Juan Iovanna
Uktam Ismailov
Editors

Pancreatology

From Bench to Bedside

Springer

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Preface

In September 2007, several experts in the field of pancreatic pathophysiology, supported by the NATO Science Committee, met in Tashkent, Uzbekistan, to present their most recent data and discuss basic, genetic, clinical, and surgical aspects of pancreatic diseases.

In basic research, the implication of pancreatic stress proteins in acute pancreatitis and pancreatic cancer and their possible role as therapeutic targets were reported. Also, very original results showing the unexpected role of lipids as mediators that worsen acute pancreatitis were described. Gene screening strategies allowing detection of the genes responsible for gemcitabine resistance of pancreatic cancer cells were presented. They led to the selection of several target genes to suppress the resistance of cells to gemcitabine treatment. The mechanism by which tetrahydrocannabinol is antitumoral in pancreatic cancer cells was presented and the use of tetrahydrocannabinol as a promising new therapeutic agent was discussed. Genetic data were shown concerning hundreds of families with hereditary chronic pancreatitis and the possible role of genetic factors in the pathogenesis of the disease was analyzed. Another very original study addressed the prevention and treatment of pancreatic diseases with diet. In clinical research, convincing data on the use of endoscopic sphincterotomy in the management of acute biliary pancreatitis were presented. Finally, the most recent consensus on indications for surgery in acute necrotizing pancreatitis was presented, based on the experience of a center highly specialized in pancreatic diseases.

Marseille, France — Dr. Juan Iovanna
Tashkent, Uzbekistan — Dr. Uktam Ismailov

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Heat Shock Proteins in Pancreatic Diseases

1

A.K. Saluja and V. Dudeja

Heat Shock Proteins

Heat shock proteins (HSPs) are cytoprotective molecules that help maintain the metabolic and structural integrity of cells. HSP synthesis is an evolutionarily conserved protective mechanism that ensures the survival of cells under stressful conditions. Although the HSPs were originally identified and named on the basis of their induction by elevated temperature, recent studies have shown that HSPs are expressed in response to an array of stresses, including oxygen-derived free radicals, amino acid analogs, ethanol, and heavy metals [1–7]. HSPs are also induced by a variety of physiological and pathological states, such as tissue injury and repair, aging, inflammation, and infection. Further, it has been demonstrated that HSPs protect against nonthermal injury-inducing stimuli [8]. Besides their role in protecting cells against injury, HSPs have many essential functions in physiological states, such as protein folding, transport, translocation, degradation, and assembly [9].

HSPs are subclassified into various families according to their molecular mass in kilodaltons. In mammals, the major families of HSPs are HSP90, HSP70, HSP60, HSP40, and the small HSPs. Four members of 70-kD family have been identified, mitochondrial HSP70, BiP, *HSP70* (*the only inducible form of this family*), and HSC70 (constitutive form) in mammals [3, 5, 7]. This review will focus mainly on the role of HSP70 in pancreatitis and pancreatic cancer.

HSPs, Beneficial or Harmful in Pancreatic Disease: A Matter of Perspective

Recent studies from our laboratory have clearly demonstrated the role of HSPs in pancreatic diseases. HSP70 especially plays a role in the pathogenesis of both pancreatitis and pancreatic cancer. In summary, our studies have shown that HSP70 protects against injury

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and cell death in pancreatitis: injury and cell death is central to the pathogenesis of this disease, and HSP70 is beneficial and protects against and reduces the severity of pancreatitis. In contrast to pancreatitis, the pathogenesis of pancreatic cancer involves dysregulated proliferation and decreased cell death. Similar to our findings in pancreatitis, our studies suggest that HSP70 prevents cell death in pancreatic cancer cells. Thus, in pancreatic cancer, where therapies are aimed towards inducing cell death in cancer cells, HSP70 expression is deleterious but at the same time gives HSP70 inhibition the opportunity to emerge as a novel therapeutic strategy.

HSPs in Pancreatitis

Many animal models of pancreatitis have been evolved to recapitulate and study human diseases. The caerulein model of pancreatitis, in which pancreatitis is induced in either rats or mice by repeated injection of caerulein, is the most commonly used model of animal pancreatitis. Though the pancreatitis observed in this model is of mild-moderate severity, this model is the most widely used and has contributed significantly to our current understanding of the pathogenesis of pancreatitis. This model closely mimics findings in the human disease, including a rise in serum amylase levels, pancreatic edema (quantitatively measured by pancreatic water content), acinar cell necrosis, and neutrophilic infiltration of the pancreas (quantitated by measuring levels of the neutrophilic enzyme myeloperoxidase in the pancreas). Also, activated trypsinogen, which has been considered to be the hallmark of pancreatitis, is also observed in this model. Trypsinogen is activated to yield active trypsin and trypsinogen activation peptide (TAP). Other models in vogue include the arginine model and the taurocholate model.

We became interested in the role of HSPs in pancreatic diseases when we observed that HSP60 protects against pancreatitis in rats [10]. Water immersion stress induces a robust HSP60 synthesis response in rats, and we observed that inducing HSP60 by water immersion prior to inducing caerulein pancreatitis decreases all the markers of pancreatic acinar injury, viz., pancreatic edema, serum amylase levels, and pancreatic necrosis [10]. Further, HSP60 not only protected acinar cells against injury, it also reduced trypsin activation, which is the fundamental early event in the initiation of pancreatitis, as observed by reduced trypsin activity and TAP levels in the pancreas.

To ascertain whether this protective effect of HSPs in pancreatitis is limited to HSP60 or whether it is current with other HSPs also, we evaluated the effect of HSP70 induction in pancreatitis. In contrast to HSP60, HSP70 is induced by thermal stress as well as by sodium arsenite. We observed that, similar to the protective effect of HSP60, HSP70 protects against pancreatic acinar injury and pancreatitis. Thus, thermal-stress-induced HSP70 markedly reduces serum amylase levels as well as other markers of pancreatic severity, such as pancreatic edema, pancreatic necrosis, and neutrophilic infiltration [11]. In fact, as observed with HSP60, HSP70 also protects against fundamental early intra-acinar events, viz., trypsin activation, which suggests that the protective effect observed is specific to pancreatitis [11]. Although these findings strongly suggest that HSP70 reduces the severity of pancreatitis and prevents intra-acinar trypsinogen activation, the fact that the thermal stress used to induce HSP70 expression may have other non-HSP70-related effects begs

additional measures to unequivocally prove that it is in fact HSP70 which mediates the protective effect of thermal stress. This has been answered by us by use of multiple approaches. Firstly, the effect of HSP70 induction on the severity of pancreatitis was evaluated by using sodium arsenite to induce HSP70 [12]. Similar to the protective effect of thermal stress induced by HSP70, HSP70 induction by sodium arsenite protects against both early and late events in pancreatitis. Thus, sodium arsenite prevented the activation of trypsin and the formation of TAP, serum amylase elevation, pancreatic edema, acinar cell necrosis, and the neutrophilic infiltration of the pancreas [12].

The specific role of HSP70 was further confirmed by use of state-of-the-art antisense technology and use of knockout mice. Antisense HSP70 but not sense HSP70 specifically reduces the thermal-stress-induced HSP70 expression but will not prevent other nonspecific effects of heat. Thus, if the protective effects of thermal stress against pancreatitis are due to HSP70, the protective effect should be obviated by antisense HSP70. We observed that in fact antisense HSP70 prevented the induction of HSP70 by thermal stress and restored the ability of supramaximal caerulein stimulation to activate intrapancreatic trypsinogen even in thermally stressed animals, and negated the protective effect of prior thermal stress against acinar cell injury in pancreatitis [13] (Fig. 1). Also, in the negative control sense HSP70 (which does not reduce HSP70 expression in response to thermal stress) did not change the protective effect of thermal stress (Fig. 1).

