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INITIATION OF EXPERIMENTAL ACUTE PANCREATITIS AND MODULATION OF INFLAMMATORY RESPONSE

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Initiation of experimental acute pancreatitis and modulation of inflammatory response

Abstract

Heparan sulfate (HS), a substance common in the extracellular matrix on epithelial cells is present in the pancreas, lining the duct lumen. During pathological conditions, we propose that it can be shed from the ECM and bind to receptors on the cell surface, thus triggering an inflammatory response, which if excessive can cause acute pancreatitis (AP).

Infusion of HS in the pancreatic duct in rats results in an inflammatory response without cellular damage, in similarity with lipopolysaccharide (LPS) infusion. The response differs though between the HS- and LPS-infusion both in regards to expressed chemokines and infiltrating cell types. HS-infusion predominantly causes early expression of CCL2 [monocyte chemoattractant protein-1 (MCP-1)] and subsequent early influx of monocytes. Increased expression of CXCL [cytokine-induced neutrophil chemoattractant-1 (CINC-1)] was not seen and neutrophils were shown to appear in the tissue later during the process. LPS, on the other hand, caused a rapid increase of CXCL (CINC-1) and also in early influx of neutrophils, in addition to similar patterns as HS-infusion of CCL2 (MCP-1) and monocytes.

Taken together, the results indicate a receptor mediated innate immune response. Both HS and LPS has been shown to signal via the same receptor. We therefore suggest a difference in signaling pathways induced by the two ligands.

Furthermore, the AP-induced systemic inflammation was studied. The anti-coagulant active site-inhibited factor VII (fVIIai) was shown to drastically decrease the inflammatory response. The effects in reducing neutrophil infiltration differed substantially between different organs. Nuclear factor kappa B (NFκB) activation was also shown to be affected by fVIIai. Pronounced differences between treatment effects was noted both depending on the studied organ and time point chosen.

In conclusion a novel concept of the initiation of pancreatitis has been studied. The ducal epithelial cells are capable of signaling as a response to HS and LPS and to recruit inflammatory cells. This shows the importance of ductal cells and as a basis of new future pancreatitis specific interventions.

Key words: active site-inhibited factor seven, acute pancreatitis, ductal cells, heparan sulfate, innate immune response, lipopolysaccharide, monocytes, neutrophils, nuclear factor kappa B

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Do not try to short-change the Muse. It cannot be done.
You can't fake quality any more than you can fake a good meal.

William S Burroughs

The truth is what is, not what should be. What should be is a dirty lie.

Lenny Bruce

Who needs answers? One good question would be a relief.

Dan Bern

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CONTENTS

LIST OF PUBLICATIONS

This thesis is based on the following papers, which will be referred to in the text by their Roman numerals. The papers are appended at the end of the thesis.

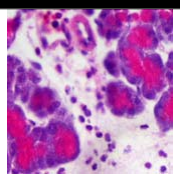
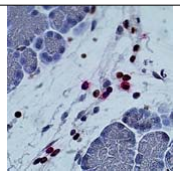
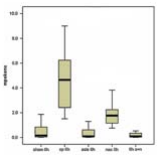
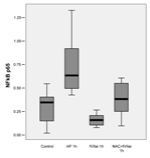
I. Axelsson J, Norrman G, Malmström A, Weström B, Andersson R. Initiation of acute pancreatitis by heparan sulfate in the rat. Scandinavian Journal of Gastroenterology 2008; in press.

II. Axelsson J, Akbarshahi H, Said K, Malmström A, Andersson R. Mechanism of cell-recruitment following heparan sulfate- and lipopolysaccharide-induced acute pancreatitis in the rat. Manuscript in preparation 2008.

III. Andersson E, Axelsson J, Pedersen LC, Elm T, Andersson R. Treatment with anti-factor VII in acute pancreatitis in rats - blocking both coagulation and inflammation? Scandinavian Journal of Gastroenterology 2007;42:765-70.

IV. Axelsson J, Andersson E, Andersson R, Lasson Å. NFκB activation and anticoagulant treatment during acute pancreatitis in rat. Journal of Organ Dysfunction 2008; in press.

THESIS AT A GLANCE

	Question	Method	Result		Conclusion
I	Is HS capable of causing local inflammation of the pancreas?	HS- and TDC-infusion, histology, ELISA, enzymatical measurements	Clear pancreatic and systemic inflammation, but no acinar cell destruction was evident.		HS-induced AP is a receptor-mediated inflammatory response.
II	How is HS-induced pancreatitis mediated?	HS- and LPS-infusion, immuno-histochemistry, ELISA	Rapid MCP-1 and MΦ response were seen. No CINC-1 increase and late neutrophil infiltration.		Early HS-induced AP is mediated primarily via monocyte recruitment.
III	Is fVIIai capable of reducing systemic inflammation during AP?	TDC-infusion, Enzymatical measurements, ELISA	Tissue neutrophil recruitment and systemic inflammation were halted after fVIIai administration.		FVIIai inhibits AP-induced systemic inflammation.
IV	Does fVIIai affect NFκB phosphorylation in distant organs during AP?	TDC-infusion, ELISA	FVIIai administration decreased NFκB activation in a time/organ dependant manner.		NFκB is reduced after treatment with fVIIai.

POPULARIZED SUMMARY IN SWEDISH

(Populärvetenskaplig sammanfattning)

Akut bukspottkörtelinflammation (akut pankreatit, AP) är en allvarlig och vanligt förekommande åkomma för vilken det i nuläget saknas specifika terapier. Årligen drabbas ungefär 300 personer per miljon invånare. Av dessa patienter utvecklar uppåt 20 procent svår akut pankreatit hos vilka både sjuklighet och dödlighet är hög.

Rådande paradigm säger, att det framför allt är pankreas enzymproducerande s.k. acinära celler, som är inblandade vid initieringen av sjukdomen. Vi har visat att de celler som bygger upp bukspottkörtelns gångar på ett mycket tidigt stadium är aktiva. Mycket av de efterföljande skeendena beror på vilka ämnen som initierar inflammationen. Både bakterieprodukter såväl som kroppsegna ämnen är möjliga kandidater för denna initiering.

Pankreas producerar förutom en basisk väska, som är till för att neutralisera magsyran som kommer ut i tolvfingertarmen, även enzym som bryter ner föda. Dessa enzym bryter ner såväl svalda växtdelar som animalisk vävnad. De är med andra ord kapabla att bryta ner den egna kroppens vävnad. Man kan på så vis säga att pankreas är en ”tidsinställd bomb” som, om den inte kontrolleras på ett både snabbt och effektivt sätt, skulle kunna bryta ner även den egna vävnaden. Därtill producerar levern galla som leds via samma gång som pankreas enzymer och även denna skulle vid sjukdomstillstånd vara kapabel till att skada kroppens egna celler.

Detta kräver ett snabbt försvar och mobilisering av celler, vilka tar hand om skadade celler och även eventuella bakterier från tarmen, som tagit sig mot strömmen upp i pankreas. De första celler som denna potentiellt skadliga vätska kommer i kontakt med är pankreasgång-celler. Ytan på dessa celler bekläds av extracellulär matrix som innehåller sulfaterade sockerstrukturer, s.k. heparansulfater. Dessa skulle kunna klyvas från cellytan av både gallans gallsalter och pankreas enzymer. Som fria molekyler skulle de kunna binda till receptorer på den egna eller närbelägna gångceller och signalera ”fara” vilket i sin tur skickar dit celler från immunförsvaret, som kan rensa upp skadade pankreasceller samt är kapabla att döda bakterier. I händelse av en kraftig stimulering, överaktivering, av dessa receptorer skulle en allt för stor ansamling av inflammatoriska celler, monocyter och neutrofiler, kunna ske. Detta skulle i så fall kunna leda till en i överkant tilltagen inflammatorisk respons och utveckling av akut pankreatit.

Vi har visat, att tillförsel av heparansulfat i pankreasgången ger en inflammation med rekrytering av inflammatoriska celler. Vilka inflammatoriska celler som rekryteras först beror på vilken substans som tillförs. Heparansulfat, som finns på de epiteliala duktcellerna, ger en ansamling av monocyter medan lipopolysackarider, som finns på utsidan av Gram-negativa bakterier, först orsakar en ansamling av neutrofiler. De av heparansulfat rekryterade monocyterna rekryterar i sin tur neutrofiler. Vilka orsaker till pankreatiten och vilka cellpopulationer som dominerar kan säkerligen ha mycket stor betydelse för utgången för dessa patienter och även avgöra hur behandlingen bör anpassas. Dessa fynd skulle i framtiden kunna användas som underlag till behandling av en grupp patienter som i nuläget bara erbjuds symptomatisk behandling d.v.s. ingen behandling mot de egentliga orsakerna till sjukdomen.

Patienterna som drabbas av svår akut pankreatit utvecklar ofta också systemisk inflammation med sviktande funktion av ett eller flera andra organ som följd. Denna inflammation påverkar koagulationen och tvärt om. Koagulationen består av kedja av olika ämnen som påverkar

varandra som en kaskad. I slutet av denna kaskad bildas en blodpropp som stoppar eventuell okontrollerad blödning. Eftersom koagulation och inflammation är intimt sammankopplade har vi undersökt om blockad av koagulationskaskaden kan påverka den systemiska inflammationen vid akut pankreatit. Vi tillförde en icke funktionell mediator, active site-inhiberad factor VII, och kunde då påvisa en minskad systemisk inflammation, men ingen påverkan på inflammationen i pankreas. Utöver detta framkom det även att aktiviteten hos en för inflammation central mediator minskade. Det som dödar patienterna med svår akut pankreatit inte är den ursprungliga inflammationen i pankreas, utan det faktum att ett eller flera av kroppens andra organ slutar fungera. En behandling av detta slag skulle kunna vara till stor nytta för denna patientgrupp.

ABBREVIATIONS

AP	acute pancreatitis
CINC-1	cytokine-induced neutrophil chemoattractant-1 (CXCL1)
CRP	C-reactive protein
ECM	extra-cellular matrix
ELISA	enzyme-linked immunosorbant assay
EMSA	electrophoretic mobility shift assay
ERCP	endoscopic retrograde cholangiopancreatography
fVIIai	active site-inhibited factor seven
H_2O_2	hydrogen peroxide
HS	heparan sulfate
HSPG	heparan sulfate proteoglycan
Ig	immunoglobulin
IHC	immunohistochemistry
IL	interleukin
IL-6	interleukin-6
IL-8	interleukin-8 (CXCL8)
IP	intraperitoneal
LPS	lipopolysaccharide
MAL	MyD88 adaptor like
MCP-1	monocyte chemoattractant protein-1 (CCL2)
MIP-2	macrophage inflammatory peptide-2 (CXCL2)
MΦ	macrophage
MODS	multiple organ dysfunction syndrome
MPO	myeloperoxidase
MyD88	myeloid factor 88
NAC	*N*-acetyl cystein
NFκB	nuclear factor kappa B
PAMPs	pathogen-associated molecular patterns
PBS	phosphate buffered saline
PFA	phosphate-buffered formaldehyde
PG	proteoglycan
PSCs	pancreatic stellate cells
SIRS	systemic inflammatory response syndrome
TIR	Toll/IL-1 receptor
TRAM	TRIF-related adaptor molecule
TRIF	TIR-related adaptor protein inducing interferon
TLR	Toll-like receptor
TNF-α	tumor necrosis factor-α

INTRODUCTION

Acute pancreatitis (AP) is a serious and, in its severe form, life-threatening condition. No specific treatment is available so far but only symptomatic treatment of the secondary events is targeted.

Much knowledge of mechanisms underlying AP has been gained using a variety of animal models. Still today much of the same concepts regarding causes and mechanisms are discussed as they were 100-150 years ago. Many issues need to be clarified in order to give a more tailored treatment to the AP patients.

A majority of the research has been focusing on the acinar cells as the cells of origin in the initiation, as these are the first damaged observed cells in the opossum ligation model of AP and also human biopsies taken at later stages of the disease. Although not seemingly damaged, the ductal cells may very well be the cell initiating the events leading to AP. In this thesis a novel hypothesis on the origin of pancreatitis involving the ductal cells is presented. The pancreas produces a variety of proteases and lipases, all capable of causing severe damage to the tissues if their activities are not tightly regulated. These defense and surveillance mechanisms need to be most efficient and react very rapidly to any sign of malfunction of the epithelium or invading pathogens. The “ductal defense” hypothesis presented herein answers several of these questions as well as gives a reasonable explanation of the initiation of AP, when this defense system is overly activated.

In this proposed novel hypothesis, heparan sulfate (HS) is shed from the ductal epithelium in response to noxious stimuli such as the action of activated proteolytic enzymes or bile salts. This in turn binds to receptors on the same, or neighboring, cells, causing intracellular signaling and expression of chemoattractants for, particularly, monocytes as a physiological response of clearing the immediate danger of damaged ductal cells. Dysfunctional ductal cells as well as invading bacteria in the pancreatic duct could quickly lead to damage of the gland and uncontrollable release of proteolytic enzymes. Therefore a rapid response, such as provided by the innate immune system, is required. During pathological conditions, this physiological response could be aggravated causing excessive cell infiltration leading to inflammation, pancreatitis. The cellular response to HS differs from the response elicited by lipopolysaccharide (LPS), possibly through different signaling pathways. This interesting finding may have great importance when designing future AP-specific therapies and treatment regimes.

Another issue facing the medical service personal is systemic inflammation as a consequence of severe AP. This condition, often called systemic inflammatory response syndrome (SIRS), can lead to one or more failing remote organs, another condition termed multiple organ dysfunction syndrome (MODS). Counteraction of these life-threatening conditions is essential in the emergency care.

Systemic inflammation affects the coagulation and the other way around. For example, activated protein C is widely used in the severe cases of sepsis. Inhibition of the early events of the intrinsic pathway of the coagulation pathway has shown to be the most influential when attempting to reduce the inflammation. We therefore investigated the impact of active site-inhibited factor VII (fVIIai) on the AP-induced systemic inflammation in a model of severe AP in rats. Dramatic effects on reduction of the inflammatory mediators released systemically were demonstrated while no changes locally in the pancreas could be seen.

Many aspects of the “ductal defense” theory need to be elucidated. Current research in our lab focuses on the signal transduction pathways initiated by HS. Some preliminary data suggest other cell types to be involved in the initiating events and should be further investigated.

GENERAL BACKGROUND

Clinical acute pancreatitis

Definition
Acute pancreatitis (AP) is a clinical term used for an inflammatory process in the pancreas (Bradley 1993). It involves, to varying degrees, local inflammation of the pancreas and may also progress into failure of one or several distant organs. (Beger et al. 1997).

AP is diagnosed by pain in the upper part of the abdomen followed by elevated plasma concentrations of pancreatic amylase and lipase (Steinberg et al. 1994), as well as C-reactive protein (CRP).

Incidence and etiology
Incidence of AP is, in several studies, around 300 per 10^6 in North-western Europe (Wilson et al. 1990, Appelros et al. 1999, Andersson et al. 2004).

