is usually the rate-controlling factor in polyamine catabolism.

Changes of polyamines in acute pancreatitis have been studied in experimental settings. In a transgenic animal model with inducible SSAT overexpression, spermidine and spermine levels decreased and putrescine levels increased, indicating activation of polyamine catabolism. In this model severe acute pancreatitis was initiated soon after the induction of polyamine catabolism [1–4]. Polyamine catabolism

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(Received 2 December 2008; accepted 10 April 2009)

ISSN 0036-5521 print/ISSN 1502-7708 online
DOI: 10.1080/00365520902964713

has taken place also in other experimental pancreatitis models, such as that induced by caerulein [2] as well as intraductal infusion of 2% or 6% taurodeoxycholate [6]. Pancreatic polyamine levels decrease more in severe than in moderate pancreatitis [6]. Interestingly, the changes in pancreatic tissue polyamine levels are reflected in blood polyamine levels, so that erythrocyte spermidine levels decrease most in animals with the most severe pancreatitis [6].

It is difficult to obtain pancreatic tissue specimens from patients with acute pancreatitis, which is why there have been so few studies of polyamine changes in pancreatic diseases using human subjects. Although there are no reports on correlations between red blood cells (RBCs) and pancreatic polyamine changes in human subjects, recent experimental studies have shown significant correlations, pancreatic necrosis being reflected in RBC polyamines. Against this background, we conducted a human study to investigate whether there are any changes in blood polyamines between patients with acute pancreatitis compared with other acute abdominal diseases, in patients with severe compared with mild acute pancreatitis and in patients with acute pancreatitis of alcoholic, gallstone or unknown etiology.

Materials and methods

Patients

Initially, 30 patients with acute pancreatitis were included in this observational study during the period 2006–07. The inclusion criteria were: first episode of pancreatitis with clinical symptoms and signs consistent with this disease; serum amylase activity more than three times the upper limit of the normal value; or acute pancreatitis in imaging (ultrasonography (US) or computed tomography). The patients were considered to have a probable alcoholic etiology when they or their families reported excessive consumption of alcohol or the Alcohol Use Disorders Identification Test (AUDIT) yielded a score of more than 8 points [7,8]. Patients with gallstones observed during US or with increased alanine transaminase or bilirubin levels underwent magnetic resonance cholangiopancreatography (MRCP) or endoscopic retrograde cholangiopancreatography (ERCP) to detect stones in the bile ducts. The etiology was considered to be of gallstone origin when either gallstones or biliary sludge was detected in imaging studies (US, ERCP, MRCP) [9,10]. The etiology was defined as unknown when neither an alcoholic nor gallstone etiology was suspected and when trauma, post-ERCP and hereditary origin were excluded through history and hypercalcemia and hyperlipemia with serum tests.

We initially included 10 consecutive patients in each of the three etiology subgroups (alcoholic, gallstone, unknown). From the initial 30 patients, one was later excluded because of simultaneous prostate cancer, since cancer can increase the blood polyamine levels [11–13]. Another three patients were excluded because amylase activities or imaging could not confirm the initial clinical diagnosis of pancreatitis, as required. Two further patients were excluded because it became obvious that these patients had had previous episodes of pancreatitis. Thus the final pancreatitis population consisted of 24 patients (Table I).

Of the 24 cases, 17 were defined as mild acute pancreatitis, and 7 were defined as severe acute pancreatitis owing to local complications (pancreatic necrosis or pseudocyst) or organ failure at any time during the hospitalization, as defined in the Atlanta criteria [14]. The sequential organ failure assessment (SOFA) score was calculated on admission for the acute pancreatitis patients, as previously described [15,16].

For comparison, 20 patients with other acute abdominal diseases without symptoms, signs and laboratory or imaging findings of acute pancreatitis were included as controls. Two patients were excluded because they had undergone an operation within one month of hospital admission, since surgery can have an effect on the blood polyamine levels [11,17–19]. Furthermore, 6 healthy adult volunteers were recruited for the analysis of normal blood polyamine levels. The study populations are summarized in Table I.

Methods

Blood samples were drawn from both acute pancreatitis patients and the controls on five consecutive days after admission to hospital, and thereafter on every second day throughout hospitalization. Furthermore, after recovery 14 patients with acute pancreatitis came back to the clinic within 41 days (median, range 30–58 days) from the onset of symptoms, by which time they had fully recovered.