As discussed, HSP70 is induced by a multitude of cellular stresses, including inflammation. Since pancreatitis in itself is a source of stress and animals with experimental pancreatitis overexpress HSP70 in pancreas, this increased HSP70 expression appears to protect

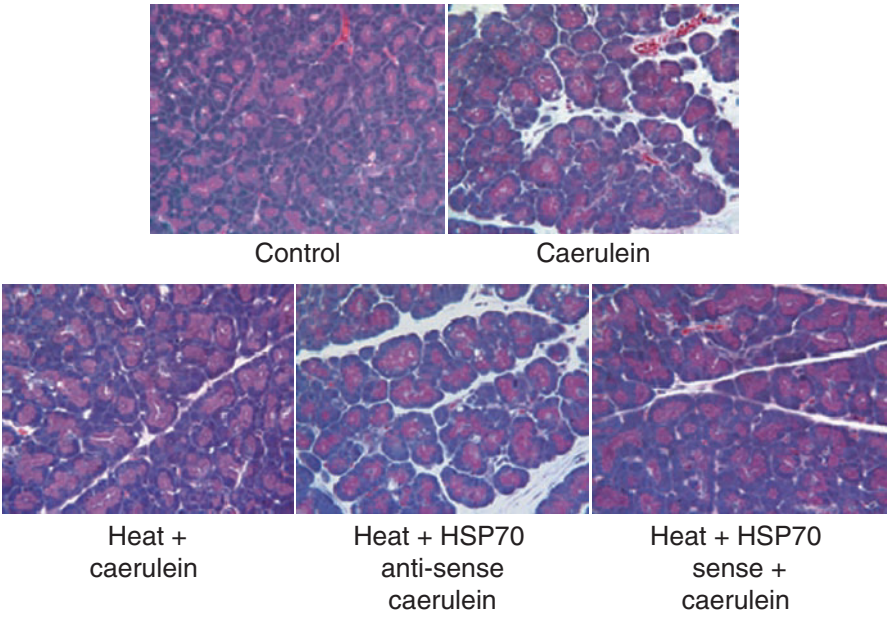


Fig. 1 Heat shock protein 70 (*HSP70*) reduces the severity of pancreatitis. Rats in which HSP70 had been induced by thermal stress prior to caerulein administration demonstrate minimal pancreatic necrosis as compared with the caerulein-only group. Administration of antisense HSP70 and not sense HSP70 reverses the protection offered by thermal stress

acinar cells from further injury, since pretreatment with antisense HSP70, which prevents induction of HSP70 in response to pancreatitis, exacerbates all the parameters associated with pancreatitis [13], again reiterating the role of HSP70 in protection against pancreatitis-induced acinar cell injury.

The protective role of HSP70 in pancreatitis was further proven by using heat shock factor 1 (HSF-1) knockout and transgenic mice overexpressing HSP70. HSF-1 is the transcription factor for HSP70 and is activated in response to thermal and other stresses; it leads to increased HSP70 synthesis. HSF-1 knockout mice are unable to express HSP70 in response to stress. This offers another avenue for examining the specificity of HSP70 in thermal-stress-induced protection against pancreatitis. We observed that since HSF-1 knockout mice lack the ability to synthesize HSP70 in response to stress of pancreatitis, they develop a more severe pancreatitis in response to caerulein administration as compared with wild-type mice. Similarly, transgenic mice overexpressing HSP70 are protected against cellular injury during pancreatitis. These experiments collectively demonstrate the protective effect of HSP70 against pancreatitis.

How Does HSP70 Protect Against Pancreatitis?

The mechanism by which HSP70 protects against pancreatitis is unclear. However, the studies in our laboratory have identified some key steps in the pathogenesis of pancreatitis which may be influenced by HSPs. A rise in intracellular calcium is an elementary intra-acinar event in the pathogenesis of pancreatitis that has been clearly demonstrated in the acinar cells in the caerulein model of pancreatitis. Remarkably, studies in our laboratory have demonstrated that HSP60 reduces caerulein-induced intra-acinar calcium elevations, thus attenuating both the peak levels as well as sustained levels of calcium. Whether this interaction with calcium is specific to HSP60 or occurs with other HSPs too is of great significance given the role of elevated calcium in many disease processes, including pancreatitis and cancer.

Lysosomal colocalization with secretory zymogen compartments has been observed early on in pancreatic acini and has been proposed as the mechanism of intra-acinar trypsin activation. Briefly, supramaximal pancreatitis-causing doses of caerulein redistribute lysosomal enzymes and lead to the colocalization of lysosomes and zymogen granules. This brings the trypsinogen from zymogens and cathepsin B from the lysosomes into contact, and cathepsin B helps activate trypsinogen. We have observed that thermal-stress-induced HSP70 prevents colocalization and could be one of the mechanisms by which HSP70 prevents intra-acinar trypsin activation and acinar cell injury, and ultimately reduces the overall severity of pancreatitis.

Simply put, the pathogenesis of cancer involves dysregulated apoptosis and inhibiting cell death mechanisms. The fact that HSP70 protects against cellular injury and cell death encouraged us to evaluate the role of HSP70 in pancreatic cancer, a deadly cancer with a poor prognosis. Our hypothesis was that pancreatic cancer cells overexpress HSP70, which prevents cell death and response to conventional therapy. To begin with, we analyzed the HSP70 protein expression in various pancreatic cancer cell lines and compared it with that of normal pancreatic ductal cells, the cells from which pancreatic carcinoma is believed to originate. Excitingly, HSP70 is markedly overexpressed in pancreatic cancer cells as compared with normal ductal cells (Fig. 2a) [14]. Similarly HSP70 messenger RNA levels are

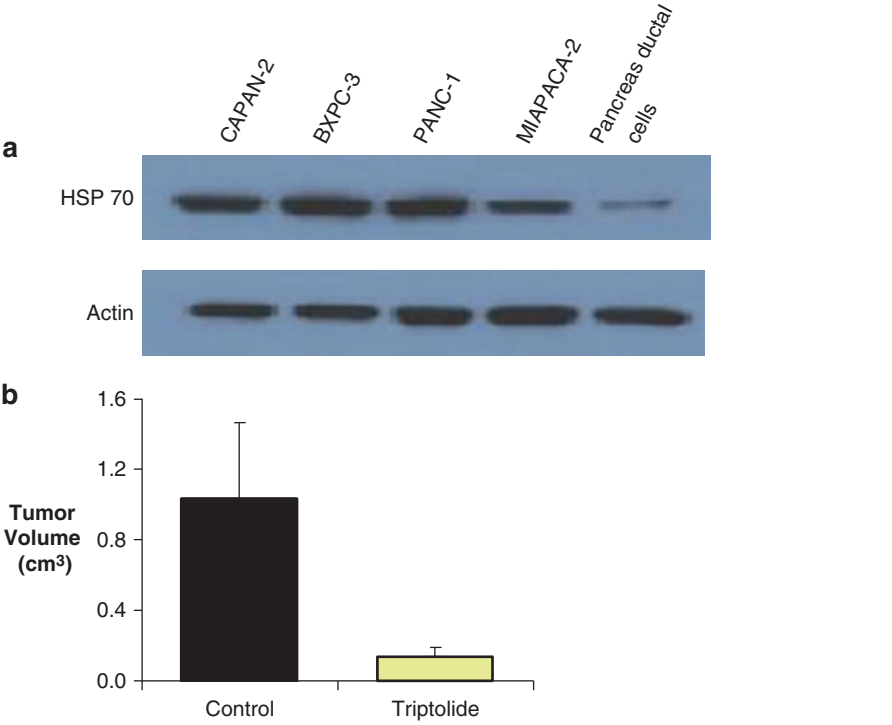
markedly high in pancreatic cancer cells as compared with normal ductal cells [14]. To place this finding in the right clinical perspective, we compared the levels of HSP70 expression in human pancreatic cancer specimens with those in normal pancreatic tissue margins of the same subject. Corroborating the data from in vitro experiments, we found the HSP70 levels in pancreatic cancer tissue were markedly high compared with those in normal margins, thus suggesting that HSP70 could play a role in the resistance to apoptosis demonstrated by pancreatic cancer cells. These facts point towards the possible role of HSP70 in the pathogenesis of pancreatic cancer as well as its relevance to human disease.

We next ascertained whether or not the inhibition of HSP70 expression can be used as a therapeutic strategy against pancreatic cancer. If HSP70 protects pancreatic cancer cells against cell death, inhibiting HSP70 should either lead to their death or should make them more susceptible to cell death. We used two different but complementary strategies to inhibit HSP70 expression: one pharmacological and the other state-of-the-art small interfering RNA (siRNA) technology.