The most common cause of AP in men is alcohol abuse and in women the predominant cause is gall stones (Dufour et al. 2003). The overall main causes can be summarized as follows; gall stones (45%), alcohol abuse (35%), idiopathic (10%), as well as other more rare causes such as post-operation, post-myocardinal infarction and trauma (10%) (Steinberg et al. 1994). Of the latter 10% of miscellaneous causes post-endoscopic retrograde cholangio-pancreatography (ERCP) examinations represent about 3% (de Beaux et al. 1995).

About 80% of the cases of AP are regarded as mild AP and revert by itself, while the rest are severe AP with a considerable morbidity as well as high mortality. Death rates around 5% have been reported in Western Europe and a trend of decreasing frequencies (Wilson et al. 1990, Jaakkola et al. 1993, de Beaux et al. 1995), probably due to earlier diagnosing and more efficient intensive care.

Pathological findings
Histological specimens of AP biopsies are characterized by necrotic acinar cells, vasculature and adjacent adipocytes. Destruction of the vascular endothelium causes hemorrhaging, a consistent feature of AP. Elevated levels of pancreatic elastase and lipase are associated with pancreatitis and more specifically with the destruction of pancreatic acinar cells. During ERCP-induced AP, triggered possibly by barotrauma and osmotic insult, it has been found that pancreatic amylase and lipase increase rapidly after the intervention, peaking after around 8 hours. Lipase both increases and decreases somewhat earlier than amylase (Okuno et al. 1985, Panteghini et al. 1991), the more rapid decrease of lipase depending mainly on its shorter half-life (Junge et al. 1985).

Characteristic of inflammatory infiltrate is a high count of neutrophils. Also macrophages as well as CD4+ and CD8+ cells have been recovered from necrotic tissue of AP patients (Bhatnagar et al. 2001).

Comparative anatomy and physiology of the pancreas

Pancreas anatomy and morphology
Generally, in both man and rodents, the pancreas consists of two parts with two very different functions. The exocrine part, consisting of enzyme producing acinar cells and electrolyte

producing ductal cells, contributes to secretion of digestive enzymes and HCO_3^- rich fluid and is emptied in the duodenal lumen. The endocrine pancreas, which is scattered as islets in the organ, is involved in the regulation of glucose in the blood by responding to glucose levels and secretion of insulin and glucagon to the blood stream.

The gross anatomy of rats and mice are very different from man and pigs (Fig. 1). In human, the pancreas is a well defined organ. In the rat and mouse it is a more diffuse organ exhibiting several lobes (Krinke 2000, Hedrich et al. 2004) and is embedded in the dorsal mesentery. The organ is, in the rat, subdivided into three major lobes, without well-defined borders; right lobe (parabiliary and duodenal segments), body (gastric and splenic segments) and left lobe (terminal part of the splenic segment) (Hebel et al. 1986, Eustis et al. 1990). Such subdivision is claimed not to be apparent in the mouse (Hedrich et al. 2004).

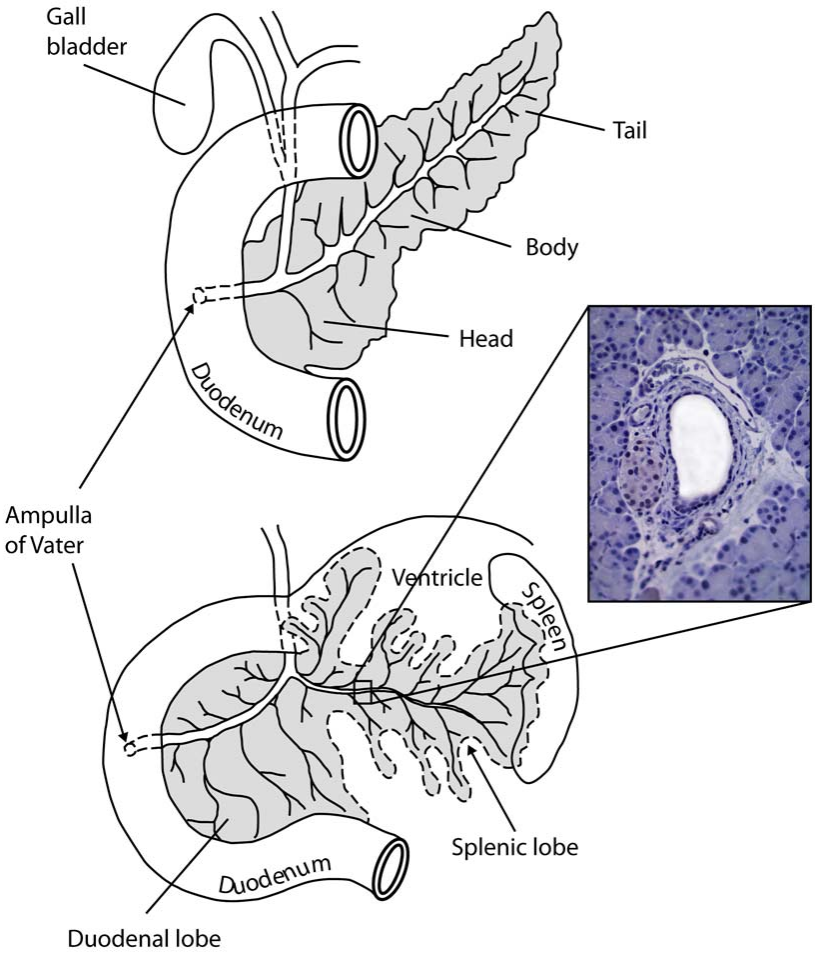


Figure 1. *Gross anatomy of man (above) and rat (below). In the organs from both species similar parts can be found; head (duodenal loop), body (ventricular loop) and tail (splenic lobe).*

The exocrine pancreas is the part of the pancreas affected during AP. Exocrine tissue makes up the major part of the entire organ. The exocrine pancreas mainly consists of acinar cells forming acini, which are drained by larger and larger ducts and finally drain into the common bile duct, ductus choledocus. In man the main pancreatic duct is single and in most cases drains into the common bile duct. In more rare cases, 5-10% of the population, the pancreatic duct is not fully fused and the head of the pancreas drain into the common biliary duct while the body and tail of the pancreas drain directly into the duodenum without any connection to the common bile duct (Kamisawa 2004).

In the rodent it is different. The rat has at least two main pancreatic ducts, posterior and anterior pancreatic ducts, and there can sometimes be as many as 5-8 ducts (Krinke 2000). The anterior pancreatic duct drains the gastrosplenic part of the pancreas and the posterior pancreatic drains the duodenal part. They have a large number of smaller assessory ducts emptying into the biliopancreatic ducts as well (Kara 2005). There is a considerable variation in the ductal system in the rat, but this was not found to be correlated to different strains when SD and Wistar rats were compared (Kara 2005). Strain dependant differences have been observed when comparing different strains of mice (Lena Kvist, Lund University, Lund, pers. comm.).

Morphologically, the pancreas consists mainly of acinar cells among which different other cell types are unevenly distributed. The exocrine pancreas contains as well as acinar cells, ductal epithelium, vascular endothelium and fibroblasts. The acinar cells are arranged in clusters, acini, which have an empty center and are surrounded by connective tissue (Fig. 2). From the cavity of the acini, ductal epithelial cells form ducts, which also are surrounded by connective tissue. In humans, secretions from the acini first drain into small intralobular ducts, which deliver the secretions to larger interlobular ducts, which finally drain into the main pancreatic duct. In rats and mice, the interlobular ducts empty directly into the common bile duct (Mann et al. 1920). The intralobular ducts proximally consist of intercalated ducts which are lined with flattened cuboidal epithelium. The first part of the intercalated ducts project into the lumen of the acini, where the epithelial cells are called centroacinar cells. The distal part of the intralobular ducts consists of classical cuboidal epithelium and is found within the lobules. From the intralobular ducts the secretions are drained into interlobular ducts. They vary in size and the smaller ducts consist of cuboidal epithelium while the larger ducts consist of columnar epithelium. The interlobular ducts are localized within the connective tissue septae and deliver the secretions to the main pancreatic duct. The main pancreatic duct consists of a single layer of columnar epithelial cells on basal lamina.

The morphology of ductal cells, in contrast to acinar cells, differs greatly between human and rat. In human the ductal cells constitute approximately 14% of the pancreatic tissue volume, while the ducts in the rat constitute only 2% (Githens 1988).

Fibroblasts and resident macrophages are found among the acini as well as in the interstitial space in close proximity to the ducts.

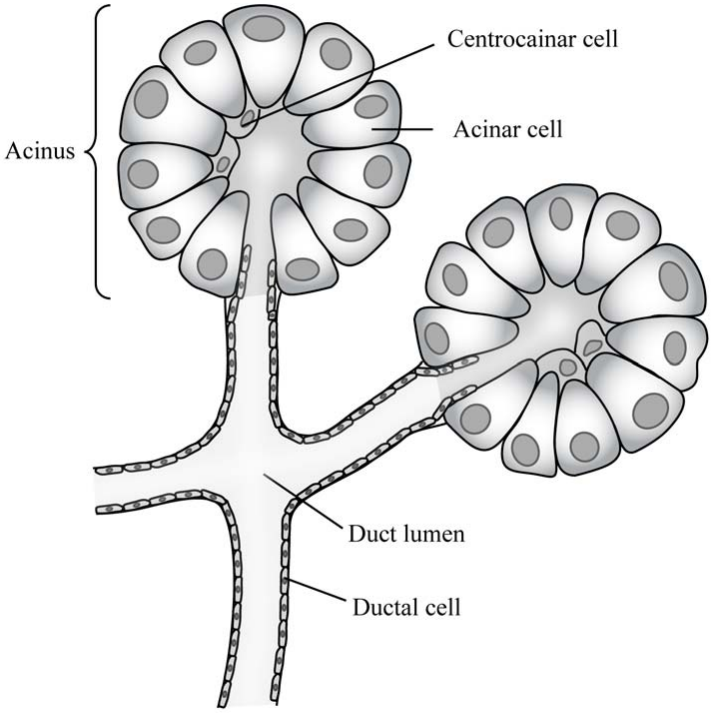


Figure 2. *Morphology of the acini and adjacent ducts. Each acinus consists of a cluster of acinar cells surrounded by connective tisse. Between the acinar cells and the ductal epithelium, centroacinar cells are found.*

Pancreas physiology

The exocrine part of the pancreas has two main functions, to deliver digestive enzymes, proteases, lipases and saccharidases, as well as HCO_3^- fluid to the jejunum. Enzymes are synthesized and secreted by the acinar cells of the pancreas and transported via the ducts to the common bile duct where it is transported together with bile to the intestine.

The secretion of enzymes from the acinar cells is regulated by cholecystokinin (CCK), but the importance of CCK as a stimulator differs between man and rat, as it has been shown that CCK receptors of the acinar cells play a minor role in the regulation of the secretion from acinar cells. Furthermore, there is a difference in the CCK receptor set in man and rat. The acinar cells of rats and mice have high- and low-affinity CCK-A receptors, signaling for enzyme secretion when stimulated. In human acinar cells, CCK-A receptors are not present. This difference is important in the caerulein model of AP and is discussed in this paragraph.

In man, CCK stimulates secretion of enzymes from acinar cells but has not been reported to enhance fluid secretions. In contrast to the human, rats respond by secretion of large volumes of liquid. Another unique property of the acinar cells in rats, and probably mice, is that CCK stimulation results in large Cl^- secretion. This is not seen in either human or in pig.

The HCO_3^--rich secretion is produced by the ductal epithelial cells lining the ducts in response to secretin in all species studied. Differences between the function of the ductal cells in humans and rats have though been shown. In humans the intercalated ducts are responsible of HCO_3^- secretion, while in the rat the interlobular ducts are probably the most important in this aspect (Case 2006). After maximal secretin-induced HCO_3^- secretion, the concentration measured in the rat is only half of that found in man.

Coagulation
The coagulation cascade and related events have numerous times been reviewed elsewhere and will not be repeated here. Some interesting and most likely relevant differences between man and rodents, such as concentration differences of different mediators of the coagulation cascade, exist (Walker 2004). These differences may be important when interpreting the results in rat and should be carefully considered before attempting to extrapolate findings from one species to another.

Pancreatic tissue and inflammatory response

The involvement of both resident and infiltrated inflammatory cells in AP were recently reviewed (Vonlaufen et al. 2007a). The involvement of different resident and infiltrating cells during the early events of AP are purely learned from animal studies. In the case of the later events involving the inflammatory cells present at this time, some knowledge has been derived from patient biopsies. Bearing in mind all the limitations of animal models of AP, I would like to present a summary of the AP pathophysiology relevant to the topics of this thesis.

Resident pancreatic cells

Acinar cells
Production of secreted digestive enzymes is restricted to the acinar cells of the pancreas. The acinar cells are arranged in acini, opening into a space that is drained by pancreatic ducts. Results from duct-ligation studies of the American opossum have suggested acinar cells to be involved in the initiating events of the onset of AP. They are capable of expressing CCL2 (MCP-1) but not CINC (Bhatia et al. 2002).

Ductal cells
The cells lining the pancreatic ducts have so far attracted little recognition in the field of AP, and have until recently been considered merely to be involved in the regulation and secretion of electrolytes. Ductal cells have been shown to produce CXCL8 (IL-8) in humans as well as CXCL2 (MIP-2) and CXCL1 (CINC-1) in rats (Osman et al. 1999), therefore being able to recruit neutrophils directly. The same cell type has recently been shown to be capable of expressing CCL2 (MCP-1) upon stimulation with LPS and HS (Axelsson et al. 2008a), making them able to attract monocytes as well.

Resident macrophages
It has been proposed that resident pancreatic macrophages are not involved in the initiation of AP (Gloor et al. 1998, Pastor et al. 2006). This is based on the observation that inhibition of macrophage activation did not reduce the pancreatic tissue destruction in the caerulein model. Compared to other organs such as the Kupffer cells of the liver, the resident pancreatic macrophages are very few in the rat.

At later stages of the disease, macrophages probably play a more active role. It has been shown that macrophages from AP patients are more activated and respond more readily to LPS stimulation than macrophages from healthy tissue (Bhatnagar et al. 2001).

Fibroblasts/pancreatic stellate cells
Recently, myofibroblast-like cells, coined stellate cells, have been characterized in the liver. Similar cells have been shown in the pancreas and are believed to be important, just as their hepatic counterparts, in fibrosis. Most research has therefore been focused on chronic pancreatitis where extensive fibrosis as well as sub-acute inflammation are important components of the clinical picture of the disease.

Extracellular matrix

The vast majority of cells in the body express extracellular matrix (ECM) molecules on their outer surface. These molecules are synthesized in the cell and secreted to the outer side of the cell to make up a complex framework of cross-linked proteins and saccharide structures. The "classical" function of ECM is as a supporting structure but this is far from the only function it has. Many of the essential functions of the body are dependent on ECM, such as chemotaxis, proliferation, coagulation, and several other vital body functions.

ECM is present on the epithelial cell surface, between the cells, inside the cells and beneath the cells as the basal lamina. The acini of the pancreas are divided by septae consisting of connective tissue, basal lamina, which also is present in large amounts around the pancreatic ducts (Fig. 3).