Blood samples were collected in tubes containing 9NC coagulation sodium citrate 3.2% as an anticoagulant. The plasma and RBCs were separated and stored in aliquots at -70°C until analyses. Plasma was analyzed for C-reactive protein (CRP) concentration in accordance with the hospital laboratory routine, interleukin-6 (IL-6) and interleukin-10 (IL-10) using ELISA kits (Quantikine; R&D Systems, Minneapolis, Minn., USA) [20]. RBC polyamine levels were determined by high-performance liquid

Table I. Demographic data of patients with acute pancreatitis, with other acute abdominal diseases and of healthy volunteers. There was no significant difference in age or gender between the groups.

	Acute pancreatitis				
	Alcoholic	Gallstone	Unknown etiology	Control[†]	Healthy volunteers
No. of patients	7*	10	7	18	6
Age years (range)	52 (32–69)	75 (44–82)	67 (45–79)	63 (44–89)	57 (51–64)
Male/female	4/3	4/6	3/4	9/9	3/3
SOFA[‡] on admission (range)	1 (0–5)	1.5 (0–3)	3 (0–10)		
No. of patients with SOFA ≥ 3 on admission	1	2	4		
No. of patients with severe acute pancreatitis according to the Atlanta criteria	2	1	4		
No. of patients with local complications	2	1	3		
No. of patients with pancreatic necrosis	0	0	3		
No. of patients with OF[§]	0	0	3		
Hospital stay, days (range)	10 (5–19)	11.5 (5–48)	14 (3–54)	4 (3–11)	0 (0–0)
Mortality, *n* (%)	0 (0)	0 (0)	1 (14)	1 (6)	0 (0)

*One patient stayed at home for 2 weeks prior to admission; [†]patients with other acute abdominal diseases served as controls, including acute cholecystitis ($n=2$), cholecystolithiasis ($n=2$), gastric ulcer ($n=2$), diverticulitis ($n=6$), intestinal occlusion ($n=2$), intestinal necrosis ($n=2$), esophageal ulcer ($n=1$) and reflux esophagitis ($n=1$). None had increased amylase activity or pancreatitis in imaging; [‡]sequential organ failure assessment (SOFA) score; [§]patients with organ failure (OF) were also treated in the ICU (Intensive Care Unit).

chromatography (HPLC), as previously described [21]. A 20 or 30 µl sample was applied to a reversed-phase column equilibrated for 5 min with buffer A. The column was then eluted with buffer A for 5 min, followed by a linear gradient to 75% buffer B over 15 min, then holding this mixture, 25% buffer A, 75% buffer B for 5 min, and back to the starting conditions over 3 min at a flow-rate of 0.5 ml/min at 37°C, using an HP 1090 liquid chromatograph. The polyamines were detected by a fluorescence detector after post-column derivatization with *0*-phthalaldehyde. The *0*-phthalaldehyde solution was mixed under nitrogen with 1L of 1M borate buffer, pH 10.4. The mixture was allowed to stabilize under nitrogen for 1 h. The fluorescence reagent was pumped at a flow-rate of 0.4 ml/min and the detection was carried out with a 340 nm excitation filter and a 425 nm emission filter.

Ethics and statistics

The study was approved by the Ethics Committee of Tampere University Hospital and was registered at ClinicalTrials.gov, number NCT00484042. The statistical significance of the differences between the groups was tested with the Mann-Whitney U-test. The paired Wilcoxon signed-rank test was used when comparing data between different time-points in the same patients. Correlation was calculated by Pearson test. The multivariate analysis was carried out using multinomial logistic regression for the cut-off values of spermine and putrescine, respectively. Statistical significance was set at $p<0.05$ (two-tailed) and the statistical analysis was performed using the SPSS 11.0 version.

Results

On admission

The spermidine level in the RBC of healthy volunteers was about 2-fold compared with the spermine level, while the putrescine level was about one-tenth of that of spermine (Figure 1a–c). On admission to the hospital, there was no difference in these levels in acute pancreatitis patients and in the patients with other acute abdominal diseases compared with those in healthy volunteers (Figure 1a–c).