Two pharmacologic inhibitors of HSP70 expression are known: quercetin and triptolide. As discussed previously, HSF-1 is the transcription factor for HSP70. Quercetin is a naturally occurring flavonoid which inhibits heat shock response by interacting with the HSP70 transcription pathway at the level of HSF-1 [15]. Dihydroquercetin is an inactive analog of quercetin. We observed that quercetin but not dihydroquercetin inhibits HSP70 expression in pancreatic cancer cells in a dose-dependent manner. Remarkably, in accordance with HSP70's protective role against cell death, inhibiting HSP70 expression by quercetin leads to pancreatic cancer cell death. Dihydroquercetin neither decreases HSP70 expression nor does it kill pancreatic cancer cells. Remarkably, normal pancreatic ductal cells are not killed by either quercetin or dihydroquercetin. We believe that this is due to the fact that normal pancreatic ductal cells express only a minimal amount of HSP70 and that the expression of HSP70 is not reduced by quercetin. We believe that anti-HSP70 therapy will specifically target the cancer cells and will not influence normal cells. Since HSP70 is an inducible protein which is expressed only in the presence of stress, normal cells of the body express very little of it and thus are immune to the anti-HSP70 therapy, thereby targeting HSP70 to cancer cells.

Since cancer cells dying in response to HSP70 inhibited by quercetin demonstrate markers of apoptotic cell death such as annexin V positivity and TUNEL positivity, it suggests that inhibiting HSP70 activates apoptotic cell death instead of necrotic cell death [14]. Also, this apoptotic cell death is caspase-dependent, as is apparent from the fact that caspases are activated on the inhibition of HSP70 in pancreatic cancer cells. Quercetin has other effects besides inhibiting HSP70 expression, for example, antioxidant effects. To prove that the effect of quercetin on pancreatic cancer cell viability is in fact due to its effect on HSP70 expression and not due to its effect on other targets, we used HSP70 siRNA to specifically evaluate the phenotypical effect of inhibiting HSP70 on pancreatic cancer cells. The specific inhibition of HSP70 by HSP70 siRNA also induced apoptotic cell death with caspase activation in pancreatic cancer cells. Together these findings suggest that although quercetin may have effects independent of the effect on HSP70 expression, its primary effect is in fact due to the inhibition of HSP70 expression [14].

We further evaluated the efficacy of quercetin at decreasing pancreatic tumor growth in animal studies. In in vivo studies also, quercetin was found to reduce the growth of subcutaneous pancreatic tumors and to effectively reduce HSP70 protein levels in tumors as compared with the control group, which points to the efficacy of anti-HSP70 therapy. However,


a
CAPAN-2
BXPC-3
PANC-1
MIAPACA-2
Pancreas ductal cells
HSP 70
Actin
b
Tumor Volume (cm3)
1.6
1.2
0.8
0.4
0.0
Control
Triptolide
c
Controls
Triptolide Treatment

nonspecific toxicity and a necessarily high dosage (50 mg/kg per day) precludes the use of quercetin in a clinical setting. Recently, a new compound, triptolide, has been identified as an inhibitor of heat shock response [16]. Triptolide is a diterpene triepoxide from the Chinese plant *Tripterygium wilfordi* and has been used as a natural medicine in China for hundreds of years, particularly in the treatment of autoimmune and inflammatory diseases, including rheumatoid arthritis. We examined whether or not triptolide could inhibit HSP70 expression in pancreatic cancer cells. Remarkably, triptolide inhibits HSP70 messenger RNA and protein expression in pancreatic cancer cells in a dose-dependent manner at nanomolar doses [17]. Also, it is able to initiate a massive apoptotic cell death response by virtue of its effect on HSP70 expression. Treating pancreatic cancer cells with triptolide activates caspase 3/9, which then activates the apoptotic cascade as confirmed by the positivity of cells for the apoptotic markers annexin V and TUNEL [17]. Triptolide markedly reduced the growth of pancreatic tumor in an orthotopic model of pancreatic cancer. In this model, treatment with triptolide for 60 days at a small dosage of 0.2 mg/kg per day drastically reduced tumor growth in the treatment group as compared with the control group (treated with vehicle alone) (Fig. 2b, c), so much so that no tumor was observed in two mice in the treatment group at gross or microscopic examination [17]. Not only did triptolide reduce the tumor growth, it also reduced the locoregional spread of the pancreatic tumor. Seven out of eight animals in the control group had spread of tumor to multiple other organs, whereas only one animal in the triptolide treatment group had locoregional spread, and that only to one organ [17]. Also, animals treated with triptolide had no signs of toxicity. In these experiments, triptolide showed great potential as a novel therapeutic strategy against pancreatic cancer. Triptolide is soluble in organic solvents such as dimethyl sulfoxide and efforts are under way to make analogs of triptolide with better water solubility and better oral bioavailability.

Summary

- HSP70 has a dual role in pancreatic disease. It has a protective role in pancreatitis but has a deleterious role in pancreatic cancer.
- HSP70 protects against cellular injury and acinar necrosis in pancreatitis, which leads to reduced severity of pancreatitis.
- HSP70 is overexpressed in pancreatic cancer. This overexpression is responsible for the cancer's resistance to cell death.
- Inhibiting HSP70 expression leads to apoptotic cell death in pancreatic cancer cells and is a promising therapeutic strategy against pancreatic cancer.

Fig. 2 Inhibition of HSP70 as a potential therapeutic strategy for pancreatic cancer. **a** Cell lines derived from pancreatic cancer (Capan-1, BXPC3, Panc-1, MiaPaCa-2) overexpress HSP70 as compared with normal pancreatic ductal cells. Actin was used as a loading control. **b** Triptolide reduces the growth of tumor in an orthotopic model of pancreatic cancer as compared with a no-treatment group. **c** Representative tumor specimens from triptolide and control groups demonstrating that the tumors in the control animals were remarkably bigger than those in the triptolide group

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Cellular Stress in Acute Pancreatitis

2

J.L. Iovanna

Introduction

Acute pancreatitis is the most frequent disease of the pancreas. The spectrum of acute pancreatitis can range from mild edematous to severe necrotizing [1]. One of the most interesting hypotheses for the pathogenesis of the disease is that autodigestion of the gland occurs when hydrolytic enzymes (e.g., trypsinogen, chymotrypsinogen, proelastase, and prophospholipase A) are unduly activated within the pancreas rather than into the intestinal lumen [2, 3]. Activated enzymes, especially trypsin, not only digest pancreatic and peripancreatic tissues but can also activate other zymogens, such as proelastase and prophospholipase A. The active enzymes then digest cellular membranes and lead to edema, interstitial hemorrhage, vascular damage, coagulation necrosis, fat necrosis, and parenchymal cell necrosis.

Living organisms respond at the cellular level to stress or pathological aggression by altering the normal pattern of protein synthesis [4–8]. That change is characterized by a dramatic induction of stress proteins with concomitant inhibition of the normal array of cellular proteins. Stress proteins are not novel components of the stressed cells since most of them are expressed to some level in cells grown under normal conditions [9, 10]. Because most attacks of acute pancreatitis are mild and self-limiting, it is possible that the pancreatic cells respond with a rapid adaptation of their phenotype, which eventually stops the progression of pancreatitis. However, in 10–20% of cases, a severe disease with multiple local and systemic complications develops [11]. In these cases, we can speculate that the pancreatic defense mechanisms fail to protect the gland and therefore the organism. Studies in animals and humans, performed during the acute phase of pancreatitis, demonstrated that the content and secretion of pancreatic enzymes, which are potentially harmful, were generally reduced, as part of a defense mechanism. Conversely, other genes were strongly activated during the acute phase of the disease [12, 13]. Therefore, like the liver [14] and other

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organs [6, 7], the pancreas exposed to acute stress seems to trigger a stringent emergency program that helps the gland fight the aggression and, consequently, protects the organism from the deleterious effects of pancreatitis.

The aim of our research is to characterize at the molecular level the pancreatic emergency program set up in response to pancreatitis. We developed a strategy in which the phenotype of the pancreas with acute pancreatitis was established by characterization of a large number of its transcripts. Such a complementary DNA (cDNA) collection represents a reservoir from which transcripts involved in the emergency response can be identified on the basis of their expression patterns. In this report, we describe a novel membrane protein, named "vacuole membrane protein 1" (VMP1), that is strongly and rapidly induced in pancreas during acute pancreatitis.