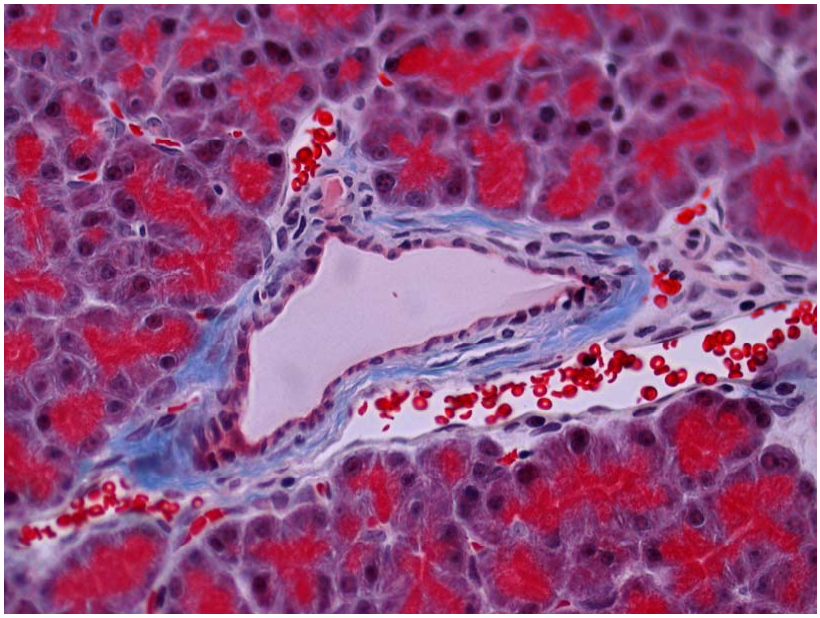


Figure 3. *Collagen (light blue) surrounding the pancreatic duct visualised by Masson's trichrome staining. Also visible in the picture are veins containing erythrocytes (red), as well as cytosol (red) and nuclei (dark purple) of acinar cells.*

ECM consists of a complex structure made up from glycoproteins and proteoglycans (PG) (Hay 1991). Depending of cell type and localization, different PGs are present.

Collagen

The most abundant protein of the ECM is collagen (Di Lullo et al. 2002). It consists of three polypeptides bound together in a helix. It provides connective tissue, tendons and vasculature its strength and flexibility. The pancreas in humans as well as in rats consists of 2-3% collagen as measured as percent of total protein content and is found evenly distributed over the gland i.e. no major differences between head, body or tail of the pancreas (van Suylichem et al. 1995). In the exocrine pancreas of rats, collagen is found in the largest amounts surrounding the ducts and is thicker around the main ducts and becomes increasingly thinner as the diameter decreases (Hosoyamada et al. 2003). Smaller amounts are found in both the inter- and intralobular septa, as well as surrounding all acinar cells in several species, including rats, pigs and humans (van Suylichem et al. 1995). In pig, collagen type VI has been shown to dominate over type I, IV and V in both inter- and intralobular septae (White et al. 1999).

Proteoglycans

PGs are made of a core protein and one or more bound sulfated glycosaminoglycan (GAG) chains. Numerous PGs are present in the pancreas and are synthesized by a variety of cells.

As mentioned above ECM components are present at various locations and the constituents vary depending on the localization. PGs are present both intracellularly and extracellularly. The extracellular PGs are found in the ECM of the cell surface and between cells as well as in the basal lamina (Fig. 4).

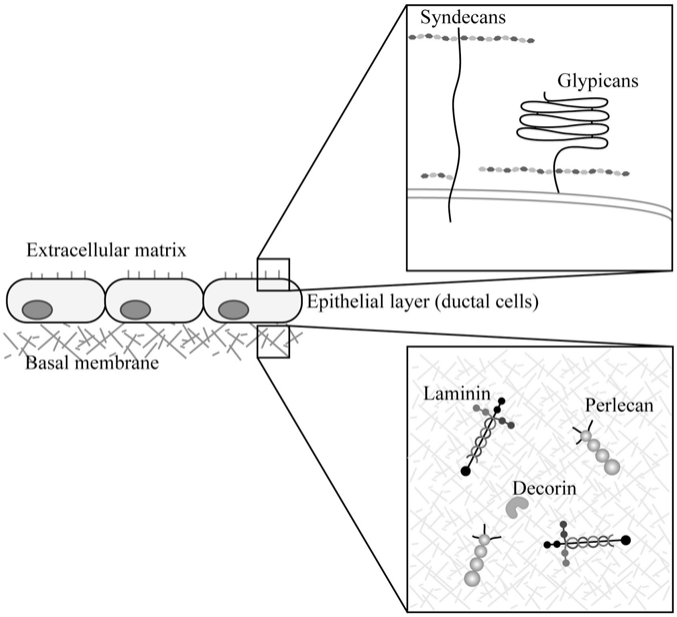


Figure 4. *Proteoglycans surrounding the ductal epithelium of the pancreas.*

Two main families of cell surface ECM PGs contain HS, the heparan sulfate PGs (HSPG) syndecans and glypicans. Syndecan-1 (SDC1), is one of the most common HSPGs in

humans and has well conserved homologues in mice and rats. It is expressed in the ductal cells of the pancreas and in lower amounts in acinar cells (Conejo et al. 2000). Glypican-1 is present in pancreatic fibroblasts and is also up-regulated on the cell surface of ductal-like cancer cells (Kleeff et al. 1998).

The basal lamina beneath the epithelial layer contains several different PGs as well. Decorin is present around the pancreatic ducts and vasculature but is absent from acinar cells and islets (Koninger et al. 2006). The basement membrane surrounding the acini is rich in perlecan (Murdoch et al. 1994).

Among the various roles of HSPGs chemotaxis is essential in the inflammatory response. The role of PG-mediated chemotaxis is of vital importance in migration of inflammatory cells.

PGs are capable of capturing and loosely binding chemokines, e.g. CCL2 (MCP-1). The chemokines then bind firmly to its receptor and trigger signaling events. This is important in vasculature and lymph ducts where otherwise flow and diffusion would prohibit high enough concentrations of chemokines to elicit the inflammatory response, which was first proven as late as 2003 (Proudfoot et al. 2003).

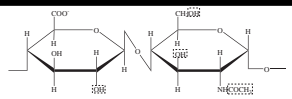
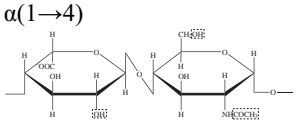
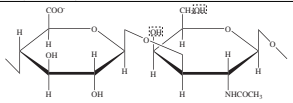
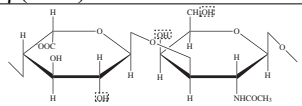
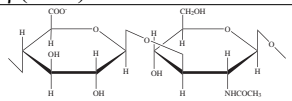
Apart from being passive carriers of chemokines on the cell surface, they can also be cleaved off and themselves trigger inflammation and cell recruitment. When damage to the cell surface occurs, PG components can be shed and bind to TLRs of the innate immune system.

Heparan sulfate

The HSPGs contain 2-3 HS chains. HSs are, just as chondrotin sulfate and dermatan sulfate, negatively charged acidic GAGs, consisting of repeating disaccharide utits, which to varying degrees are sulfated. The main disaccharide units in PGs are shown in table 1. The most common disaccharide units of HS is glucuronic acid (GlcA) linked to N-acetylglucosamine (GlcNAc). HS is able to bind a huge variety of ligands and therefore interact in numerous biological processes. Among many other, HS is capable of binding; IL-8, antithrombin III, as well as fibroblast and vascular endothelial growth factors.

A growing body of evidence points to the important role of HS in inflammation. The acidic and heavily charged properties, and probably as well as more specific structural properties, HS is able to trap various cytokines when bound to PGs on the cell surface. When it its soluble form it can interact with inflammation in other ways, namely acting as receptor ligands and initiating inflammatory cell recruitment. Shedding of HS has been shown after tissue injury (Subramanian et al. 1997, Kato et al. 1998) and has been proposed to be monitoring tissue injury and repair (Johnson et al. 2002). Initiation of SIRS has been shown to be possible by intraperitoneal administration of soluble HS via TLR4 (Johnson et al. 2004). Biglycan is a chondrotin/dermatan sulfate containing PG. It has been shown to activate NFκB, via MyD88 by triggering TLR4 as well as TLR2 (Schaefer et al. 2005). Structural differences between the sulfated polysaccharides exist and HS has so far not been shown to signal via TLR2.

Table 1. Selected polysaccharides of glycosaminoglycans. The degree of sulfation can vary considerably and sulfate groups are therefore excluded from the structures. The positions of possible sulfation are encircled with a dashed line.

GAG	PG	Hexuronic or Iduronic acid	Hexosamine	Disaccharide composition
Heparan sulfate	Syndecans Glypicans Perlecan	D-glucoronic acid (GlcA) L-iduronic acid (IdoA)	D-glucosamine (GlcNAc)	 GlcAβ(1→4) GlcNAc α(1→4)  IdoA α(1→4) GlnNAc α(1→4)
Chondrotin sulfate	Decorin Biglycan	D-glucoronic acid (GlcA)	D-galactosamine (GalNAc)	 GlcAβ(1→3) GalNAc β(1→4)
Dermatan sulfate	Decorin Biglycan	D-glucoronic acid (GlcA) L-iduronic acid (IdoA)	D-galactosamine (GalNAc)	 IdoA β(1→3) GalNAc β(1→4)
Hyaluronan		D-glucoronic acid (GlcA)	D-glucosamine (GlcNAc)	 GlcAβ(1→3) GlcNAc β(1→4)

Cell signaling and inflammatory response

Many cells of the body are capable of initiating an innate immune response by signaling for recruitment of immune cells. The innate immune system is a very rapid way for the organism to deal with injured cells and invading pathogens. The most important group of receptors signaling for “immediate danger” is the TLRs. The response is very rapid as the signaling is transducted via transcription factors already transcribed and available in the cytosol of the cell. They are translocated over the membrane of the nucleus, stimulating transcriptions or various mediators of inflammation, including chemokines. The chemokines are then secreted, bound to extracellular HS structures and hence providing a gradient facilitating chemotaxis of immune cells.

Toll-like receptors

The innate immune system is the organism's defense against invading pathogens. TLRs are highly conserved surveillance receptors alerting the innate immune system of unknown threats. They all share a conserved extracellular leucin-rich domain and an intracellular Toll/IL-1 receptor (TIR) domain (Rock et al. 1998) that interacts with TIR domains of a variety of adaptor proteins. This group of receptors was first discovered in *Drosophila melanogaster*, where it constitutes the organism's entire immune system, but homologues were soon discovered in humans as well (Medzhitov et al. 1997). In humans, 10 different receptors have been describes to date, all recognizing specific pathogen-associated molecular patterns (PAMPs). Bacterial, viral and endogenous components have all been shown to act as ligands to the different TLRs.

There is a distinct possibility that several of the TLRs are involved in the early events of the pathogenesis. This was proved during in the mid-1950s, though more than 50 years would pass until the signaling events of the substances used were shown. In an initial experiment Thal and Brackney (1954) showed that LPS prepared from the Gram-negative *E. coli* could initiate AP when infused into the pancreatic duct of rabbits (Korn 1963) and goats. Rats also develop AP from *E. coli* LPS infusion (Axelsson et al. 2008a), as do dogs (Egner et al. 1956), although not all studies have been able to confirm that LPS alone can trigger AP in dogs (Williams et al. 1968). *E. coli* LPS is known to specifically trigger TLR4 but not TLR2 (Tapping et al. 2000). Thal and Molestina (1955) also showed that similar results could be obtained in rabbits and dogs when toxins from the Gram-positive genus *Staphylococcus*, known to trigger TLR2 but not TLR4 (Han et al. 2003, Schroder et al. 2003), were infused. The LPS infusion in Thal's experiments was reported not to cause trauma or rupture of the ductal cells, indicating that the response is a receptor-mediated response. This clearly demonstrates the presence of functional TLR2, TLR4, LPS binding protein (LBP), CD14 and MD2 in or in close proximity of the pancreatic duct of these species. LPS signaling molecules have been shown at increased levels during caerulein-induced AP suggesting that these pathways are important (Wang et al. 2005).

LPS has been suggested to be involved in alcoholic AP by reaching the pancreatic tissue via the circulation. This is due to the observation that chronic alcoholics have increased gut permeability. Both human and rat pancreatic stellate cells (PSCs) express TLR4 and CD14 and have been shown to be activated by ethanol and LPS exposure in vivo (Vonlaufen et al. 2007b). Ethanol exposure has also been shown to increase the susceptibility of necrosis instead of apoptosis as is the case without previous ethanol exposure (Fortunato et al. 2006).

The TLRs are signaling via two main pathways, the MyD88-dependant or the MyD88-independent pathways. All TLRs but TLR3 are capable if signaling through the MyD88-dependant pathway, inducing NFκB nuclear translocation and transcription of pro-inflammatory cytokines. Both TLR4 and TLR3 can signal via the MyD88-independent pathway, mediated by TRIF. This finally leads to either translocation of the transcription factors NFκB or IRF-3 and IRF-7 into the nucleus, resulting in transcription of pro-inflammatory cytokines or IFN-α and IFN inducible genes, respectively.

Figure 5 summarizes the cellular events when the ligands of TLR2 and TLR4 bind to their receptors. TLR4 and TLR2 use several adaptor proteins in the transduction signaling pathway. The four most important of these are; myeloid factor 88 (MyD88), MyD88 adaptor like (MAL), TIR-related adaptor protein inducing interferon (TRIF) and TRIF-related adaptor molecule (TRAM).