None of the polyamine levels measured on admission was varied according to the severity of pancreatitis as assessed by the Atlanta criteria. Polyamine levels had no association with SOFA score. However, on admission, the patients who developed pancreatic necrosis had a higher level of putrescine in their RBCs (Figure 2a), while spermidine and spermine levels remained unchanged. Furthermore, spermine level was significantly higher in the pancreatitis patients with a CRP value >250 mg/l on admission to the hospital (Figure 2b). Since an increase in CRP level is exhibited with a delay of one to two days after onset of the disease, and on admission to the hospital the level may be greatly increased only in the patients with a longer interval from onset of symptoms, a multivariate analysis of the association of both CRP and the duration of symptoms before admission with the spermine level was performed. This showed that CRP, but not the duration of symptoms, had an independent significant association with the spermine level (with the cut-off ≥ 60 pmol/mg protein, $p=0.044$).

Spermidine and putrescine levels were highest among the patients with pancreatitis of unknown

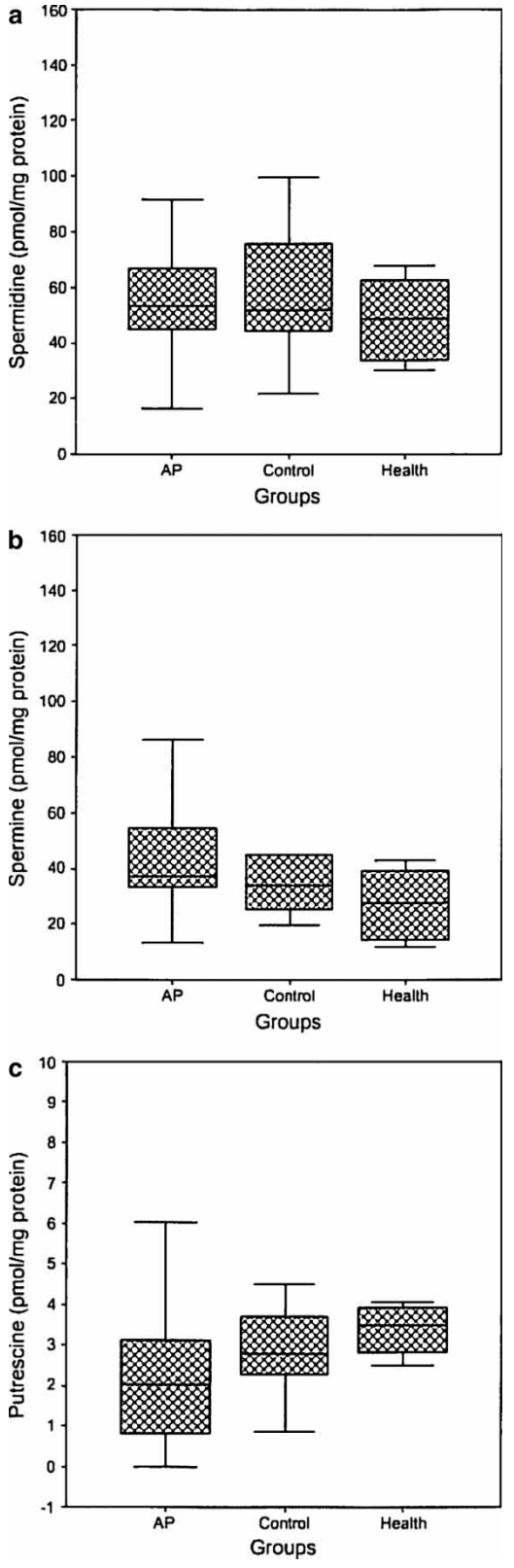

Figure 1. Red blood cell polyamine levels on admission to hospital in patients with acute pancreatitis (AP) and other acute abdominal diseases (controls) and healthy volunteers. There were not significant differences between groups. All data are expressed as medians [range].

etiology, while spermine level did not change according to the etiology. Because necrotizing pancreatitis was found only in the patients with unknown etiology (Table I) and necrosis was associated with increased blood putrescine level, multivariate analysis of the association of necrosis and etiology with the putrescine level was carried out. This confirmed that necrosis, but not the etiology, had an independent significant association with the putrescine level (with the cut-off ≥ 3 pmol/mg protein, $p = 0.024$).