Cloning the Rat VMP1 Messenger RNA

A cDNA library of 4.1×10^5 clones was constructed from the polyadenylated RNA fraction purified from a rat pancreas with acute pancreatitis. From this cDNA library, 1,536 randomly selected clones were partially sequenced and the resulting sequences were compared with the GenBank database. Among these clones, 256 could not be related to any sequence in the database. Expression of these messenger RNAs (mRNAs) during acute pancreatitis was systematically analyzed by northern blot. Expression analysis of clone 10F5 showed an interesting pattern. This mRNA is strongly and rapidly activated after the induction of experimental acute pancreatitis. The VMP1 mRNA codes for a protein of 406 amino acids, with a theoretical p*I* of 6.28. The predicted molecular mass is 45,901 Da. Analysis of the deduced VMP1 primary structure revealed the presence of six transmembrane helices which are typical of a transmembrane protein.

VMP1 mRNA Expression During the Course of Acute Pancreatitis

Pancreatic RNA was obtained from rats at different times after induction by caerulein of experimental acute pancreatitis. Northern blot analysis with the VMP1 cDNA probe revealed a very low level of expression at time zero. Significant induction was observed after 30 min. It was maximal after 1 h and remained activated during the whole study (18 h). Also, high levels of VMP1 mRNA were expressed during taurocholate-induced pancreatitis. In situ hybridization was performed to identify which cell type expressed the VMP1 transcript in pancreas. Pancreatic tissues from control and caerulein-treated rats were hybridized with the digoxigenin-labeled antisense VMP1 RNA. A strong labeling was observed in the acinar cells. By contrast, VMP1 remained undetectable in the islets of Langerhans, ducts, inflammatory infiltrate, and stromal tissue in pancreas. Pancreas from control rats showed no signal.

Induction of VMP1 mRNA Expression in Stressed Kidney

To see whether VMP1 induction was specific to the injured pancreas, we monitored its expression in the postischemic kidney. In our experimental model, the left kidney of the rats was subjected to 30-min ischemia, the right kidney being used as control, and expression of VMP1 mRNA was studied 16 h later. VMP1 mRNA expression is induced by ischemic treatment. VMP1 mRNA expression was low in the right (control) kidney, but strong in the left (ischemic) kidney. Pancreas of the same animals showed no VMP1 mRNA induction.

VMP1 Expression Induced Vacuole Formation

The intracellular localization of VMP1 was assessed by transfecting Cos7 cells with a vector allowing expression of an enhanced green fluorescent protein (EGFP)-tagged VMP1 protein and direct monitoring of EGFP fluorescence. Figure 1 shows that VMP1 is located in the Golgi apparatus and the endoplasmic reticulum area, and that its expression induces formation of vacuoles and this is why we named the protein "vacuole membrane protein 1" (VMP1). The same fusion plasmid was transfected into other cell lines with similar results. The forced expression of VMP1 induces vacuole formation in mammalian cells and its endogenous expression correlates with vacuole formation in pathological pancreatic tissue [15]. Therefore, we thought that VMP1 could be involved in the autophagic process and we tested the hypothesis that VMP1 expression triggers the formation of autophagosomes in mammalian cells.

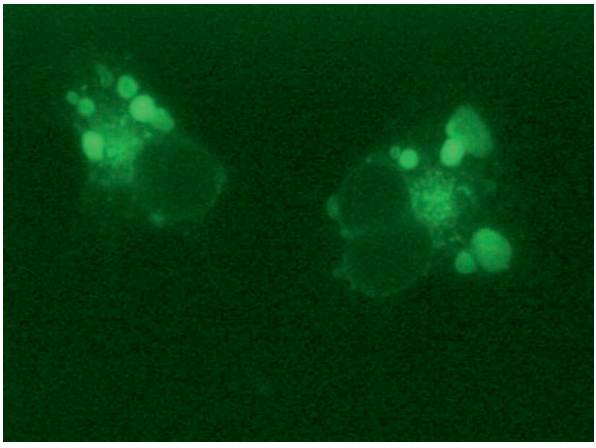

Fig. 1 Forced expression of the vacuole membrane protein 1 (VMP1)–enhanced green fluorescent protein (EGFP) fusion protein in Cos7 cells. Cos7 cells were transfected with the plasmid pEGFP–VMP1 using FuGENE reagent. VMP1 expression was evidenced by direct green fluorescence of the VMP1–EGFP fusion protein. Fluorescence microscopy images show that VMP1–EGFP expression induced cytoplasmic vacuole formation

What Is Autophagy ?

Autophagy is an evolutionarily preserved degradation process of cytoplasmic cellular constituents, which serves as a survival mechanism in starving cells [16–18]. This catabolic process is involved in the turnover of long-lived proteins and other cellular macromolecules, and might play a protective role in development, aging, cell death, and defense against intracellular pathogens [19–22]. Autophagy is also associated with nonapoptotic type II cell death, also called "autophagic degeneration" [17, 23–25]. Recently, autophagy was described to be required for apoptotic cell clearance during embryonic development [26]. By mostly morphological studies, autophagy has been linked to a variety of pathological processes. Early reports of autophagy in human disease include the ultrastructural autophagic features described in pancreas from human pancreatitis [27]. Furthermore, autophagy has been linked to neurodegenerative diseases and tumorigenesis, which highlights its biological and medical importance [19, 28, 29].

Autophagy is characterized by sequestration of bulk cytoplasm and organelles in double-membrane vesicles called "autophagosomes," which eventually acquire lysosomal-like features. During autophagy, an isolation membrane (herein referred to as "autophagosomal membrane") forms as a preautophagosomal structure, invaginates as a cup-shaped structure, and sequesters cytoplasmic constituents, including mitochondria, endoplasmic reticulum, and ribosomes. The edges of the membrane fuse to form a double-membrane or multimembrane structure, known as the "autophagosome," or "autophagic vacuole." The outer membrane of the autophagosome fuses with the lysosome to deliver the inner membranous vesicle to the lumen, thus forming the autolysosome. The final degradation step takes place within these structures, where lysosomal hydrolases digest the luminal content of the autophagic vacuole to turn it into recyclable breakdown products [30, 31].

Autophagy is inhibited by a serine threonine protein kinase originally recognized as a target of rapamycin and therefore named "TOR" [16, 32], which is inhibited under starvation-induced autophagy. The progression of the autophagy is sensitive to the phosphatidylinositol 3-kinase (PI3K) inhibitors such as 3-methyladenine (3-MA), with the target being the class III PI3K [33]. Most of the detailed molecular mechanistic work on autophagy has been carried out in the yeast *Saccharomyces cerevisiae* as a cellular response for survival during nutrient-limited conditions [34]. Autophagosome formation is mediated by a set of evolutionarily conserved autophagy-related proteins (Atg proteins) [32]. One of the best-defined Atg proteins is microtubule-associated protein 1 light chain 3 (LC3). LC3, the mammalian homolog of yeast Atg8, undergoes complex C-terminal proteolytic and lipid modifications, upon which it translocates from the cytosol to the autophagosomal membrane [35–37]. The recruitment and proper localization of critical Atg complexes to the autophagosomal membrane is mediated by a lipid kinase complex formed by VPS34 and autophagy-specific subunits such as Atg6, which is eventually followed by the translocation of Atg8/LC3 to the autophagosomal membrane [38]. In mammalian cells, the initial step of autophagic process is controlled by Beclin 1 [39], the mammalian ortholog of the yeast Atg6. Beclin 1 is a Bcl-2 interacting protein [18, 40, 41] that promotes autophagosome formation when it functions as part of a complex with hVsp34, the class III PI3K [42]. Subcellular distribution to a membrane structure and generation of phosphatidylinositol 3-phosphate by the Beclin 1–class III PI3K complex is thought to be important in mediating

the localization of other autophagy proteins to autophagosomal membranes. However, despite the advances in understanding autophagy, autophagosome formation in mammalian cells is a complex process and neither the molecular mechanism leading to its formation nor all the implicated genes have been fully elucidated [43, 44].