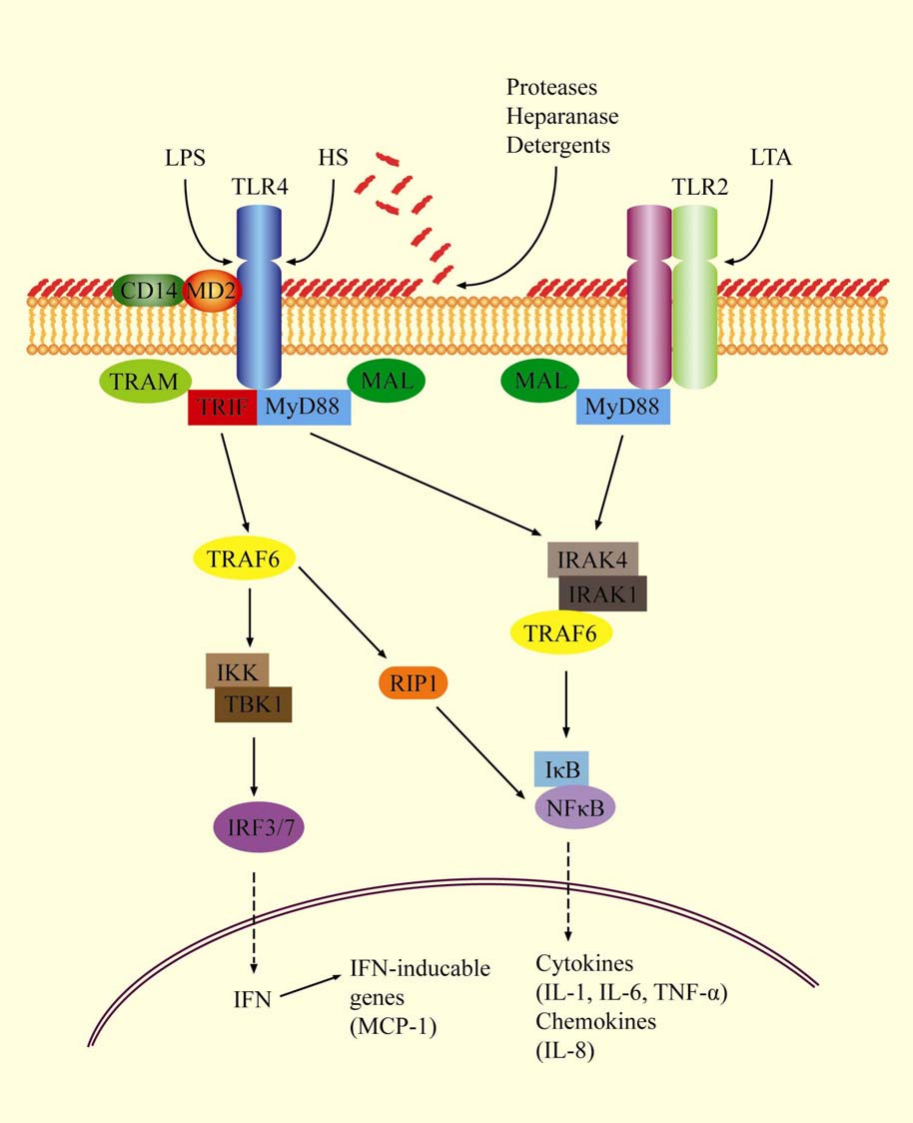


Figure 5. *Signaling pathways of TLR4 and TLR2. Ligands to TLR4 include endogenous heparan sulfate shed from the cell surface as well as lipopolysaccharides from Gram-negative bacteria. TLR4 utilizes both the MyD88 and TRIF pathways. TLR2 is mainly triggered by products of Gram-positive bacteria and uses exclusively the MyD88 pathway.*

TLR4

TLR4 was the first human TLR to be discovered (Medzhitov et al. 1997). The first described and most well known of the ligands of TLR4 is LPS (Poltorak et al. 1998). The LPS response is mediated through several associated proteins. In serum, LPS is bound to LPS-binding

protein (LBP) and transported to CD14 (Takeda et al. 2003). The CD14 in turn lets LPS bind to the complex of TLR4 and MD2, which triggers the signal. Numerous other ligands, both exogenous and endogenous, have been described. Recently ECM components have been shown to be ligands of TLR4. Both hyaloranan oligosaccharides (Termeer et al. 2002), fibronectin fragments (Okamura et al. 2001), produced in response to tissue injury, biglycan (Schaefer et al. 2005), a chondrotin/dermatan sulfate containing PG, mindin (He et al. 2004), and HS (Johnson et al. 2002) have been shown to trigger TLR4 signaling.

TLR4 has been shown to be present in the ductal cells in the rat, but in no other parts of the exocrine pancreas, as well as in vascular endothelium and islets (Li et al. 2005). The receptor is not only expressed but fully functional, as discussed above, in response to LPS. We have also shown that it is very likely that HS stimulation of the pancreatic cells using HS also signals via TLR4. This will be discussed later in the thesis.

TLR4 is a monomeric receptor capable of signaling both via the MyD88-dependant and MyD88-independent pathways. Which pathways that dominates is depending on the ligand responsible for the signaling.

TLR2

The TLR2 receptor is able to recognize a variety of substances of Gram-positive bacteria and *Mycoplasma*. Unlike TLR4, which functions as a monomer, TLR2 always exerts its actions as a dimer (Triantafilou et al. 2006), either in combination with TLR1 (Ozinsky et al. 2000) or TLR6 (Takeuchi et al. 1999a, Takeuchi et al. 1999b). This contributes to its wide range of PAMPs recognized. Ligands include bacterial lipoproteins and lipopeptides (Khor et al. 2007), fungal zymozan as well as lipotechoic acid (LTA) of Gram-positive bacteria (West et al. 2006).There is a specificity of the two different dimers both in regards of ligands and adaptor proteins. Gram-positive bacteria express LTA as well as diacylated and triacylated lipoproteins. LTA and diacylated lipoproteins are specific ligands for the TLR2/TLR6 complex (Takeuchi et al. 2001), while triacylated lipoproteins are ligands for the TLR2/TLR1 complex. Just as CD14 is necessary for LPS stimulation of the TLR4, CD36 greatly enhances LTA signaling via the TLR2/TLR6 complex (Hoebe et al. 2005). CD14 instead facilitates the signaling of the TLR2/TLR1 complex (Triantafilou et al. 2006).

TLR2 mRNA expression has been shown in canine (Ishii et al. 2006) and human (Zarember et al. 2002) pancreatic tissue.

MyD88

Most TLRs so far described are capable of interacting with MyD88 via hemophilic TIR-TIR interaction, TIR domains being present on both MyD88 and the intracellular portion of the TLRs.

Upon ligand stimulation of the TLR, MyD88 is recruited and binds to the cytoplsmic domain of the TLR by TIR-TIR interaction. This, in turn, recruits IL-1R-associated kinase 4 (IRAK4) and activated IRAK4 phosphorylates and thereby activates IRAK1 (Suzuki et al. 2002, Khor et al. 2007). IRAK1 binds to and activates TNFR-associated factor 6 (TRAF6) (Gohda et al. 2004). Both disassociate from the complex and together activates transforming growth factor (TGF)-β-activating kinase 1 (TAK1) by phosphorylation (Jiang et al. 2002). TAK1 phosphorylates and hence activates IκB kinase (IKK) and MAP kinase kinase 6 (MKK6) (Wang et al. 2001). Inhibitor of κB (IκB) is phosphorylated and degraded, which releases and enables nuclear translocation of NFκB. The NFκB induces transcription of pro-inflammatory cytokines.

Besides NFκB activation, MyD88 also is able to initiate activation of MAPKs, such as p38 and JNK, activating activator protein 1 (AP-1), as well as activating the transcription factor interferon regulatory factor 5 (IRF-5).

MAL

MyD88 adaptor like (MAL), also called TIR domain containing adaptor protein (TIRAP) (Fitzgerald et al. 2001, Horng et al. 2001), is an adaptor protein involved in the MyD88-dependant signaling of TLR4 and TLR2 (Horng et al. 2002, Yamamoto et al. 2002). It is believed to facilitate delivery of MyD88 to TLR4 and TLR2 and thereby making possible to elicit a signal (Kagan et al. 2006).

TRIF

The MyD88-independent pathway is mediated via TRIF and is utilized by TLR4 and TLR3 (Hoebe et al. 2003).

TRIF activates TRAF family member-associated NFκB activator (TANK) that binds, via TRAF3 and IKK (Yamamoto et al. 2002), binds TANK-binding kinase 1 (TBK1). In turn TBK1 phosphorylates the transcription factors IRF-3 and IRF-7. Both translocate into the nucles and activate transcription of IFN and IFN inducible genes (Sato et al. 2003).

TRIF also interacts with TRIF-recruited kinases, receptor-interacting protein 1 (RIP1) and TRAF6. This complex then activates NFκB and subsequently inflammatory cytokines are expressed, thus providing a link between the MyD88-indipendant pathway and NFκB (Cusson-Hermance et al. 2005).

TRAM

TRIF-related adaptor molecule (TRAM) is an adaptor protein bridging between TLR4 and TRIF, facilitating TLR4 to signal (Fitzgerald et al. 2003).

NFκB

Expression of cytokines and chemokines in response to inflammatory stimuli is predominantly regulated by transcription factors, among which nuclear factor kappa B (NFκB) plays a key role (Steinle et al. 1999, Vaquero et al. 2001, Bhatia et al. 2002, Ramudo et al. 2005, Shi et al. 2006).

NFκB is a very well conserved gene, being expressed in higher animals as well as in insects and other lower organisms (Waterhouse et al. 2007). It is a transcription factor capable of inducing various mediators such as cytokines, iNOS, COX-2, and several others.

NFκB consists of several subunits (Rel/NFκB proteins) in different constellations. Five Rel proteins have been characterized; p50, p52, RelA (also called p65), RelB and c-Rel, all containing a conserved N-terminal region, called the Rel homology domain. The RelA/p50 complex is the predominant heterodimer in most cells and is what is commonly referred to simply as NFκB. Homo- and hereodimers of p50 and p52 have been shown to inhibit transcription by competing with active dimers such as RelA/p50 (Lernbecher et al. 1993).

IRF-3/7

Interferon regulatory factor (IRF) transcription factors are important in regulating interferon (IFN) transcription. IFNs in turn, induce transcription of other inflammatory mediators as well, such as the chemokine CCL2 (MCP-1). IFN response during AP has been shown to be an important part of the caerulein AP model in mice. Interestingly, IFN-γ KO mice had increased pancreatic damage compared to the WT mice after administration of caerulein (Hayashi et al. 2007).

Expression of IFR-3 is constitutively and ubiquitously expressed, while IFR-7 expression varies in different tissues. In human, a high degree of expression is found in the ductal cells while it is present in lower levels in the rest of the exocrine pancreas. IFR-7 is also expressed in the exocrine pancreas of rats and mice.

Chemokines

Chemokines consists of a large family of structurally related cytokines. Four groups of chemokines have been described and are named based on cystein sequence; CXC, CC, C, and CX_3C (Rollins 1997, 2003).

Chemokines are chemotactic cytokines that attract numerous inflammatory cells important to the progression of AP. Recruitment of inflammatory cells are necessary both for clearing the inflamed area of damaged cells and for activation of cells involved in the tissue repair.

CCL2 (MCP-1)

CCL2 [monocyte chemoattractant protein-1 (MCP-1)] belongs to the CC chemokines and was first isolated in 1973 (Altman et al. 1973) but later characterized in detail (Leonard et al. 1990). There is a 55% homology between the human and murine MCP-1 (Rollins et al. 1989). It is synthesized and secreted by a wide variety of cells, including monocytes, vascular endothelial cells and smooth muscle cells.

CCL2 (MCP-1) binds and activates the chemokine receptors CCR2 and CCR4. It is highly chemotactic for monocytes, T lymphocytes, NK cells and basophils, but shows no effect on neutrophils and eosinophils. It is known to be one of the most potent recruiters of monocytes/macrophages (Fuentes et al. 1995, Lu et al. 1998). In addition to its chemotactic properties, it also activates monocytes, causes Ca^{2+}-influx, and stimulates respiratory burst (Rollins et al. 1991) and stimulates them to secrete a variety of different cytokines (Miller et al. 1992). In both the human and rodents CCL2 (MCP-1) is dramatically up-regulated during different inflammatory states.

IFN-β is a powerful inducer of CCL2 (MCP-1) in peripheral blood monocytes (Fantuzzi et al. 2001). It has been shown in peritoneal macrophages that IFN-γ but not LPS was able to induce CCL2 (MCP-1) expression (Bauermeister et al. 1998). Other inducers of CCL2 (MCP-1) are IL-1, IL-6 and TNF-α.

The CXL8 (IL-8) group

CXCL8 [interleukin-8 (IL-8)] is one of the most important recruiters of neutrophils in man. Two receptors have been described so far that both bind CXL8 (IL-8) with high affinity (Holmes et al. 1991, Murphy et al. 1991).

Although mice and rats do not express CXL8 (IL-8), both species produce proteins showing high degree of similarity to the human growth related oncogenes (GROs), CXCL1-3 (CINCs) in the rat and CXCL1 (KC) and CXCL2 (MIP-2) in the mouse. Besides the sequence homology, functional similarities exist. It has been shown that both CXCL1 (CINC-1) and CXL8 (IL-8) bind to the same CXCR2 receptor (Wuyts et al. 1998), as do the rest of the CINCs (Shibata et al. 2000), the GROs (Ahuja et al. 1996) and the mouse orthologs KC and MIP-2 (Bozic et al. 1994).

The GRO group shows a large sequential similarity to the rat CINCs and is therefore often regarded orthologs. In turn CXCL1 (CINC-1) and CXCL2 (CINC-3) are regarded orthologs to the mouse KC and MIP-2 (Nakagawa et al. 1994). The CXCL8 (IL-8) orthologous genes in the human, rat and mouse are summarized in Table 2.

Table 2. Orthologs of a selection of neutrophil and monocyte chemoattractants in the human, rat and mouse. The human CXL8 (IL-8) has no known orthologs in either species. The mouse ortholog of the rat CXCL3 (CINC-2) remains to be described as well.

Human	Rat	Mouse	Receptor
CXL8 (IL-8)	-	-	CXCR1 (IL-8RA) CXCR2 (IL-8RB)
GRO α	CXCL1 (CINC-1)	KC	CXCR2
GRO γ	CXCL3 (CINC-2)	-	CXCR2
GRO β	CXCL2 (CINC-3)	MIP-2	CXCR2
MCP-1	CCL2 (MCP-1)	JE	CCR2

CXCL1 (CINC-1)

The CXC chemokine CXCL1 (CINC-1) was first isolated in 1989 (Watanabe et al. 1989a) and the CINC group of chemokines was later further characterized (Nakagawa et al. 1994). It belongs to a family of which three more proteins have been shown in the rat (CINC-2α, CINC-2β, CINC-3/MIP-2), all of which are potent neutrophil chemoattractants (Nakagawa et al. 1994). CXCL1 (CINC-1) shares 63-67% sequence homology with the rest of the group. Another group of proteins sharing a high degree of sequence similarity of CXCL1 (CINC-1) are the human GROs, of which it shared 68%, 71% and 69% of the sequence with GROα, GROβ and GROγ. It has therefore been suggested that CINCs are the rodent homologues of the human GROs (Zagorski et al. 1993) and well as the mouse KC and MIP-2 (Watanabe et al. 1989b).

CXCL1 (CINC-1) is chemotactic for neutrophils, recruiting them to the site of inflammation. The other members of the group, CINC-2 and CINC-3, have been shown to possess as powerful chemotactic properties as CINC-1, while CINC-3 in addition to its chemotactic properties also is capable of inducing Ca-influx (Shibata et al. 1995).

In conclusion, the innate inflammatory response initiated by the TLRs rapidly results in synthesis and secretion of chemoattractants necessary to recruit inflammatory cells. Via the TLR pathways both chemoattractants necessary for monocyte and neutrophil influx are produced.

Infiltrating inflammatory cells

Monocytes/macrophages

Monocytes are leucocytes found in the circulation but can migrate to inflamed tissue. Monocytes that migrate into inflamed tissue are activated and converted into macrophages (MΦ). Monocytes are capable of phagocytosing foreign matter and protect the body against bacteria. They also clear inflamed areas from malfunctioning endogenous cells and rid the tissue of cell debris.

Monocytes are recruited and extravasate from the circulation by CCL2 (MCP-1). Already one hour after caerulein administration, up-regulation of mRNA expression of CCL2 (MCP-1) can be seen (Grady et al. 1997). We have shown that an up-regulation of the protein is seen 1 hour after HS administration (Axelsson et al. 2008a).

Although resident macrophages seem to play a minor role in the initiation of AP, monocytes are recruited rapidly in several models of AP, including the caerulein (Adler et al. 1979), TDC (Axelsson et al. 2007), LPS (Axelsson et al. 2008a) and HS (Axelsson et al. 2007, Axelsson et al. 2008a) models of experimental AP.