During hospitalization

RBC polyamine levels did not change during hospitalization in acute pancreatitis patients when compared with control patients with other acute abdominal diseases (Figure 3a–c). Occasionally, some polyamine levels showed a statistically significant difference between acute pancreatitis and the control groups, but the ranges overlapped extensively.

Spermidine and spermine levels appeared to remain significantly higher during hospitalization in the patients with acute pancreatitis of unknown etiology compared with patients with acute pancreatitis of alcoholic etiology. Putrescine level was also significantly elevated in patients with acute pancreatitis of unknown etiology compared with those with acute pancreatitis of alcoholic or gallstone etiology. Again, extensive overlaps in variation were seen in all polyamine levels between the groups. When correcting the result in a multivariate analysis according to etiology and presence of necrosis, only necrosis had a statistically significant association with the putrescine level ($p = 0.015$).

After recovery

After recovery, the spermidine and spermine levels had increased significantly from the levels found on admission to hospital, but with huge overlaps of ranges (Figure 2c–d), while the putrescine level remained unchanged. The putrescine level of unknown etiology, however, was significantly higher after recovery compared with other etiologies. Owing to the limited number of samples from patients with necrotizing pancreatitis revisiting the clinic, the statistical significance of the presence of necrosis could not be evaluated by multivariate analysis.

IL-6, IL-10 and CRP

On admission, IL-6 and CRP levels were significantly elevated in patients with acute pancreatitis compared with the control and healthy groups. Both IL-6 and IL-10 had returned to normal levels by the