VMP1 Is an Autophagy-Involved Protein

To find out if VMP1 triggers autophagy, we transfected HeLa cells with the expression plasmid pcDNA4–VMP1, which codes for the VMP1–V5 fusion protein. Cells were cultured in nutrient-replete conditions and fixed in glutaraldehyde 24 h later so we could perform transmission electronic microscopy. We observed that cells expressing VMP1 showed multiple autophagic features. We found cup-shaped structures, double-membrane structures containing cytoplasmic material (autophagosome-like structure), as well as single-membrane structures containing cytoplasmic constituents at different stages of degradation (autolysosome-like structure) [45, 46]. Autophagy features did not differ from those obtained in rapamycin, a well-known inductor of authophagy, treated cells. The same morphological features were obtained when 293T or AR42J pancreatic acinar cells were transfected with the VMP1 expression plasmid. During autophagy, the precursor LC3 is cleaved by the proteolytic enzyme atg4 (LC3-I) and then undergoes C-terminal lipid modifications (LC3-II), and translocates from the cytosol to the autophagosomal membrane [36, 47] as expected. With some limitations [48], LC3 is currently used as a specific marker of autophagy [49]. To confirm the extent and specificity of VMP1 autophagosome induction, we first immunostained pcDNA4–VMP1-transfected cells with a specific LC3 antibody and we observed the signal of endogenous LC3 in punctate structures. Then, we investigated LC3-I and LC-II forms by western blot analysis and we found induction of LC3 with increased LC3-II form signal in pcDNA4–VMP1-transfected cells as expected. Since intra-autophagosomal LC3-II is degraded by lysosomal proteases, we blocked its proteolysis using the lysosomal protease inhibitor E64d [50] and we found the enhancement of LC3-II signal in VMP1-expressing cells. In another series of experiments, HeLa, 293T, and AR42J pancreatic acinar cells cultured under nutrient-replete conditions were concomitantly transfected with an expression plasmid encoding for the red fluorescent protein (RFP)–LC3 fusion protein and pcDNA4–VMP1 or pcDNA4-empty plasmids. We found the recruitment of LC3 fluorescence fusion protein in punctate structures in VMP1-transfected cells in contrast to the diffuse RFP–LC3 fusion protein signal observed in control cells. Finally, we investigated the potential for inhibiting the pathway with an agent well documented to inhibit autophagy such as the PI3K inhibitor 3-MA [33]. Although 3-MA was reported to have side effects apart from inhibiting autophagy [24], the inhibition of LC3 recruitment is due to its ability to inhibit autophagy. HeLa, 293T, and AR42J pancreatic acinar cells were treated with 3-MA before the cotransfection with pRFP–LC3 and pcDNA4–VMP1 expression plasmids and the percentage of RFP–LC3 cells with punctate staining was low and almost the same as that observed in pcDNA4-empty transfected cells. These results collectively demonstrate that VMP1 expression triggers autophagy in mammalian cells, even under nutrient-replete conditions.

VMP1 Expression Is Required for Extracellular-Stimuli-Induced Autophagy

The autophagy trafficking pathway was first described as a cellular adaptation to starvation [51]. To investigate whether starvation activates endogenous VMP1 expression, we developed a rabbit polyclonal anti-VMP1 antibody. We subjected HeLa cells to a standard starvation protocol (amino acid/serum-deprived medium) and then analyzed the time course of VMP1 mRNA expression by reverse transcription PCR assay and of VMP1 protein expression by western blot analysis. The expression of VMP1 mRNA and that of VMP1 protein are activated under starvation. VMP1 expression was evident after 2 h of treatment. Moreover, we localized VMP1 in punctate structures by immunofluorescence. To confirm the above-described findings, we induced autophagy by pharmacological means. mTOR kinase plays a central role in the amino acid pool sensing mechanism. In response to starvation, mTOR is inhibited, resulting in the induction of autophagy [52] through a downstream mechanism, which is still unknown. Since mTOR can be inhibited by rapamycin, this compound is routinely used as a pharmacological agent to induce autophagy. We treated several cells with rapamycin and, using reverse transcription PCR, western blot analysis, and immunofluorescence, we found that mTOR inhibition induces VMP1 expression as expected. Thus, extracellular-stimuli-induced autophagy activates VMP1 expression. To establish whether VMP1 is required for autophagy, we reduced the expression of VMP1 using the small interfering RNA (siRNA) strategy. HeLa cells were transfected with VMP1 siRNA and then subjected to a starvation standard protocol or rapamycin treatment. We found that autophagosome formation was almost completely inhibited in VMP1 siRNA cells under both treatments, as evidenced by the distribution of the RFP–LC3 fluorescent fusion protein. These findings demonstrate that VMP1 expression is required for autophagosome formation.

VMP1 Is an Autophagosomal-Membrane Integrated Protein

To analyze the role of this putative transmembrane protein in the molecular mechanism of autophagy, we hypothesized that VMP1 is an integrated protein of the autophagosomal membrane. We noticed that the VMP1–EGFP fusion protein remains in the vacuole membrane induced by its own expression in cells transfected with pEGFP–VMP1. Consequently, to find out whether endogenous VMP1 induced during autophagy remains as an integrated membrane protein, we performed subcellular fractioning of HeLa cells undergoing rapamycin-induced autophagy and investigated VMP1 in membrane preparations by western blot analysis. While the protein is not detectable in untreated cells, VMP1 is detected in membrane preparations of cells undergoing autophagy, and the signal persists when the cell lysate is treated with 1.5 M NaCl or is exposed to pH 11.0 before membrane fractioning. These results indicate that VMP1 functions as an integrated membrane protein in autophagic cells. Then, to investigate if VMP1 is an autophagosomal membrane protein, we analyzed whether VMP1 colocalizes with endogenous LC3 in the autophagosomes. We performed inmunofluorescence using the anti-LC3 antibody and anti-V5 antibody in HeLa cells transfected with VMP1–V5 expression

plasmid. We found a remarkable colocalization between VMP1–V5 fusion protein and endogenous LC3 in the VMP1-induced vacuoles. These results indicate that VMP1 is a transmembrane protein integrated into the autophagosomal membrane.

VMP1 Is a Beclin 1 Binding Membrane Protein

To obtain a mechanistic insight as to how VMP1 triggers autophagy, we analyzed its function in the molecular pathway of autophagosome formation. The initial steps of the autophagic process in mammalian cells are controlled by Beclin 1 [39], which promotes autophagosome formation when it works as a complex with the class III PI3K [33, 38, 53]. During autophagy the Beclin 1–class III PI3K complex, apparently originating from the *trans*-Golgi network [53], is thought to undergo subcellular distribution to the autophagosomal membrane, which eventually leads to the recruitment of autophagy proteins and the proper conjugation of LC3 to membrane phospholipids [42, 54]. However, the transmembrane protein of the autophagosomal membrane with which the Beclin 1–class III PI3K complex interacts remains elusive. We therefore investigated whether VMP1 interacts with Beclin 1. First, we analyzed if Beclin 1 localizes in the membrane of the VMP1-induced vacuoles. To this end, we concomitantly transfected 293T cells with pEGFP–VMP1, pRFP–LC3, and pCFP–Beclin 1 expression plasmids. We found a remarkable colocalization between VMP1, LC3, and Beclin 1 fluorescent fusion proteins, suggesting that Beclin 1 could attach to VMP1-induced vacuole membranes, which are marked by endogenous LC3 as autophagosomes. To determine if VMP1 is the autophagosomal membrane protein target to which Beclin 1 attaches to allow the initiation of autophagy, we studied whether VMP1 interacts with Beclin 1 in cells transfected with the VMP1–V5 expression plasmid by conducting coimmunoprecipitation experiments. We investigated whether endogenous VMP1 interacts with endogenous Beclin 1 in cells developing rapamycin-induced autophagy. Immunoprecipitates of either VMP1 or Beclin 1 prepared from Triton X-100 solubilized rapamycin-treated HeLa cells were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and detected by immunoblotting with anti-Beclin 1 and anti-VMP1 antibodies. Endogenous Beclin 1 and VMP1 induced by rapamycin treatment were detected in immunoprecipitates using anti-VMP1 antibody. Interaction of endogenous VMP1 with endogenous Beclin 1 was also confirmed by immunoprecipitation using anti-Beclin 1 antibody. Similar results were found in starved cells. We then analyzed whether VMP1 expression is able to induce LC3 recruitment in autophagy-deficient, low Beclin 1 expressing MCF7 cells. We transfected MCF7 cells with pcDNA4–VMP1 expression plasmid and found that the percentage of RFP–LC3 cells with punctate staining was low and similar to the percentage found in starved and control MCF7 cells. These results collectively show that VMP1 interacts with Beclin 1 to trigger the autophagic process and suggest that VMP1–Beclin 1 interaction is involved in the molecular mechanism of autophagosome formation.