Monocytes, just as neutrophils, cause tissue damage during AP by releasing reactive oxygen species. Just like the neutrophils monocytes are recruited early after the onset of AP. In stark contrast to neutrophils, targeted depletion of monocytes or its chemotactic cues have not been shown to reduce the local damage of the pancreas but only reducing secondary effects such as AP-induced lung injury (Gerard et al. 1997). Instead it has been proposed that monocytes activate other cells such as PSCs (Zhao et al. 2005) and as we propose in this thesis recruits neutrophils.

Neutrophils

Neutrophil granulocytes, or neutrophils, are found in the circulation but extravasate and migrate to sites of inflammation. They exert an important part of the immune system and are capable of phagocytose and neutralize bacteria and foreign material. They also release proteins such as myeloperoxidase (MPO) and elastase. They are believed to be the inflammatory cells predominantly inflicting damage to the pancreatic tissue. Depletion of neutrophils in caerulein-induced pancreatitis dramatically reduces the severity (Gloor et al. 1998, Pastor et al. 2006). Myeloperoxidase (MPO), an iron-containing heme protein localized in the azurophilic granules of neutrophil granulocytes and in the lysosomes of monocytes, is involved in the killing of several micro-organisms and foreign cells, including bacteria, fungi, viruses, red cells, and malignant and nonmalignant nucleated cells. Although it is expressed by other cells, it occurs in a greater amount in neutrophils than other cells.

Neutrophils are recruited by CXCL8 (IL-8) in man and mainly by CXCL2 (MIP-2) and CXCL1 (CINC-1) in rodents but also by other chemoattractants such as IFN-γ. Inhibition of the chemotaxis of neutrophils has been shown to reduce pancreatic injury, as depletion of CXCL2 (MIP-2) reduced pancreatic injury in the caerulein model of AP (Pastor et al. 2003).
Infiltration of neutrophils has been reported in the caerulein model to coincide with the influx of monocytes (Adler et al. 1979). Different results have been shown when comparing the HS and the LPS models of AP-induction. In the case of HS neutrophils have been shown to be a secondary event to the influx of monocytes into the pancreas (Axelsson et al. 2008a). LPS on the other hand induces a response with rapid neutrophil infiltration that coincides with monocytes or even precedes them.

Lymphocytes

Lymphocyte infiltration has been shown to be a prominent feature in the development of AP. Both CD4 and CD8 positive T cells are present in low numbers in the healthy pancreas in mice and particularly CD4+ cells are recruited already 6 hours after the start of caerulein injections (Demols et al. 2000). CD4+ cells seem to be particularly important during the AP development as depletion of CD4+, but not CD8+, decreases the pancreatic injury during caerulein-induced AP (Demols et al. 2000).

The fact that CD4+ but not CD8+ cells effects the AP, suggests that not cytotoxic effects of CD8+ cells are dominating the process but rather secondary effects of CD4+ cells. The effects of CD4+ cells during pancreatitis are not know but may include secretion of pro-inflammatory cytokines or activation of monocytes to exert their inflammatory properties.

The etiology may be crucial for the properties of lymphocytes during AP. It has been demonstrated that in biliary-induced AP, the numbers of CD4+ cells were increased compared to controls, while in alcoholic AP, the numbers were reduced. CD8+ cells also show difference between the groups. They were shown to produce reduced amounts of IFN-γ in biliary AP, while its ability to secrete IFN-γ was increased in the group of alcoholic AP patients (Bhatnagar et al. 2001).

Systemic inflammatory response and coagulation

The local pancreatic inflammation can in the severe form also progress into involving systemic and remote organ inflammation. This condition is called systemic inflammatory response syndrome (SIRS). Characteristic of the severe form of AP is failing of the affected organs, termed multiple organ dysfunction syndrome (MODS).

Inflammation and coagulation have been shown to be intimately connected and blockage of the extrinsic pathway has been shown to reduce inflammation, including AP. The initiating event of the extrinsic pathway is the binding of tissue factor (TF) and activated factor VII (fVIIa). Blocking this interaction has been shown to dramatically reduce systemic inflammation.

Active site-inhibited factor seven
FVIIai is a substance synthesized by incorporation of chloromethyl ketone D-Phe-L-Phe-L-Arg into the active site of activated fVII (fVIIa) (Sorensen et al. 1997). FVIIai is a competitive inhibitor of TF / fVII complex formation. In equilibrium studies it has been shown that fVIIai possess the same high affinity for TF as fVIIa (Sorensen et al. 1997) and it has also been suggested that it has an even higher affinity in other bioassays (Jang et al. 1995). It is established that the fVIIa/TF complex is able to signal via protease activated receptor-2 (PAR-2).

PAR-2
Although the biological functions of active PAR2 signaling differ greatly in different tissues, this receptor is probably important both locally in the pancreas and in the peripheral circulation. PAR2 is activated by proteolytic cleavage of the N-terminal by serine proteases, such as trypsin (Nystedt et al. 1995, Bohm et al. 1996), a proteolytic enzyme found at elevated concentrations in the circulation both during clinical and experimental AP (Le Moine et al. 1994, Hartwig et al. 1999). Trypsin has been shown not only to be elevated but also responsible for part of the NFκB activation during cerulein-induced AP (Tando et al. 2002).

PAR2 has been implicated in AP (Gorelick 2007) and is also, as mentioned above, involved in TF/fVIIa signaling. Further studies are needed to elucidate the effects of fVIIai on the PAR system, in particular PAR2, during experimental AP.

Experimental models of acute pancreatitis

The events underlying human AP are rather unexplored for several reasons. First, patients usually arrive at the hospital at the earliest several hours after the onset of an AP attack, making the early events impossible to investigate in humans. Secondly, pancreatectomy is not performed, except for removal of necrotic and infected areas at later stages of the disease, which makes access to relevant tissue in order to explore the events leading to pancreatitis scarce. The retroperitoneal localization of the organ makes it inaccessible in patients and healthy volunteers. Therefore numerous animal models have been developed to address different scientific questions related to pancreas physiology and inflammation (Fig. 6). The different models of AP have been reviewed several times in the past (Lerch et al. 1994, al-Mufti et al. 1999, Foitzik et al. 2000, Saluja et al. 2004, Su et al. 2006, Chan et al. 2007, van Minnen et al. 2007) and in order to be able to evaluate the relevance of the results from various experimental models a thorough understanding of their physiological mechanisms is needed.

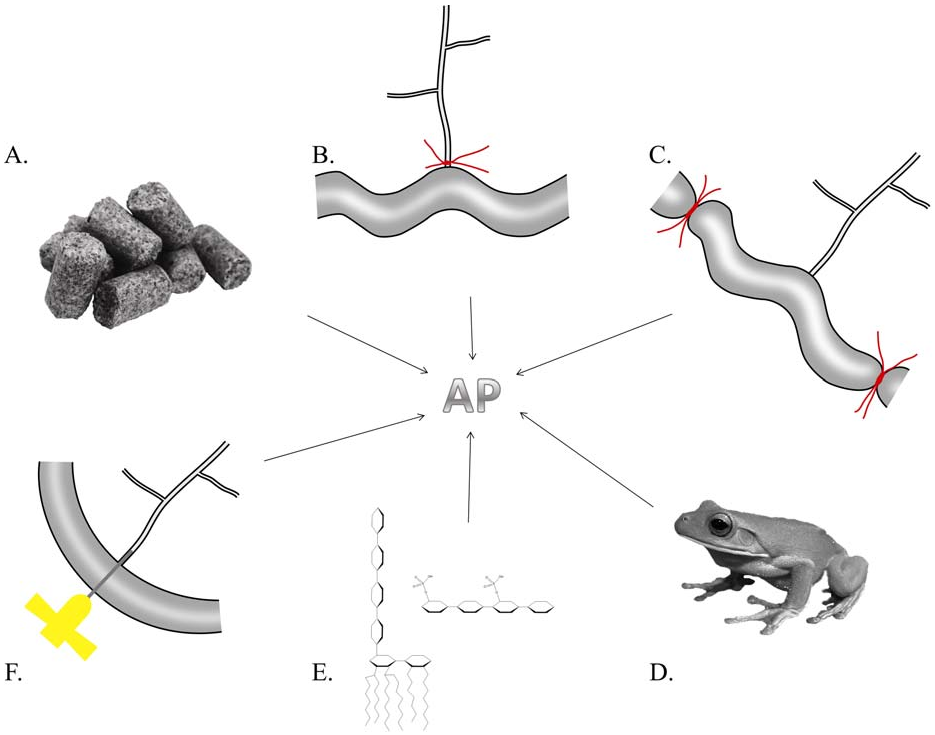


Figure 6. *Experimental models of acute pancreatitis. A) Diet-induced AP. B) Biliary duct ligation. C) Closed duodenal loop. D) Caerulein. E) HS and LPS retrograde biliopancreatic duct infusion. F) TDC retrograde biliopancreatic duct infusion.*

Based on the observations of two AP patients, who also had gall-stones impacted into the papillae of Vateri, Opie (1901a) proposed the "common channel" theory in 1901. He proposed that gall-stones obstructed the flow of bile causing it to reflux into the pancreas. This theory has never been fully proven and several objections to this theory exist. Despite this, the hypothesis is widely accepted as a valid initiation cause of AP. This theory initiated the starting point of several animal models of pancreatitis based on retrograde infusion of bile or bile components into the pancreas and biliary duct obstruction models.

Retrograde biliopancreatic duct infusion / prograde pancreatic duct perfusion

One of the most widely used duct-infusion model was developed by Flexner in 1906, where he showed that infusion of a sodium taurocholate (TDC) solution into the pancreas caused necrotizing AP (Flexner 1906). Despite his comments on his own experiments that this model caused too much damage to mimic the clinical situation, this model has been one of the most frequently used methods for studying AP and associated remote organ damage. Over the years the TDC model has been modified to control variability and severity. Consistent early findings in the TDC model are focal hemorrhage, necrosis of acinar cells and neutrophil infiltration (Kudari et al. 2007).

Controlling the intra-ductal pressure is crucial when inducting AP by ductal perfusion. In order to be able to perfuse the pancreatic duct at low pressure, prograde infusion of various agents has been used. This is accomplished by cannulating the pancreatic duct at the tail of the pancreas. In many animal species the duct has to be permeabilized using detergent, e.g. sodium taurocholate, or using prostaglandin E2 to cause severe AP (Widdison et al. 1992). Depending on the pressure and concentration of detergent used, several mechanisms for initiation of inflammation in this model can be proposed.

Biliary duct ligation

Biliary duct obstruction is a technique logically developed on the "common channel" theory. It involves surgical ligation of the common bile duct. In most species, including primates and pigs, this only induces pancreatic regeneration or a mild edematous pancreatitis. In rats the progression of the inflammation is a slowly increasing event and both macrophages and neutrophils first appear at 24 hours after ligation (Meyerholz et al. 2007). To aggravate the situation to severe AP, a secretagogue must the administered. In contrast to most other species investigated so far, the American opossum develops a severe necrotizing AP as a result of ligation without any additives (Lerch et al. 1992, Lerch et al. 1993). This fact has made it a favorite model in the studies of initiating events of the disease. It is thought that bile-reflux cause premature activation of pancreatic enzymes, which are responsible of the initiation of the induced inflammation.

Closed duodenal loop

The same year as Opie proposed his famous "common channel" theory, he also suggested an alternative hypothesis. This hypothesis proposed the passage a gall stones through the sphincter of Oddi causing it to malfunction (Opie 1901b). This would in turn permit duodenal content to more freely pass into the bile duct and possibly making its way to the pancreatic ductal system. To include the possible bacterial contribution of the initiation of AP the closed duodenal loop (CDL) method was developed by Pfeffer et al (1957). This initial finding generated subsequent models based on the same principle; both the closed duodenal loop (CDL) and variations of the same model. Suggested mechanisms underlying inflammation caused in this model are discussed in detail in the discussion section of this thesis.

Immunological/inflammatory models

Immunological models have been used since 1907 when Williams and Busch (1907) showed that retrograde infusion of duodenal content, but not sterile-filtered duodenal content, into the pancreatic ducts of cats, caused acute pancreatitis.

Retrograde infusion of live bacteria into the pancreatic duct has been shown to cause inflammation with a rapid onset. Both the innate and the acquired immune system can cause AP and necrosis resembling what is found clinically (Thal et al. 1954, Thal 1955). Thal and Brackney (1954) showed that AP could be triggered by the innate immune system by intraductal infusion of LPS prepared from the Gram negative bacteria *E. coli* and meningococci. In a later study they also showed that similar results could be obtained by the acquired immune system by infusion of ovalbumin in rabbits sensitized to the foreign protein (Thal 1955). Thal and Molestina (1955) further demonstrated similar reactions using toxin preparations from *Staphylococcus* sp, a genus of Gram positive bacteria.

Although the presence of LPS in the pancreatic duct is somewhat doubtful, the presence of HS is indisputable as both syndecan-1 and glypican-1 are expressed on the surface of the ductal epithelium (Kleeff et al. 1998, Conejo et al. 2000). If the amounts necessary to elicit a response powerful enough to trigger an attack of AP are high enough is still not proven but suggested by Axelsson et al (2007, 2008a). HS is shown to induce a rapid CCL2 (MCP-1)

mediated inflammatory response of the ductal cells and a subsequent infiltration of monocytes/macrophages into the pancreas.

Other models of immune-mediated AP include intraductal infusion of foreign serum (Nevalainen 1978). Although it is interesting to note that foreign substances in the serum are able to elicit an inflammatory response, these models are not even remotely connected to clinical AP.

Caerulein

It has long been known that excessive neuronal stimulation can cause damage to the exocrine pancreas. Over-stimulation of the pancreas using cholecystokinin (CCK), or substances similar in action, causes acute pancreatitis. Cerulein, found in the Australian Hylid, *Pelodryas caerulea* (De Caro et al. 1968), as well as other amphibians (Anastasi et al. 1970), is a decapeptide exhibiting the same properties as CCK on regulation the pancreas enzyme secretion. When administered, odematous pancreatitis results in rats (Lampel et al. 1977, Adler et al. 1979).

The theories of mechanisms underlying the inflammatory response following caerulein administration were recently reviewed (Saluja et al. 2007). Caerulein administration results, just as its endogenous counterpart CCK, in an increase of enzyme recreation when given at physiological concentrations. When administered at doses of a 50-fold excess of the maximal stimulatory dose, enzyme secretion is instead inhibited. This is explained by two affinity states of the CCK receptor; high- and low-affinity. At physiological concentrations caerulein and CCK bind to the high-affinity receptors, which elicit enzyme secretion. At supramaximal concentrations, also the low-affinity receptors are occupied and signaling of these receptors inhibits enzyme secretion. This results in premature intraacinar activation or zymogens. Actually, activated trypsin can be measured in the acinar cells just 15 minutes after caerulein stimulation (Mithofer et al. 1998).