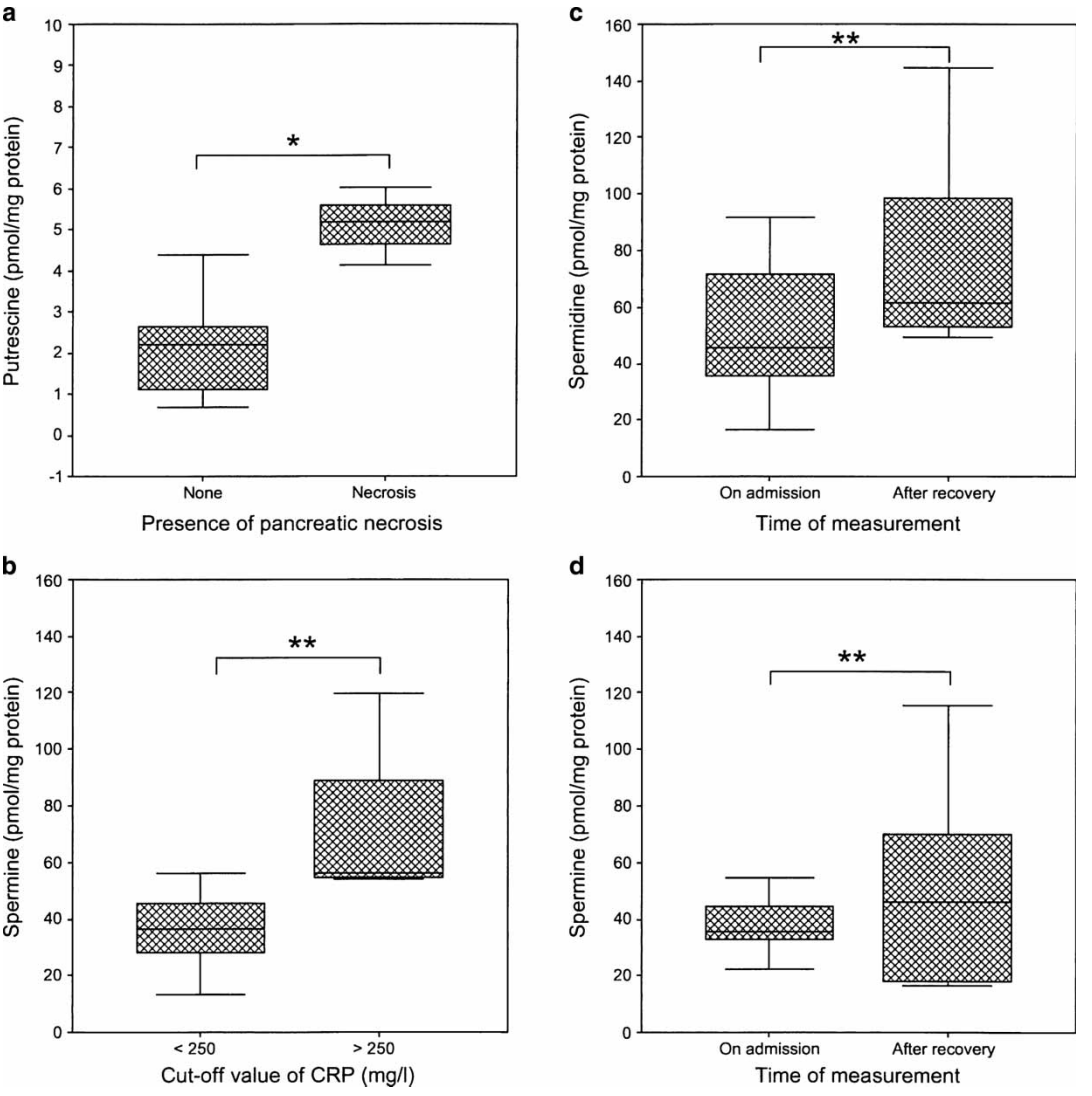

Figure 2. Statistically significant changes in red blood cell polyamine levels according to the presence of pancreatic necrosis (a), plasma C-reactive protein (CRP) level (b), and the timing of sampling (c, d) in patients with acute pancreatitis. All data are expressed as medians [range]. *$p<0.05$; **$p<0.01$.

follow-up visit. The levels of IL-6 and CRP, but not that of IL-10 increased during the first week of hospitalization in patients with acute pancreatitis compared with controls (Figure 3d–f). IL-6 and IL-10 levels, but not levels of CRP, appeared to be highest in acute pancreatitis of unknown etiology.

The level of both IL-6 and IL-10 correlated significantly with the level of putrescine in acute pancreatitis patients (Figure 4a–b), but not with the level of spermidine or spermine. CRP values correlated significantly with the spermine levels (Figure 4c) but not with the level of putrescine or spermidine.

Discussion

In experimental acute pancreatitis induced by taurodeoxycholate, the depletion of polyamines (spermidine and spermine) has been observed not only in pancreas but also in blood, and this change was dependent on the severity of the pancreatitis [6]. Therefore we investigated whether there is a change in RBC polyamines in human acute pancreatitis. Such changes were observed in this clinical study.

RBC polyamine levels were measured with HPLC, as previously described [6,21,22]. This method is laborious and time-consuming and is not appropriate for the clinical emergency setting. Currently, the lack of standardization in HPLC also makes it difficult to transfer from a research setting to the practical clinical laboratory [23]. This study was not primarily intended to develop a clinical marker, but to investigate whether there are similarities or dissimilarities between experimental and clinical acute pancreatitis.

RBC did not contain measurable activity of SSAT (the rate controlling enzyme in polyamine catabolism) and hence RBC polyamine levels might indicate