To further delineate the interaction between VMP1 and Beclin 1, we tested VMP1 hydrophilic domains for Beclin 1 binding. VMP1 is predicted to contain six transmembrane domains. Four recombinant glutathione *S*-transferase (GST)–VMP1 hydrophilic

peptides, VMP1(aa1–75), VMP1(aa187–247), VMP1(aa324–366), and VMP1(aa378–406), and full-length His6–Beclin 1 were produced in *Escherichia coli*. We incubated the recombinant His6–Beclin 1 fusion protein from *E. coli* lysates with the purified GST–VMP1 peptides and retained protein was eluted with imidazole. Eluates were separated by SDS-PAGE followed by immunoblotting with anti-Beclin 1 and anti-GST antibodies. We observed that only GST–VMP1(aa378–406) was found in the eluates, indicating that VMP1 interacts with Beclin 1 through the aa378–406 hydrophilic domain, which we named "autophagy-related domain" (AtgD). To obtain further molecular insights into the proposed function of VMP1 in autophagosome formation and to evaluate the relevance of VMP1–Beclin 1 interaction, we constructed pEGFP and pcDNA4 plasmids containing the VMP1ΔAtgD defective mutant, in which the VMP1(aa378–406) peptide was specifically deleted. First, we performed a pull-down assay using the VMP1ΔAtgD–V5 fusion protein. Lysates from HeLa cells transfected with pcDNA4-VMP1ΔAtgD were incubated with nickel nitrilotriacetic acid agarose beads and retained proteins were eluted with imidazole, separated by SDS-PAGE, and immunoblotted with anti-Beclin 1 or anti-V5 antibodies. We found no signal of endogenous Beclin 1 in the elution fraction from VMP1ΔAtgD–V5 fusion protein, whereas, as expected, control experiments showed that both VMP1 and Beclin 1 were detected in the elution fraction from VMP1–V5 fusion protein. Then, we performed subcellular fractioning of HeLa cells transfected with pcDNA4–VMP1ΔAtgD and investigated VMP1ΔAtgD–V5 in membrane preparations by western blot analysis using anti-V5 antibody. We found that VMP1ΔAtgD was detected in membrane preparations, and the VMP1 signal persisted in the membrane fraction even after 1.5 M NaCl or pH 11.0 treatments. These results indicate that the VMP1ΔAtgD defective mutant still functions as an integrated membrane protein. In other series of experiments, HeLa cells were transfected with pEGFP–VMP1 or pEGFP–VMP1ΔAtgD, which encodes the VMP1ΔAtgD–EGFP fluorescent fusion protein, and cells were cultured in nutrient-replete conditions for 24 h. Surprisingly, we found that cells expressing VMP1ΔAtgD—EGFP fluorescent fusion protein do not show the characteristic vacuolization observed in VMP1–EGFP-expressing cells. To evaluate if the Atg domain of VMP1 is required for autophagy, HeLa cells were concomitantly transfected with the expression plasmids encoding the RFP–LC3 and VMP1ΔAtgD–V5, or VMP1–V5 fusion proteins and cells were cultured in nutrient-replete conditions for 24 h. We found that VMP1ΔAtgD expression failed to trigger autophagy as it is evidenced by the diffuse distribution of the RFP–LC3 fluorescence fusion protein observed in pcDNA4–VMP1ΔAtgD transfected cells in contrast to the recruitment of the RFP–LC3 observed in the full-length VMP1-expressing cells. The percentage of RFP–LC3 cells with punctate staining in VMP1ΔAtgD-expressing cells was highly reduced in comparison with that in those expressing the full-length VMP1. The results described above show that the VMP1 Atg domain is essential for autophagosome formation and suggest that VMP1 is an autophagosomal membrane protein, which interacts with the Beclin 1 complex, allowing the initiation of the autophagic process.

VMP1–EGFP Targeted to Pancreatic Acinar Cells in the Pancreas of Transgenic Mice Triggers the Formation of LC3-Positive Vacuoles

To gain in vivo insight into how stable expression of VMP1 is able to induce autophagy, we developed transgenic mice in which the pancreatic acinar cell specific elastase promoter drove VMP1–EGFP expression. Expression of the VMP1–EGFP transgenic fusion protein was detected by western blot analysis in the pancreas of transgenic mice, but not in the liver, kidney, spleen, heart, or lung as expected. Pancreatic tissue from VMP1–EGFP transgenic mice showed numerous vacuoles in acinar cells whose membranes were immunostained with anti-EGFP (Fig. 2). VMP1-induced vacuoles in pancreas from transgenic mice were assayed for LC3 by immunofluorescence using anti-LC3 and anti-GFP antibodies. We found that VMP1 colocalizes with endogenous LC3 in the membrane of VMP1-induced vacuoles, showing that stable expression of VMP1 in pancreatic tissue from transgenic mice is able to induce the formation of LC3-positive vacuoles. These data indicate that the in vivo expression of VMP1 triggers autophagosome formation and strongly support the in vitro findings.

VMP1 Localizes in the Membrane of Pancreatitis-Induced Autophagic Vacuoles

Finally, we investigated whether VMP1 is involved in the autophagy during a pathological process using an experimental animal model of pancreatitis. Autophagy has been described as an early cellular event in human and experimental acute pancreatitis [55–57]. Pancreatic tissue from rats treated with caerulein, a widely used experimental model of pancreatitis,

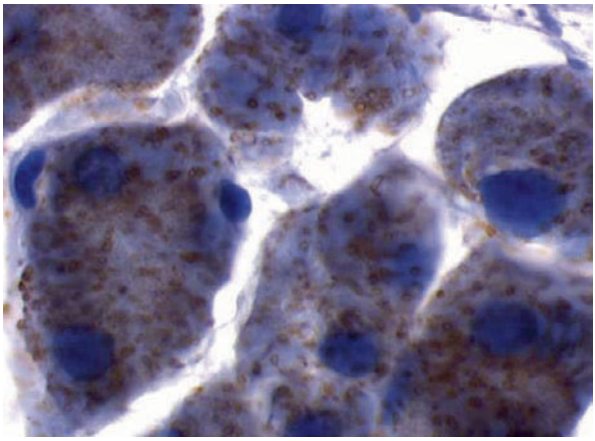

Fig. 2 VMP1–EGFP expression in the acinar cells of the pancreas of transgenic mice induces autophagic vacuoles. Immunohistochemistry of EGFP in pancreatic tissue from VMP1–EGFP mice (original magnification 100×) using mouse anti-EGFP and anti-mouse horseradish peroxidase antibodies

developed cytoplasmic vacuolization with ultrastructural features of autophagy [55, 56]. We analyzed the expression of the VMP1 protein by western blot in pancreas tissue during the development of the experimental pancreatitis and found the maximal VMP1 expression after 6 h of treatment. To determine if VMP1 expression is related to the autophagic process in pancreas undergoing pancreatitis, we analyzed VMP1, LC3, and Beclin 1 in pancreas specimens from the animal model by immunofluorescence using anti-VMP1, anti-LC3, and anti-Beclin 1 antibodies. We found that endogenous VMP1 highly colocalizes with endogenous LC3 in vacuolated structures. This in vivo result confirms the autophagosomal localization of VMP1. Moreover, we found that endogenous VMP1 also colocalized with endogenous Beclin 1 in the vacuole membrane of the autophagic tissue, showing the vacuolar localization of Beclin 1 during in vivo induced autophagy. These results strongly support the findings obtained in vitro and suggest that the early expression of VMP1 could be related to the autophagy induction in tissue suffering from a pathological process.