When it comes to clinical applicability, the caerulein model seems to mimic pancreatitis caused by the sting of the Trinidad scorpion, *Tityus trinitatis*, (Bartholomew 1970, Bartholomew et al. 1977) or anti-cholinesterase poisoning (Dressel et al. 1979) most closely. Furthermore, the CCK_A receptors on the rodent acinar cells, claimed to cause the events, are not present on human acinar cells (de Weerth et al. 1993). In line with these findings is also the fact that CCK does not evoke any effects on human acinar cell function (Ji et al. 2001, Ji et al. 2002). Despite the obvious poor correlation to common causes of clinical AP and large variability within the model, the caerulein model has become the most commonly used model in rodents and a substantial part of the knowledge in pathophysiology of AP is derived from it. It is claimed to be particularly useful when investigating AP-induced pulmonary injury.

Diet-induced AP

The commonly used choline-deficient, ethionine-supplemented (CDE) diet was developed by Lombardi et al. (1975a) using young female mice fed this particular diet. By varying the diet composition, desired mortality and severity rates can be obtained.

The mechanism of initiation of the inflammatory events in this model remains to be elucidated. Excluding ethionine from the diet does not cause pancreatitis (Lombardi et al. 1975b). Ethionine is a homologue of methionine exhibiting an ethyl group as substituent instead of a methyl group. It inhibits protein synthesis and interferes with ATP metabolism. This probably harms cells producing large amounts of proteins such as pancreatic acinar cells.

Ethanol-induced AP

To more closely mimic the clinical situation of a large part of the AP patients, models based on ethanol exposure have been developed. These have been reviewed in depth previously

(Schneider et al. 2002, Pandol et al. 2003). The AP attacks elicited by ethanol most commonly happens after many years of abuse and are not easily simulated in the experimental situation. Several attempts have been made though.

Various less commonly used models

Arginine-induced AP has been employed rarely in animal systems and involves administering a single large dose of L-arginine to rats (Tani et al. 1990). The clinical relevance is very limited and, to my knowledge, only a single patient diagnosed with arginine-induced AP has been reported in the litterature (Saka et al. 2004). The mechanism of which arginine induce AP is not known, but several mediators such as reactive oxygen species and NO have been proposed. NO is a most likely candidate as arginine is a known substrate of iNOS.

After extensive surgery, e.g. cardiac surgery, it is been reported cases of AP. This is thought to be due to hypovolemic shock and has been mimicked experimentally. The main objections to this model are the dramatic trauma inflicted on the animal and the un-specificity of the model, causing injury not only to the pancreas but several other organs as well.

Very similar changes as in the TDC model can be seen during ischemia/reperfusion-induced AP (Hoffmann et al. 1995).

"Mixed models"

In order to more accurately mimic the clinical findings several "mixed" models have been developed. The most reputable of these is the Boston model of AP (Schmidt et al. 1992a, Schmidt et al. 1992b). It is based on the TDC model but with the addition of caerulein injections. It can be adjusted to superficially correlate to clinical findings when comparing histological scores, infection frequencies, etc. It has therefore been particularly useful when studying bacterial translocation secondary to AP as well as interventional studies (van Minnen et al. 2007). However, it suffers from the same disadvantages as the original models it was developed from, e.g. that the initiating noxae have little in common with the causes in humans, as well as being invasive.

AIMS

The overall purpose of this work was to investigate the mechanisms of initiation of AP and modulation of the subsequent systemic inflammatory response. In more detail the following was investigated:

- HS is capable of inducing systemic inflammation when administered intraperitoneally in mice and is readily shedded from injured tissue. Does the pancreas respond with an inflammatory response when administered intraductally?

- HS administration induces rapid influx of monocytes and neutrophils without signs of acinar or other cell damage. HS is a known ligand of TLR4. What cells are the primary initiators in this particular model of AP? How is the influx of inflammatory cells regulated? Does the inflammation induced by HS differ from the response induced by LPS, another known TLR4 ligand?

- FVIIai has been shown effective in reducing the inflammatory response in sepsis. Does pre-treatment of fVIIai reduce the AP-induced systemic inflammatory response?

- The transcription factor NFκB has been shown to be an important mediator of inflammation in numerous inflammatory conditions. Does pre-treatment of fVIIai affect NFκB activation in remote organs during AP-induced SIRS?

GENERAL ASPECTS OF MATERIALS AND METHODS

The outline of the methods used can be found in Fig. 7. For detailed information about the different techniques used, the reader is referred to the individual articles. Below a discussion on the practical matters of the experiments is given.

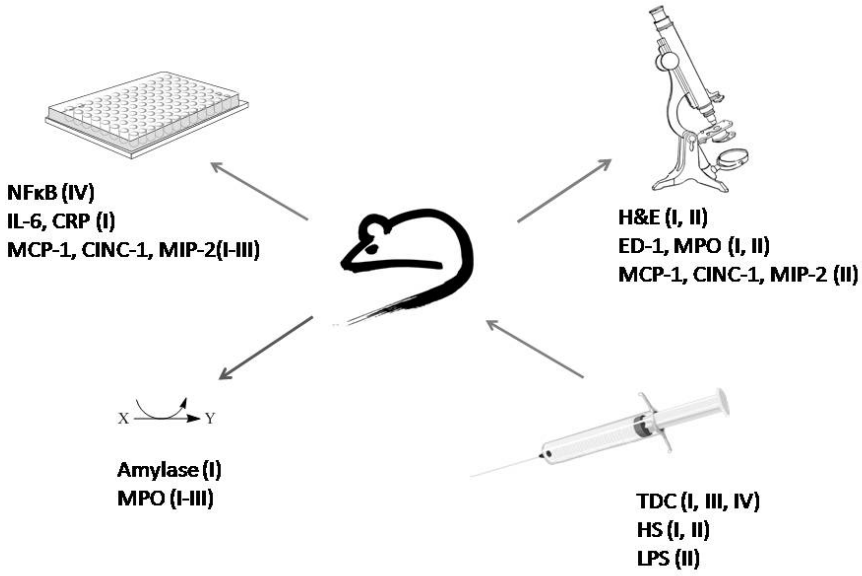


Figure 7. *Methods used for the investigations. The roman numbers in parentheses refer to the studies the particular method was used in.*

AP models used

Two models of AP were used in the studies, both chosen for particular reasons. In order to study the effect of HS on the ductal epithelium, a HS-induced AP model was developed. No previous models studying HS effects on the pancreas has been published and therefore a novel model was used.

In order to study AP-induced systemic inflammation and remote organ injury the well characterized TDC model of severe necrotizing AP was chosen. It was chosen over other available models as it produces severe systemic inflammation is claimed to be more reproducible than other models of severe AP.

Some considerable variation of the results has been noticed in all the models of AP used. This may be due to the administration of a comparably small volume of solution, 200 µl in the rat and 50 µl in the mouse, to a ductal system with a larger capacity. A control of the filling of the pancreatic ductal system using the appropriate volume of black ink was performed in the rat and showed that the duodenal lobe was completely filled (Fig. 8). The more inaccessible

splenic lobe was not inspected. The variation may be due to varying degrees of the filling of the splenic lobe.

In addition to the anatomical and practical considerations, the in vivo environment is a complicated one to work in. Despite long selective inbreeding of strains, each individual is unique and different from the other ones. As noted in the introduction, there is a wide variation in the anatomy of the ductal system. The same variability is probably applicable to the individuals at a molecular level as well. The setup and the density of different surface receptors, e.g. the TLRs, may differ and contribute to the observed variation.

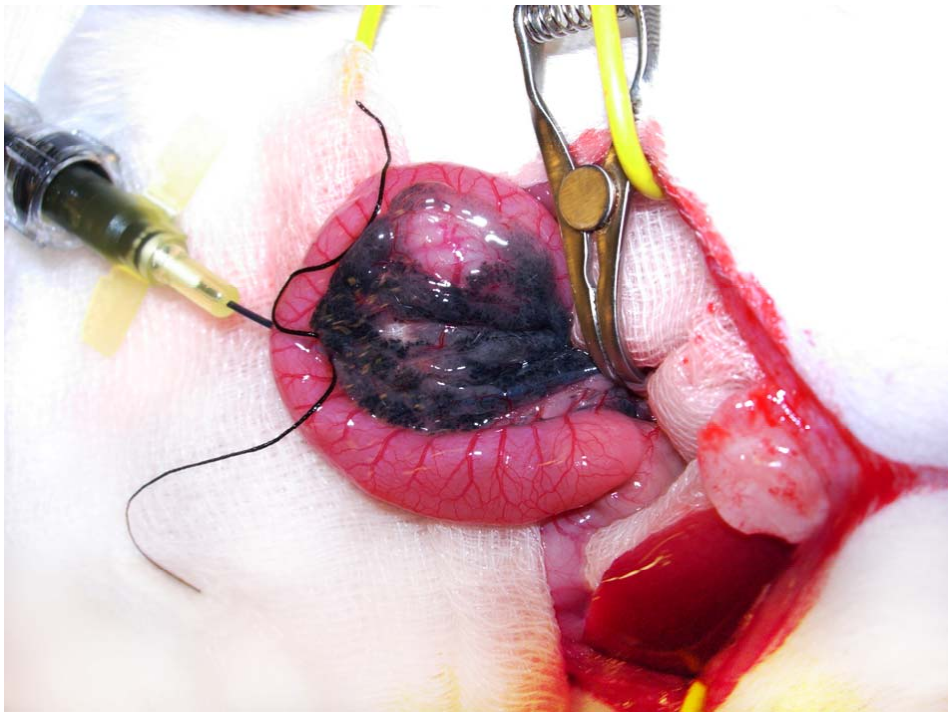


Figure 8. *Black ink injected into the pancreas showing the degree of filling of the duodenal lobe of pancreas.*

RESULTS

Initiation of AP

Introduction
Initially we wanted to investigate if HS would induce an inflammatory response in the pancreas, just as had previously been shown systemically (Johnson et al. 2004). Consequently, HS was infused into the biliopancreatic duct and tissue and plasma was harvested 6 hours after infusion. Two different fractions of HS were used to discriminate effects of HS of different degrees of sulfation from each other. Two controls were also used in the initial experiments. TDC was used as a reference group as an example of a commonly used model of a very severe necrotizing model of AP. Intraperitoneal administration of the same amount of HS as infused intraductally was used as a negative control to show that the changes in the pancreas were not secondary to systemic inflammation.

HS-induced inflammation and tissue damage
Infusion caused pronounced signs of inflammation of the pancreatic tissue. Histology showed that six hours after infusion significant differences ($p=0.010$ and 0.018 respectively for HS3 and HS6) of edema were present between the two HS groups and the control.

Influx of monocytes/macrophages was also significantly increased ($p<0.001$ in both groups) as evaluated using IHC. At the 6 hour time point, elevated MPO activity could be demonstrated in pancreatic tissue homogenates ($p= 0.024$ and $p<0.001$, respectively, as compared to PBS).

No statistically significant differences could be seen when evaluating hemorrhage and necrosis in any of the HS groups but only in the TDC group. To further back up the results of the necrosis evaluations, plasma amylase concentrations were measured during the first 9 hours after HS and TDC infusion. This showed no increase in amylase concentration in any of the HS group but a steadily increasing concentration in the TDC group. Both these analyses show that no acinar cell damage was present in the HS groups during the first 9 hours. The group receiving HS IP did not show any statistically significant changes in any of the variables analyzed.

No systematic difference between the HS3 and HS6 groups could be proved statistically. However the MPO data suggests some differences in neutrophil amounts at 6 hours, which may indicate a difference of transduction pathways.

It was now shown that HS was capable of inducing pancreatitis with inflammatory cell counts and systemic inflammation (see below) comparable to the severe TDC model of AP. These profound changes were shown to be induced without acinar cell destruction, leading us to believe that this response was a receptor mediated response.

In order to investigate the mechanisms underlying the rapid cell infiltration in more detail, a second set of experiments were undertaken. Two of the inflammatory cells arriving earliest on the scene of a site of inflammation are monocytes and neutrophils. Monocytes are mainly recruited by CCL2 (MCP-1) and one of the more important recruiters of neutrophils is CXCL1 (CINC-1). Therefore, these two chemoattractants and two cell types were studied during one to nine hours after HS- and LPS-infusion. The variations of monocytes/ macrophages, neutrophils and their corresponding chemokines during the first 9 hours after infusion are summarized in Figure 9.

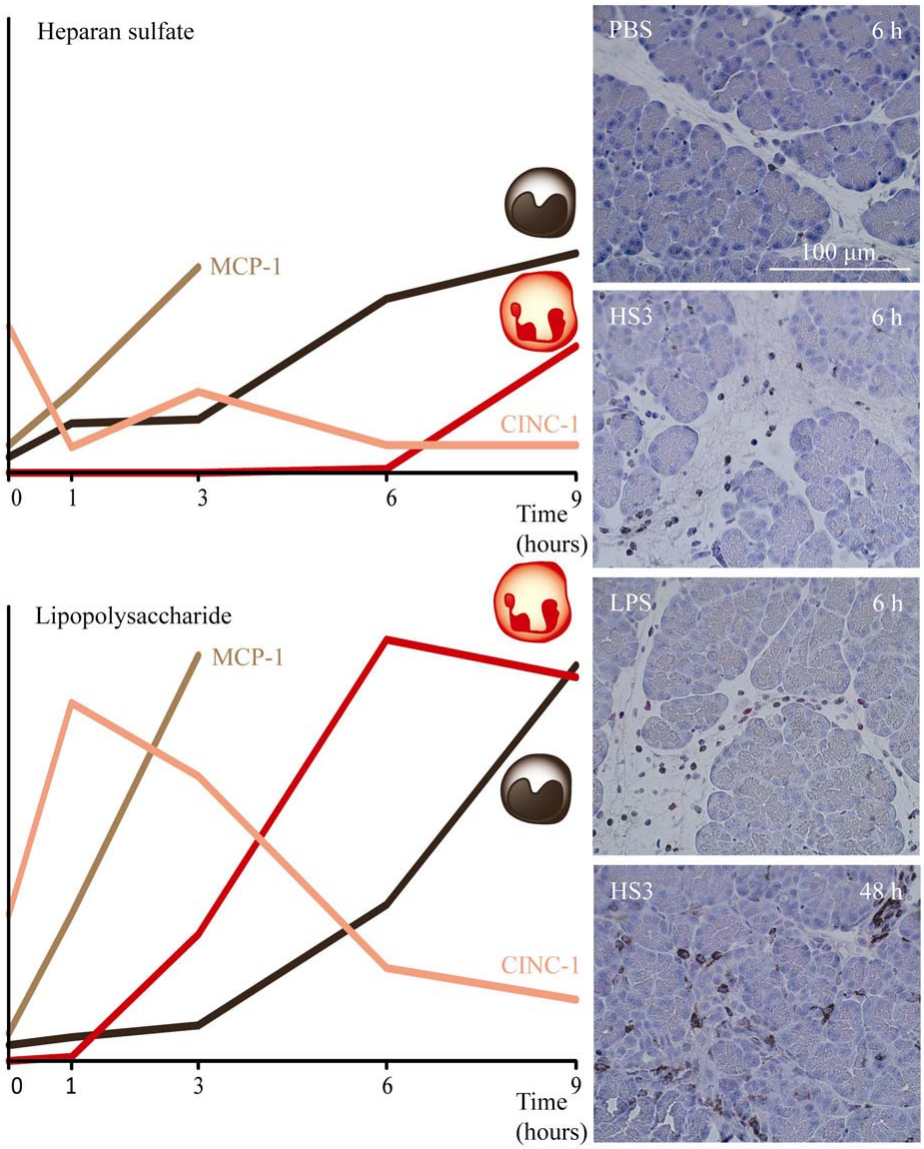


Figure 9. *Variation of monocytes/macrophage and neutrophil infiltraion as well as corresponding concentraions of their chemoattractants in the pancreas 1-9 hours after heparan sulfate- and lipopolysaccharide-infusion.*
Left: The diagrams show the monocytes/macrophage (brown) and neutrophil (red) counts of the HS and LPS group together with their corresponding chemokines; CCL2 (MCP-1) (light brown) and CXCL1 (CINC-1) (light red) respectively.
Right: The histological images show representative selections of tissue sections of the PBS, HS and LPS groups at 6 hours and 48 hours after AP-induction. All sections are double stained for monocytes (ED1, brown) and neutrophils (MPO, red).