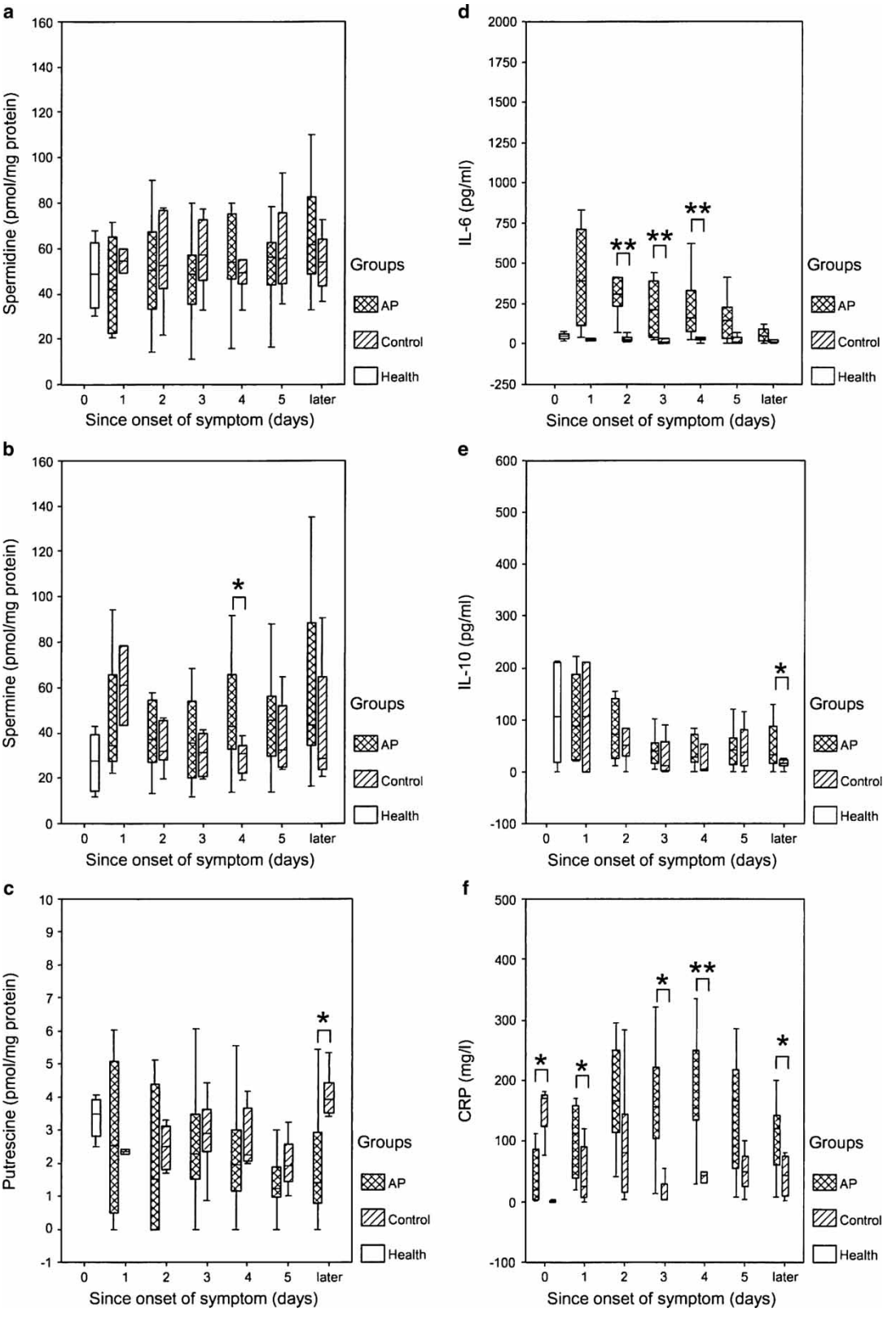

Figure 3. Daily plasma levels of red blood cell polyamines, interleukin (IL)-6, IL-10 and C-reactive protein (CRP) in patients with acute pancreatitis (AP) and other acute abdominal diseases and healthy volunteers during hospitalization. Polyamine values (a–c), all values of interleukin (IL)-6, IL-10 and CRP (d–f). AP, acute pancreatitis; control; other acute abdominal diseases; health; healthy volunteers. All data are expressed as medians [range]. *$p<0.05$; **$p<0.01$.