Discussion and Conclusions

We have previously reported that gene expression is strongly altered in pancreas during the acute phase of pancreatitis [13, 58]. These phenotypic changes could enable the pancreas to protect itself against an acute attack of pancreatitis or, conversely, participate in the pathophysiological mechanism of the disease. Therefore, identifying the genes involved in cell response to pancreatitis could lead to new strategies in the treatment of the disease. Using a systematic approach, we identified by sequencing its cDNA a new protein that we named VMP1. It was strongly expressed in the pancreas of rats suffering from acute pancreatitis. Our studies indicated that VMP1 is a transmembrane protein located at the Golgi apparatus and endoplasmic reticulum area and that its overexpression induces vacuole formation. Our functional studies present experimental evidence that the sole expression of VMP1 triggers autophagy, even under nutrient-replete conditions. VMP1 expression in cells cultured under nutrient-replete conditions presents multiple ultrastructural features of autophagy, including cup-shaped structures, autophagosomes, and autolysosome-like features. In addition, VMP1 induces the almost total recruitment of transient expression of RFP–LC3 fluorescent fusion protein. In all the experiments more than 85% of RFP–LC3 cells showed punctate staining. Consistent results were found when endogenous LC3 was assayed. Moreover, VMP1 induces processing of endogenous LC3-I to LC3-II, which is enhanced in the presence of hydrolase inhibitors. Although transiently overexpressed LC3 protein could be prone to aggregate in an autophagy-independent manner, the processing of the endogenous LC3-I to LC3-II was reported to be unaffected by the overexpression and remains a hallmark of autophagy [48, 49]. On the other hand, VMP1-expression-induced LC3 recruitment was inhibited by 3-MA, which, although not specifically, inhibits autophagy [24, 33]. Moreover, VMP1 is involved in the extracellular-stimuli-induced autophagy since treatments currently used to trigger autophagy such as starvation and pharmacological mTOR inhibition induce VMP1 expression. Furthermore, the knockdown of VMP1 expression abolishes starvation as well as rapamycin-induced autophagosome formation, suggesting that VMP1 expression is required for autophagy. On the other hand, our results have shown that VMP1 is an integral protein of

the autophagosomal membrane. VMP1 remains in the membrane fraction of cells undergoing rapamycin-induced autophagy and it colocalizes with LC3 in the vacuole membrane. Finally, the transgenic mice for VMP1 expression targeted to pancreatic acinar cells allowed us to confirm, within a physiological setting, the results discussed above. The in vivo stable expression of VMP1 induces the formation of numerous vacuoles in acinar cells, where it colocalizes with endogenous LC3.

VMP1 is involved in the initial steps of the autophagic process. We have obtained experimental data indicating that VMP1 interacts with Beclin 1 and this interaction is essential for autophagosome formation. Both VMP1 and Beclin 1 endogenous proteins coimmunoprecipitated from rapamycin-treated cells. On the other hand, VMP1-induced autophagy is dependent on Beclin 1, since VMP1 expression fails to induce autophagy when it is expressed in autophagy-deficient, low Beclin 1 expressing MCF7 cells. VMP1–Beclin 1 interaction was confirmed using recombinant peptides. Beclin 1 directly interacts with the VMP1–AtgD peptide (aa378–406) and the VMP1ΔAtgD mutant failed to precipitate endogenous Beclin 1 in transfected cells. Our results also show that the VMP1 Atg domain is essential for the formation of autophagosomes. We found that the mutant protein VMP1ΔAtgD, which is also a transmembrane protein, does not induce autophagosome formation since it failed to promote vacuole formation and LC3 recruitment. These findings also suggest that the mechanism by which VMP1 induces autophagy is likely to involve the interaction of Beclin 1 with the VMP1–Atg domain. Beclin 1 is a haploinsufficient tumor-suppressor gene [18], and is involved in the autophagosome formation mediating the localization of other autophagy proteins to the autophagosomal membrane [42]. Beclin 1 activity seems to be dependent on its partners and its subcellular localization. Two major Beclin 1 interactors have been described affecting its autophagic activity, Bcl-2 and class III PI3K. While Bcl-2 inhibits Beclin 1-dependent autophagy and this complex was localized at the endoplasmic reticulum [41], the Beclin 1–kinase complex was localized in the *trans* Golgi network in nutrient-replete HeLa cells and was shown to be essential for early stages of autophagosome formation [53]. The data presented here show the colocalization of LC3 and Beclin 1 in VMP1-induced vacuoles. In contrast, this triple colocalization was abolished in VMP1ΔAtgD-expressing cells. Furthermore, we found endogenous VMP1–Beclin 1 colocalization in vacuole membranes when a pathological tissue undergoing autophagy was assayed. These findings support the hypothesis that the distribution of Beclin 1 to the autophagosomal membrane is required for autophagy progression. To our knowledge, there are few reports showing Beclin 1 autophagosomal membrane location during autophagy. Transfected Beclin 1 was found localized in the membrane of the autophagosome in starved HEK293 cells [59] and endogenous Beclin 1 was reported to colocalize with LC3 in dorsal root ganglion cells from diabetic rats [60]. It is thought that during autophagy, subcellular distribution of the Beclin 1 complexes from the *trans* Golgi network to an autophagosomal structure would require the interaction of Beclin 1 with an autophagosomal transmembrane protein. Our results suggest that VMP1 is an autophagosomal-integrated protein where Beclin 1 interacts to initiate autophagosome formation.

The source of the autophagosomal membrane remains to be elucidated [43, 44]. Two general models have been proposed: the membrane may be derived from a preexisting cytoplasmic organelle such as the endoplasmic reticulum, or it may be assembled from constituents at its site of genesis [43, 61]. The autophagosomal membrane is rich in lipids

and poor in proteins [62, 63]; therefore, it is difficult to determine its origin on the basis of protein content. So far, only two transmembrane proteins have been described in yeast to be autophagy-related, Atg9 [64] and Atg27 [65]. Atg9, as part of a functional complex [66], cycles between the mitochondria and the preautophagosomal structure and is thought to mediate the delivery of the membrane to the forming autophagosome. Mammalian Atg9 was also described, but its subcellular distribution is different and its actual participation in the autophagosome formation remains to be delineated [67–69]. In addition, none of these transmembrane proteins have been reported to interact with Beclin 1. Our results have identified VMP1 as an autophagosomal transmembrane protein required for autophagosome formation. The findings that VMP1 has no known homolog in yeast and that its expression is required to start the autophagic process, support the hyothesis that mammalian cells regulate autophagy in a different way and suggest VMP1 as a candidate protein to further explore the source of the autophagosomal membrane in pancreatic acinar cells during acute pancreatitis.

In this study two in vivo models of VMP1 expression were used to support the data obtained in the in vitro studies: the VMP1-transgenic mice, and the experimentally induced acute pancreatitis in rats. VMP1-transgenic mice, in which VMP1 expression was targeted to pancreatic acinar cells, show that the in vivo stable expression of VMP1 is able to induce autophagosome formation in acinar cells. These results support the findings obtained when transient VMP1 expression was assayed in cell lines. During experimental acute pancreatitis, autophagic morphology of the acinar cell has been reported to appear 3 h after pancreatitis induction and remains detectable for about 15 h [55, 70]. The data presented here show that the time course of VMP1 protein expression is consistent with that of the presence of autophagic morphology during experimental pancreatitis. Our results also show that VMP1 is localized in the membrane of the acinar cell autophagic vacuoles, since endogenous VMP1 highly colocalizes with endogenous LC3 in vacuolated structures in the pathological tissue. Moreover, endogenous Beclin 1 colocalizes with VMP1 in pancreatitis-induced vacuole membranes. These findings strongly support the hypothesis that VMP1 is an autophagosomal-integrated protein where Beclin 1 interacts.

Although autophagic morphology was described in human pancreatitis in 1980 [27], the physiopathological role of autophagy in pancreatitis has not been fully elucidated. Interestingly, no other morphological event resembling acute pancreatitis was observed in pancreatic tissue from transgenic mice, suggesting that VMP1-induced autophagy per se may not induce acute pancreatitis. In addition, GFP–LC3-transgenic mice subjected to starvation developed autophagy in pancreas without visible pancreatitis morphology, suggesting that autophagy might be a mechanism of degradation of secretory granules in starved pancreas [47]. Therefore, during acute pancreatitis, autophagy could be a defense mechanism, which activates as an early cellular response to the disease. On the other hand, VMP1 mRNA basal expression was found in several rat tissues such as kidney [71]. This observation is consistent with the basal autophagy found in the same tissue from the GFP–LC3 transgenic mice [47]. Although basal VMP1 expression could be related to other physiological processes, the relationship between GFP–LC3 transgenic mice and VMP1 basal expression leads us to speculate that the expression of VMP1 might correlate with the presence of autophagy in mammalian tissue. On the other hand, the fact that this novel autophagosomal transmembrane protein is involved in autophagy

during a pathological process will allow further studies on the role of autophagy in the cellular response to disease and would be of potential clinical relevance.