CCL2 (MCP-1)

The immunohistochemical analysis of CCL2 (MCP-1) revealed up-regulation of the protein in the ductal cells already at 1 hour after infusion in the HS, as well as the LPS group compared to PBS. Constitutive expression of the protein was also noted in islet cells and vascular endothelium. At later time points expression was also seen in invading inflammatory cells.

Quantitative analysis using ELISA of whole tissue homogenates showed no statistically significant up-regulation of the protein at 1 hour in either group, but a prominent increase ($p=0.026$ and $p=0.002$, respectively, for HS and LPS compared to LPS) at 3 hours after infusion.

CXCL1 (CINC-1)

After HS-infusion no differences in CXCL1 (CINC-1) expression could be detected using IHC. Prominent constitutive expression of the islets was seen.

Protein determination using ELISA did not show any difference in the HS group either at 1 or 3 hours. LPS induced up-regulation already at 1 hour ($p=0.015$) and at 3 hours the amount was roughly the same ($p=0.041$).

Just as the different expression of the above chemoattractants suggest, there are distinct differences in the invading cell populations in the HS and LPS groups (Fig. 9). They are visualized as ED-1 positive monocytes/macrophages (black) and MPO positive neutrophils (red).

Monocytes/ macrophages

Infusion of HS and LPS both induce influx of monocytes ($p=0.002$ and $p<0.001$ when comparing HS and PLS to PBS) 6 hours after infusion. The same numbers of monocytes are seen at 9 hours in both groups. The stained cells were predominantly found in the connective tissue septae of the pancreas.

Animals infused with HS were also analyzed 48 hours after infusion. The numbers of monocytes remained constant compared to 6 and 9 hours but the localization and morphology of the cells had dramatically changed. The cells had relocated into a more periacinar localization. During the time up to 9 hours after infusion the monocytes/macrophages were round and had a monocyte-like appearance. At 48 hours after infusion, the cells had an irregular shape and were more macrophage-like.

Neutrophils

In the HS and LPS models the neutrophils do not coincide with monocyte infiltration. In the HS model a dramatic monocyte increase is followed by a much less pronounced neutrophil increase. After HS administration a small but statistically significant ($p=0.041$) increase of monocytes is seen after 9 hours. Forty-eight hours after administration the neutrophil counts are back to basal levels. On the contrary, in the LPS group, neutrophils precede monocyte infiltration and they are more numerous compared to the HS group. LPS induce a significant increase ($p=0.009$) of neutrophils already at 3 hours after infusion and reaches a plateau at 6 hours, which continues at 9 hours.

MPO cells / activity

The neutrophil numbers do not correlate with MPO activity. Just as in the neutrophil counts, the MPO activity of the MPO group reaches statistical significance at 9 hours, but it is almost twice as high as the activity of the LPS at the corresponding time point. LPS infusion causes a small, but statistically significant ($p=0.026$), increase already at 1 hour after infusion. The activity of the LPS group plateaus at 6 hours, but only at half of the activity of which the HS group reach at 9 hours.

Systemic inflammation
To indirectly evaluate the magnitude of the local inflammation of the pancreas, systemic inflammation was also investigated. Plasma concentrations of CINC-1, MCP-1, IL-6 and CRP were measured.

Concentrations of circulating CXCL1 (CINC-1) were increased in both HS groups compared to PBS, even though only HS6 reached statistical significance ($p=0.055$ and $p=0.019$, respectively). IL-6 is secreted by activated circulating monocytes and is a predictive protein of severity of AP. Concentrations of IL-6 were elevated in both HS groups ($p=0.012$ and $p=0.007$, respectively). Circulating IL-6 stimulates hepatocytes to produce the acute phase protein CRP, which is used clinically as a predictor of AP severity. Increased plasma concentrations of CRP could be demonstrated as well but showed variation between the three consecutive experiments (results not shown).

AP-induced SIRS and interplay with coagulation in the TDC model

Local inflammation of the pancreas is the first event of acute pancreatitis and subsequent SIRS. To study the SIRS and MODS following AP, a severe model of necrotizing AP was chosen, i.e. the TDC model. As many studies have shown a profound link between coagulation and systemic inflammation, we wanted to investigate if fVIIai could affect the inflammatory process during AP. Another end point was to study the NFκB phosphorylation and therefore NAC, an unspecific inhibitor of NFκB was used.

Pancreas
During AP, no increased MPO activity or NFκB activation could be demonstrated and therefore no treatment effects could be shown.

Systemic cytokine response
Six hours after TDC-induced AP, IL-6 plasma concentrations were significantly increased ($p<0.001$). These concentrations were reduced in all, fVIIai, NAC and fVIIai+NAC, treatment groups ($p<0.05$). CXCL2 (MIP-2) was significantly ($p<0.05$) increased after AP-induction. This increase was reversed in all treatment groups.

Lung
MPO activity in lung homogenates significantly ($p=p<0.05$) increase after AP-induction. The levels are restored to baseline levels in all treatment groups.

The NFκB activation in the lung increased significantly ($p=0.017$ as compared to controls) 1 hour after AP-induction and was even further increased after 6 hours ($p<0.001$). Pre-treatment with fVIIai decreased the NFκB activation at both time points to a similar degree ($p=0.002$ at both time points as compared to controls). The combination fVIIai and NAC also reduced inflammation at both time points, but to a lower degree than fVIIai alone ($p=0.081$ and $p=0.020$ at 1 hour and 6 hours, respectively, as compared to controls). In fact, at both time points the combination was significantly higher ($p=0.020$ and $p=0.001$, respectively) compared to fVIIai alone.

Liver
MPO inhibitors are present in high amounts in the liver. Therefore the MPO activity is difficult to measure in liver homogenates, was therefore excluded.

The NFκB activation in the liver showed some tendencies of increase 1 hour after AP-induction, but was restored to baseline levels after 6 hours. Despite that the increase of NFκB

did not reach significance, fVIIai pre-treatment significantly reduced the levels at both time points ($p=0.002$ and $p=0.009$, respectively) as compared to controls due to lower levels as in healthy animals. Just as in the lung at one hour after induction, the combination of fVIIai and NAC resulted in some decrease ($p=0.026$) in NFκB activity compared to controls, but was significantly increased ($p=0.041$) compared to fVIIai alone.

Distal ileum

In the distal ileum MPO activity was significantly ($p<0.05$) increased 6 hours after AP-induction. All treatment groups showed close to normal activities.

In distal ileum, NFκB activation did not increase at 1 hour after induction, but was significantly ($p=0.002$) raised after 6 hours. This coincides with the influx of monocytes/macrophages (data not shown). Pre-treatment with fVIIai, as well as in combination brought the levels back almost to levels seen in control animals ($p=0.002$ and $p=0.001$ for fVIIai and the fVIIai/NAC combination, respectively, compared to AP 6h).

DISCUSSION

The "ductal defense" hypothesis

The current paradigm suggests that initiation of AP in an event of the acinar cells. It is suggested that the first event is blockage of secretion and later co-localization of zymogens and lysosomal enzymes. The activation of trypsinogen and other zymogens are said to cause acinar cell damage, which in turn, recruits inflammatory cells. This current consensus is based on results from experiments performed by duct ligation on opossum (Lerch et al. 1992), a species of which the biliopancreatic ducts resemble the human (Haley-Russell et al. 1992).

We have shown that pancreatic inflammation accompanied by cell infiltration comparable to TDC-induced AP, can be initiated by HS and LPS without any signs of acinar cell destruction. This does not at all fit the commonly accepted theory of AP initiation. What is the biological relevance for a powerful inflammatory response to intraductal administration of HS?

Pancreas synthesizes numerous digestive enzymes which are secreted in their inactive form as zymogens. They are then transported through a ductal system consisting of a single layer of epithelial cells on a basal lamina, to the jejunum where they are activated by intestinal peptidases. Several lines of defense, such as nonoptimal pH, proteinase inhibitors, etc, are present in the pancreas to evade premature activation of the zymogens. If these systems fail, for example during pathological conditions or duct stasis, a quick cellular response is needed to remove damaged cells and induce tissue repair. The innate immune system offers just that, a very rapidly responding system with cell recruitment.

HS is present on syndicans and glypicans, which reside the surface of the ductal epithelium facing the duct lumen. Upon enzymatic cleavage, HS is released and can in its free form trigger receptors on the same cells of which they were cleaved from. When bound to the cell surface they are inactive but have, in its soluble form, been shown to trigger TLR4. TLR4 has been shown to be present only in the ductal cells of the exocrine pancreas, as well as vasculature and islets (Li et al. 2005). In the pancreas, the most obvious substances capable of cleaving HS from the cell surface are prematurely activated enzymes from the acinar cells. However several other proteins and other substances should be considered. The duct cells, as well as the acinar cells, contain heparanase-1 at low concentrations (Koliopanos et al. 2001). Upon rupture heparanase would be released and cleave HS from PGs on the cell surface and thus initiate an inflammatory response by a very local cellular damage. Retrograde bile reflux has been proposed as a possible initiator of AP, and if that is the case, it also has properties both of cleaving HS as well as rupturing ductal cells. Although a controversial cause of AP, as discussed elsewhere in this thesis, retrograde migration of bacteria would also be discontinued by recruitment of phagocytic cells, as receptors triggered both by LPS and LTA are present in the pancreatic duct.

The reasoning above can be formulated as a mechanism of "ductal defense". This "ductal defense" theory therefore offers an explanation of a physiological response to endogenous substances, which during non-pathological condition rapidly is regulated and merely causes an appropriate inflammatory response evade an uncontrollable situation of acinar cell destruction and digestion of the gland. HS can therefore be regarded as a watch dog quick to bark at any signs of danger. During pathological states, such as duct stasis, supra-physiological stimulation of secretion, an exaggerated inflammatory response could be triggered and hence a supra-physiological inflammatory response. A too large infiltration of inflammatory cells would, opposite to the physiological situation where they would prevent

further damage, instead inflict damage to ductal and acinar cells and hence trigger and AP attack.

We have shown that, during HS-induced inflammation, chemoattractants for monocytes and monocyte infiltration precede neutrophil infiltration. Chemoattractants to neutrophils are not seen up-regulated by resident pancreatic cells during HS-stimulation but are present in large amounts in the invading monocytes. We therefore propose the process to be a biphasic event, where monocytes are first recruited and then in turn recruit neutrophils (Fig. 10).

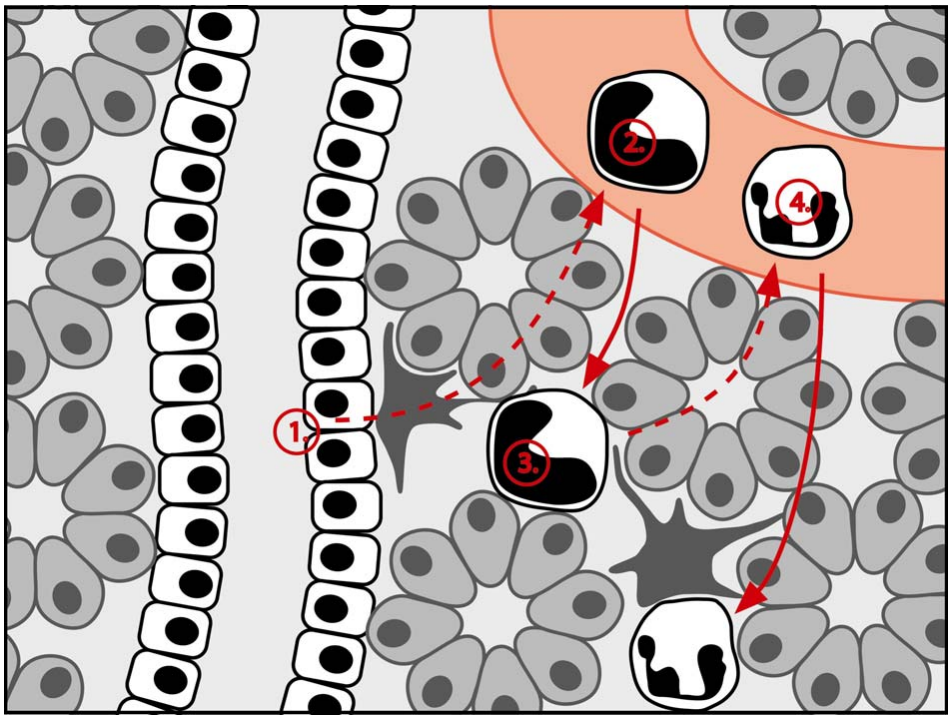


Figure 10. *Proposed mechanism of cell infiltration after intra-ductal infusion of heparan sulfate of the pancreas.*
(1) Soluble HS is present in the pancreatic duct and ductal cells respond by secretion of MCP-1 (dashed line).
(2) MCP-1 recruits monocytes (continous line) from the circulation.
(3) Monocytes thereby extravasate (continous line) from the blood circulation, entering the pancreatic tissue.
(4) Monocytes produce neutrophil chemoattractants like CINC-1 (dashed line), which in turn induce extravasation of neutrophils from the blood circulation.

TLR4 signaling upon HS stimulation

The difference in cell types, monocytes and neutrophils, at the early time point can have several reasons. It has been shown that HS causes cell recruitment to the bladder of WT mice. Inhibition could be seen in TLR4-/- and TRIF-/- but not to the same extent in MyD88 KO mice (Hans Fischer, Lund University, Lund, pers. comm.), suggesting TLR4 signaling via the

TRIF pathway. The TRIF pathway induces IFN, a powerful inducer of CCL2 (MCP-1), but not affected by LPS (Bauermeister et al. 1998). LPS is known to give IL-8 response, via the MyD88-dependant pathway. Two different transduction pathways from the same signaling receptor may thus explain the different cellular responses for the two different ligands.

As seen in Fig. 9, rapid MCP-1 expression precedes monocytes infiltration in both the HS and LPS groups. What differs is the CINC-1 and neutrophil response. HS causes no increase in CINC-1 expression, where LPS induces rapid and pronounced CINC-1 production. The subsequent neutrophil infiltration shows significantly elevated number in the HS group after 9 hours while LPS induces a significant increase already after 3 hours. This evidence strongly suggests two separate signaling pathways, of which the above suggested hypothesis is a likely candidate.

Is LPS a physiologically relevant ligand of TLR4 in the pancreas?