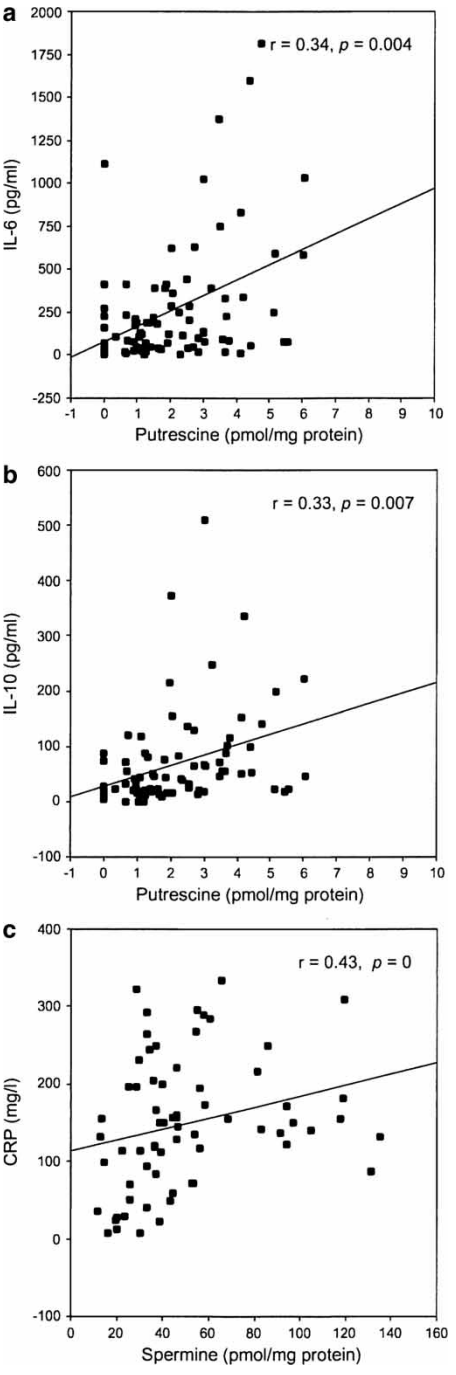

Figure 4. Correlation between plasma inflammatory mediator levels and red blood cell polyamine levels during hospitalization. IL, interleukin; CRP, C-reactive protein.

what is happening elsewhere in the body. RBC polyamine levels were measurable, in contrast to plasma levels that have been below the limit of sensitivity of the assay.

The pancreatitis population was representative based on the clinical data and IL-6, IL-10 and CRP measurements [24,25], which differed significantly between acute pancreatitis, other diseases and healthy volunteers. There was no significant difference in the RBC polyamine levels on admission to hospital between the patients with acute pancreatitis and those with other acute abdominal diseases or between the patients with acute pancreatitis and healthy volunteers. This is in accordance with experimental data where also other conclusions involving anesthesia and surgical procedures induce polyamine depletion [5,6]. Thus polyamine changes in a clinical setting are an unspecific phenomenon.

Severity of acute pancreatitis, as assessed by multiorgan failure (SOFA score) or Atlanta criteria, was not associated with changes of polyamine pool on admission to hospital. However, the higher level of blood putrescine was found in acute pancreatitis patients with pancreatic necrosis compared with acute pancreatitis patients without necrosis. It has previously been reported that pancreatic necrosis also correlated positively with blood putrescine level in experimental pancreatitis [6]. However, owing to the limited number of patients, the present result remains inconclusive. Depletion of blood spermidine and spermine levels [6], which was characteristic of experimental necrotizing pancreatitis, was not observed in the human setting.

Acute pancreatitis of unknown etiology was associated with most polyamine changes, both during hospitalization and after recovery. These changes were found, however, to be due to the more severe pancreatitis in this etiology subgroup. When corrected with necrosis, the polyamine changes did not differ according to etiology; therefore the RBC polyamine changes are not induced by the etiology but rather by the disease itself.

In patients with acute pancreatitis, the levels of IL-6, IL-10 and CRP showed a positive correlation with blood putrescine or spermine levels. Similarly, the experimental study using a polyamine catabolism-induced pancreatitis model of transgenic rats showed that the levels of serum IL-6 and IL-10 increased along with an early time-course, and the IL-6 level, in particular, showed a fluctuation trend similar to the accumulation of pancreatic putrescine at 6 h and 24 h [26]. Therefore, the changes in inflammatory mediators, such as cytokines and CRP, and RBC polyamines concur in the development of acute pancreatitis.

In conclusion, polyamines are neither early nor specific biomarkers for the diagnosis or etiology of acute pancreatitis. However, the association between blood putrescine and pancreatic necrosis and the correlations between RBC polyamines and inflammatory mediators CRP, IL-6 and IL-10 suggest that polyamines change according to the severity of the disease.

Acknowledgements

This work was supported by a grant from Tampere University Hospital Research Fund. We express our thanks to the following collaborators who contributed to this study:

Tampere University Hospital: Marja-Leena Haukkavaara and Tarja Tuomisaari, Department of Gastroenterology and Alimentary Tract Surgery; Annamari Aitolahti and Tuula Kuningas, Center for Laboratory Medicine.

University of Tampere: Taina Ahlgren and Heini Huhtala, Medical School; Leena Honkaniemi and Pirkko Rajala, Clinical Medicine, Medical School; Pekka Vilja and Marja-Leena Koskinen, Medical School; Sami Oikarinen, Department of Virology, Medical School.

The Finnish Red Cross: Arja Vallivaara.

Declaration of interests: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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