Several researchers have suggested that bacteria are not a likely trigger of AP. One of the earliest results suggesting that bacteria are not involved in AP initiation was published as early as 1919 by Achibald, who was unable to demonstrate backflow of duodenal content through the intact sphincter even at elevated pressure. The most serious objection against the hypothesis of retrograde migration of bacteria into the pancreas is the low numbers of bacteria in the proximal part of the duodenum. To trigger the innate immune system very low concentration of bacteria or bacteria derived substances are needed and there are a certain number of bacteria, even if low compared to other parts of the intestine, in the rat (Axelsson et al. 2006). The fact that early AP is a sterile condition is not any evidence against LPS triggered pancreatitis. If the cells of the innate immune system function properly, even if eliciting a supra-physiological response, the bacteria should be neutralized. This means that the bacteria originally triggering the response cannot be found in bacterial culture.

There are more facts suggesting retrograde bacterial migration to be an event the pancreas has to evade. As discussed in the introduction, the pancreas contains fully functional TLR2, TLR4, MD2 and CD14. Could this be a rudimentary set of receptors and adaptor proteins from a time when the gastrointestinal tract was possibly more prone to invasion of bacteria into the biliopancreatic ducts? It is hardly likely that the entire complex of receptors and associated proteins is conserved in its fully functional state for no obvious use. To produce surface receptors that is of no use is a load for the cell and will be selectively removed during evolution. To think that these complexes are conserved, not just in one species but several, is not only a highly unlikely coincidence, it is logically an impossibility.

Involvement of resident macrophages

The observation that resident macrophages were more or less absent from the healthy pancreas and that monocytes/macrophages started to appear first several hours after AP initiation seem to be backed up by the fact that blocking macrophage activity using gadolinium affects the local inflammation in the pancreas very little but dramatically reduces the extent of remote organ failure in caerulein-induced AP in mice (Gloor et al. 1998, Pastor et al. 2006). This suggests that possibly occurring resident macrophages play a minor role in the initiating events of AP in this particular model of panceratitis.

Clinical relevance of the "ductal defense" theory and evidence from human studies

Löhr et al (1999) showed that the ECM components, laminin, hyaloronan, a known ligand of TLR4, and procollagene-III-peptide were elevated in both the pancreatic secretions and in the circulation during acute necrotizing AP compared to edematous AP. Although failing to incorporate the findings into a biological context, they suggest that these molecules are of pancreatic origin. If these components are shed from the ductal epithelium or not is not known

but the study above shows that they reach the ductal surface and should be able to trigger an innate immune response. If shedded HS is also found in the secretions of pancreatitis patients is not known but highly likely based on the results above.

Systemic inflammation and coagulation

Clinical applicability of fVIIai treatment
Inhibitors of the fVIIai/TF interaction present a possible future treatment regime for AP-induced SIRS and subsequent MODS. Doses capable of reducing systemic inflammation needs to be considerable lower than what can cause coagulation disorders. One question that needs to be addressed is the inter-species differences. The rat has twice the amount of circulating fVII compared to man under physiological conditions. This may influence the therapeutic window. One other consideration is that recombinant human fVIIai was used during the experiments. How this modified human protein differ in regards to affinity of different mediators of coagulation and inflammation in different species compared to man is not fully known. This is another relation that needs to be investigated further.

Local and systemic effects of fVIIai
Potential differences in treatment effects locally and systemically in regards to PAR2 activation during fVIIai have been discussed elsewhere (Axelsson et al. 2008b). The lack of demonstrable effects on NFκB during AP in the pancreas could be explained by several other potential mechanisms. It is known that the effect of fVIIai varies widely among different cell types, although TF is induced in the acinar cells during AP in the rat (Ji et al. 2003). Another explaination might be that this is an effect depending on the nature of the model. In the present model, AP is induced by intraductal infusion of sodium taurodeoxycholate, which solubilizes the pancreatic duct and adjacent tissue. Because of the extensive tissue destruction and subsequent inflammation, it is unlikely to get an effect of pre-treatment, although it may be effective in other secondary inflamed tissues or, as others have shown, in cell culture. Although unable to demonstrate any differences in the model used, other models have shown such as cerulein administration and duct ligation have increase of NFκB activation also locally in the pancreas (Gukovsky et al. 1998, Samuel et al. 2006). A third explanation may be that p65 is not involved in the activation but rather p50 as shown previously in acinar cells in AP in the rat (Shi et al. 2006).

Physiological relevance of commonly used animal AP models

Many objections may be presented to each of the commonly used experimental models of AP. Most of them address the issue of the initiating noxae not resembling the initiating events in the clinical situation.

The targeting of specific pancreatic cell types believed to be the primary effector cells is a problem. One example of where the models have steered the research in a monotonous way of thinking is the introduction of genetically modified mice (Axelsson et al. 2004). The opportunity to selectively study the impact of a single gene on the development of AP is an incredibly useful tool for the physiologist. The problem is the fact that KOs and transgenic animals are predominantly available in mice and the caerulein model is the model of choice in this particular species. This doesn't make the results produced using KO mice in this model useless but far from universal to AP in general and only applicable for caerulein-induced AP and other models specifically targeting the acinar cells. This may, as shown in the proposed

"ductal defence" theory, not always be the case. Several other AP models are available for use in mice, such as the TDC and HS models, and it is advisable that these are evaluated in the same setup as the commonly used caerulein model before jumping to conclusions and presenting them as universal facts concerning the pathophysiology of AP. The same problem is encountered when setting up in vitro systems to study certain events in AP pathophysiology. Many studies have been done on isolated acinar cells and to study their properties. Apart from the difficulties of contamination of other cell types, this, of course, disregards any interaction between different cells types.

The TDC model presents several problematical properties, all of which are hard to neglect. To induce a hemorrhagic AP in rats, a standardized protocol of infusion of 5% solution of TDC is commonly used (Aho et al. 1980). Besides being a supra-physiological concentration, not found in the normal rat, this concentration solubilizes all ductal cells and adjacent acinar cells of which the solution comes into contact with. Again, this specifically targets, or rather excludes, a specific cell type, namely the ductal cells as they are the first to be destroyed, without knowing the relevance of their importance in the etiology of clinical AP. This particular model is said to be useful for the study of secondary remote organ injury during AP. When administering TDC to the common bile duct it solubilizes the duct giving the bile from the liver and the secretions from the pancreas free access to the abdominal cavity. This is something not seen in clinical AP and causes peritonitis affecting remote organs of the same magnitude as rupturing the biliary duct without inducing pancreatitis. Interventional studies therefore are done in a mixed inflammatory model with very little in common with the clinical situation intended to be simulated.

To counteract the problems involved with specifically stimulate or destroy a particular cell type, other less specific or destructive models can be used. To mimic a more physiological situation the ductal ligation or HS models are especially appealing. Both of which do not initially destroy certain cell types and are possibly pathophysiologically relevant.

With this said about targeting specific cells, it is important to stress that these models only reflects certain parts of a complex disease (Schreiber 2000). This is important to take into account when evaluating the clinical applicability of new therapies tested in animals. It is not known how many potentially useful therapies were discarded because of being tested in an animal model not suitable for the certain physiological mechanism of the particular intervention. On the other hand, the opposite problem can be seen where promising therapies have failed in large clinical studies after promising results were obtained from unsuitable experimental studies. The need for more physiologically relevant models of AP cannot be stressed enough. In the long run, it is my firm belief that the development of models better reflecting the clinical situation is of far more value than an intensified evaluation of new interventions in presently existing experimental models of AP.

To summarize the detailed discussion on specificities and limitations of animal models, it is of the utmost importance to choose the model very carefully to suit the particular situation and mechanism of the intended study, when designing studies.

General reflections of science and scientific methods

One of the main reasons that new ideas develop as slowly as they do and that old paradigms are kept despite obvious poor correlation to the human situation is, in my opinion, not purely scientific but more likely political. Several of the cornerstones of the current consensus of the initiation of AP are very doubtful. Many of the more influential researchers in the field of pancreatology have spent a considerably piece of their career working on these theories and

are repeatedly seen pleading their case in different reviews of AP. This does not encourage new ideas to be shared freely in an efficient manner although the foundation on which the old paradigms are based is very weak. New trends such as open access scientific journals may in the future promote more openness within the scientific community and thereby facilitating more efficient evaluation of new results and theories.

One thing most certainly differing today's researcher from the Renaissance man is the fraction of the total scientific knowledge known to him or her. This presents a new and ever-increasing problem. The smaller fraction of the entire area he or she is able to grasp, the higher the risk of producing irrelevant data. When considering these circumstances, the need for a deeper understanding of biological processes is obvious. Closer collaborations of representatives of the whole area of which medical science is conducted are therefore needed. More inter-disciplinary projects will not expand the knowledge of each individual within a project but will expand the total body knowledge within the project, and hence will increase the probability relevant results being produced.

SUMMARY AND CONCLUSIONS

The most important conclusions of the thesis are summarized below:

- HS was shown to be capable of inducing rapid infiltration of both monocytes and neutrophils without causing acinar cell damage. This finding suggests a receptor mediated response in pancreatic cells.

- HS-induced pancreatic inflammation is probably initiated by ducal cells, which respond to HS-stimulation by secretion of MCP-1. Influx of inflammatory cells is a biphasic process where monocytes are first recruited and in turn produce CINC-1, which is capable of attracting neutrophils. HS-induced inflammation is clearly regulated via different pathways as LPS. LPS cause simultaneous influx of both monocytes and neutrophils and thus shows a different time course regarding influx of inflammatory cells to the pancreas.

- Pre-treatment of fVIIai reduces systemic inflammation as well as neutrophil infiltration in remote organs affected by AP. This suggests fVII to be a promising target for future interventional studies of AP-induced SIRS.

- NFκB was shown to be elevated in three remote organs commonly affected during severe AP. Pre-treatment with fVIIai was shown to reduce the NFκB activity increase in an organ and time dependent manner.

FUTURE ASPECTS

Innate immune response

We are currently undertaking HS and LPS infusion studies on TLR4, TRIF and MyD88 KO mice to study the signal transduction pathways in greater detail. It has previously been shown, in models of systemic inflammation (Johnson et al. 2004) as well as in epithelium in the urinary bladder (Hans Fischer, Lund University, Lund, pers. comm.) in mice that HS signal through the TLR4. This is very likely to be the case in the pancreatic ductal epithelium as well.

It has been shown that infusion of trypsin, α-chymotrypsin, and elastase into the pancreas cause AP (Yamano et al. 1998). Elastase has also been shown to induce SIRS when administered intravenously (Johnson et al. 2004) and is claimed to cause inflammation via TLR4 (Hietaranta et al. 2004), although criticism has been raised against this, claiming LPS contamination to be the reason (Geisler et al. 2005). All these proteases are likely to release HS from the ductal epithelial wall in the pancreas by cleaving the protein anchor of the HS-containing PG, leaving the HS intact. This may be enough to trigger the TLR4 or other receptor to signal, but it would also be of great interest to investigate if smaller parts of the HS are the active components and the important parts of the ligand. It would therefore be interesting to see if infusion of heparanases and heparinases, cleaving the HS at different sites but leaving the protein anchor intact, would elicit the same inflammatory response.

In connection to the above discussion, the ductal defense can be further studied using heparanase-1 over-expressing mice. Heparanase-1 is present in low concentrations in the ductal epithelium. If the epithelium is damaged, without complete solubilization, a physiological innate response could be elicited. Infusion of a low concentration of TDC would possibly give another degree of inflammatory response in the heparanase-1 over-expressing animals as compared to WT mice.

To further investigate the relationship of the structure of HS and inflammatory activity it would be of great importance to study interaction of different HS with TLR4. Current research is therefore undertaken in our lab on HS of different degrees of sulfation and size to elucidate their inflammatory properties in a TLR4/MD2/CD14 transfected cell line (HEK-293, InvivoGen, San Diego, CA, USA).

It has not been fully elucidated if HS and LPS bind to the same active site on TLR4. This could be done in vitro by pre-treatment with a known antagonist of LPS-mediated TLR4 signaling, such as E5531 (Christ et al. 1995), and thereafter stimulating with HS.

The ducal cells are to some extent affected by both HS and LPS infusion as evidenced by light microscopy evaluation. There changed need to be studied further, preferable using transmission electron microscopy. Comparing the images to human pancreatitis biopsies will provide a possibility to morphologically investigate the biological relevance of HS as a inflammatory initiator.

To show that the initial events shown in this thesis are applicable in human, and not restricted to the rat, it is of the utmost importance to show that the human ductal epithelium responds in the same way as in the rat. Experiments of stimulating human pancreatic epithelium, excised during operation, using HS and LPS either in vitro or ex-vivo would yield evidence of the proposed “ductal defense” hypothesis is an important principle in man as well.

In combination of the experiments proposed above on the active parts of the HS molecules as well as the “true” triggering concentrations discussed under the paragraph ‘variability of the models’, it is important to investigate if the necessary concentration of free

HS to elicit an inflammatory response can be reached within the pancreatic duct. It would therefore be very interesting to sample the content of the biliary or pancreatic ducts of patients showing signs of stasis of the biliary duct. This could easily be done when doing ERCP to remove obstructing gall stones and analysis of the HS content would yield conclusive evidence of the clinical relevance of this novel hypothesis.

When the studies suggested above are performed and evaluated, clinical studies should be initiated to adapt the current knowledge of HS, LPS and TLR4 in relation to AP, into clinical practice. Several opportunities based on the conclusions from this knowledge are possible. Mixing TLR2 and TLR4 inhibitors to the contrast medium could possibly reduce the incidence of ERCP-induced AP attacks.

Coagulation

Previous studies indicated that the inflammatory properties of fVIIa are mediated though PAR2 (Camerer et al. 2000). It is therefore likely that the anti-inflammatory effects systemically are closely linked to the reduced signaling of PAR2. Other studies have shown that caerulein-induced pancreatitis is less severe in PAR2 KO mice, indicating a protective effect of PAR2 signaling locally in the pancreas. These reversed effects of PAR2 in the pancreas versus the circulation must be addressed in relation to fVIIai. Studies of fVIIai effects using PAR2 inhibitors or PAR2 KO mice would be most interesting and may answer questions concerning possible increased pancreatic inflammation after fVIIai administration.

In an attempt to investigate if fVIIai therapy is effective also after the onset of AP, fVIIai was administered 30 and 90 minutes after TDC-induced AP in the rat. These results show that, although not as dramatic effects as when the substance was given prior to AP-induction, it was effective when lowering the systemic inflammatory response. Many objections to evaluating the clinical applicability of therapy in animal models can be put forward. For example the difference in concentrations of mediators in the coagulation cascade and their possibly varying importance for the inflammatory response are questions that must be addressed before proceeding further in this direction. Despite this, the results indicate some kind of clinical usefulness as it is crucial to investigate if substances are capable of affecting the inflammation even when administered after the onset of AP.

Measurement of fVII and TF in plasma of patients of mild and severe acute pancreatitis as well as healthy volunteers has been undertaken at our lab. These studies did not conclusively show any differences in concentrations between the groups but showed an enormous variation in all groups (data not published). One theory of failing to show results is the interference of plasma lipids with the ELISA used. Further studies in this interesting area of research should prove useful in elucidating if fVII is involved in the progression of systemic inflammation during AP and therefore can be used as a biomarker to access AP severity or predict clinical outcome.

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