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Systemic Inflammatory Effects of Acute Pancreatitis; Effects of Lipid Mediators

3

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Acute pancreatitis is a serious autodigestive and inflammatory process frequently associated with a broad spectrum of systemic complications, including shock, renal failure, acute respiratory failure, and hepatic damage [1]. The severity of the disease is related to the magnitude of these disorders and ranges from mild edema to fulminant multisystemic organ failure. Its pathogenesis relates to the inappropriate activation of trypsinogen to trypsin and a lack of the efficient elimination of the active trypsin from inside the pancreas. During acute pancreatitis, the segregation of digestive enzyme zymogens and lysosomal hydrolases is perturbed and they become colocalized, resulting in the activation of trypsinogen. Therefore, trypsin is believed to be the key enzyme in the initiation and exacerbation of acute pancreatitis by activating pancreatic zymogens. Since the initial damage occurs in the pancreatic tissue, it has long been believed that activated hydrolases released from the damaged pancreas gain access to the systemic circulation and are responsible for the induction of the damage in distant organs [2]. This concept has been abandoned largely owing to the fact that protease inhibitors have not been shown to be of significant value in the treatment of acute pancreatitis. Despite the considerable experimental data to support the use of protease inhibitors, a number of clinical trials have failed to show any benefits of this therapeutical approach in decreasing morbidity or mortality of patients with acute pancreatitis [3].

Systemic Inflammatory Response in Pancreatitis

On the other hand, severe acute pancreatitis exhibits many of the features of the systemic inflammatory response induced by multiple trauma, burns, or sepsis [4]. For this reason, the new concept of severe acute pancreatitis is focused not only on the intra-acinar events, but also on the systemic generation of inflammatory mediators that could regulate the extent of local pancreatic cell damage and the systemic inflammation [1]. Several inflammatory

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molecules have been identified as potential final, common pathway mediators that induce multisystemic organ failure. In this line, a number of cytokines, chemokines, and inflammatory mediators, including tumor necrosis factor α (TNF-α), interleukin (IL)-1β , IL-6, IL-8, IL-10, monocyte chemoattractant protein 1, platelet-activating factor, C5a, and intracellular cell adhesion molecule 1, have been implicated in acute pancreatitis [5]. Consequently, initial acinar cell damage results in the release of activated hydrolases, but also of inflammatory mediators, altogether leading to the systemic inflammatory response. This mechanism is a normal response to sepsis or tissue injury and is characterized by the cytokine-mediated activation of leukocytes. Organ damage and multiple organ dysfunction syndrome appear when this response is excessive or uncontrolled. In addition to cytokines and chemokines, other mediators are known to act by modulating the progression of the inflammatory response. In the case of pancreatitis, oxygen free radicals [6] and lipid mediators [7] are known to be released in the early stages of the disease. There are data indicating that these mediators are not able to induce the systemic response but play a role modulating the severity of the process.

Lipids and Their Effect During Acute Pancreatitis

A number of studies have suggested that pancreatitis-associated ascitic fluid (PAAF) plays a role in the pathogenesis of acute pancreatitis [8–10]. Accumulation of ascitic fluid in the peritoneal cavity occurs in the severe forms of acute pancreatitis and patients with acute pancreatitis have a high morbidity owing to systemic complications and a high mortality. Removal of PAAF by peritoneal lavage has been reported to be beneficial for survival rate in experimental models of acute hemorrhagic pancreatitis [11]. However, it was not associated with a significant improvement in mortality or morbidity rates in clinical trials [12], probably owing to the fact that, at the time of hospitalization, the events leading to systemic dysfunction have already been triggered. Ascitic fluid is known to contain proinflammatory cytokines and activated hydrolytic enzymes which are released into the peritoneal cavity during acute pancreatitis. It has been reported that PAAF modulates the function of peritoneal macrophages [8], induces lung injury [9], induces direct hepatocyte cell death [10], and stimulates the generation of cytokines in acinar cells [13]. However, it is noteworthy that some of these effects appear to be mediated by factors other than pancreatic enzymes or cell-derived cytokines [10].

Analysis of PAAF revealed that pancreatic enzymes are present in concentrations 1 order of magnitude higher than that observed in plasma. For this reason, the peritoneal fat necrosis characteristic of the severe forms of pancreatitis has been attributed to the action of pancreatic lipase present in PAAF upon the neutral fat of the tissues [14]. This enzyme hydrolyzes triacylglycerols, thus producing monoacylglycerols and free fatty acids and causing tissue necrosis due to the disruption of lipid balance and the detergent properties of fatty acids. Other enzymes, such as pancreatic phospholipase A2, have also been implicated in this process [15]. The enzymatic activity of these enzymes results in the release of nonesterified fatty acids (NEFA) could account for the high concentrations of NEFA observed in PAAF. A several-fold increase in ascitic fluid NEFA compared with the plasma

values in different experimental models has been reported [16]. The metabolic fate of these NEFA could be the absorption into the portal venous system and the reesterification to triglycerides in the liver, then contributing to the hypertriglyceridemia associated with severe pancreatitis [17].

A potential role of circulating NEFA in acute pancreatitis has been suggested owing to the observed deleterious effects of these products in vitro. It is known that oxidized polyunsaturated fatty acids are highly cytotoxic to cultured cells [18] and several works have reported on the proapoptotic effects of different lipid mediators in cell lines [19]. On the other hand, oxidized lipid mediators could modulate inflammation through the activation of transcription factors such as peroxisome proliferator-activated receptor γ (PPARγ). This nuclear factor is one of the members of the PPAR family that has gained interest as it is involved in the modulation of the inflammatory response [20]. Its activity is governed by the binding of small lipophilic ligands, mainly fatty acids derived from nutrition or metabolism [21]. Altogether this suggests a potential role for NEFA in the pathogenesis of acute pancreatitis both in the local pancreatic necrosis and in the systemic inflammatory events.

As occurs with human patients, fat necrosis and PAAF have been observed in experimental models of severe acute pancreatitis. In an experimental model of acute pancreatitis in rats induced by intraductal administration of sodium taurocholate, lipase levels in PAAF reached 10,000 U/L, while in plasma lipase was present at 3,000 U/L during pancreatitis and at less than 300 U/L in control animals [22]. This high concentration of lipolytic activity could account for the levels of NEFA in PAAF when compared with the concentration observed in peripancreatic adipose tissue. Nevertheless, despite the simultaneous increase in a lipolytic enzyme and in the product of lipolysis strongly suggesting a direct relationship, clinical data supporting this hypothesis are scarce and contradictory and several experimental works do not confirm this theory [23]. In particular, it has been indicated that lipase appears to be unable to locally hydrolyze triglycerides in circulating blood, pancreatic tissue, or the peritoneal cavity.

When the levels of lipoperoxidation in these fatty acids released to PAAF are measured, the values obtained are higher than in the control white adipose tissue [24]. Interestingly, lipoperoxidation was increased in adipose tissue obtained from animals with pancreatitis. Probably, oxygen free radicals generated by xanthine oxidase or NADPH oxidase from leukocytes present in ascitic fluid affect the surface of fat tissue, resulting in a moderate lipoperoxidation index in white adipose tissue. In contrast, lipids presents in PAAF are subjected to a strong oxidative process that results in a high concentration of lipoperoxides.

We observed that in cultured cells the addition of the lipid fraction obtained from PAAF exerted a cytotoxic effect. This toxicity was higher than could be expected only from the high concentration of NEFA, suggesting that the oxidative status of these lipids could also play a role in the toxic effect of PAAF-derived NEFA. The involvement of NEFA in the toxicity of PAAF confirmed the multiplicity of deleterious mechanisms activated during acute pancreatitis, including hydrolytic enzymes, inflammatory mediators, oxidative stress, and lipid metabolites. However, in addition to their direct toxicity, the most important biological effect induced by these metabolites seems to be the exacerbation of the inflammatory response.

Regulatory Effects of Lipid Mediators

The generation of different bioactive lipids, including prostaglandins, leukotrienes, and platelet-activating factor, has been recognized during acute pancreatitis [7]. These mediators act by promoting both the local and the systemic inflammatory response. However, their importance in the progression of the disease seems to be limited when compared with the effect of cytokines released during pancreatitis. Nevertheless, there are lipid mediators that play a role in the regulation of the cell response to cytokines and other proinflammatory mediators. It has long been known that macrophage functions and gene expression can be modulated by specific nuclear receptor pathways, and some of them are under the control of lipid mediators. In particular, PPARs are nuclear receptors activated by fatty acid derivatives. PPARγ is one of the members of the PPAR family that has gained interest as it is been involved in the modulation of the inflammatory response [25]. Its activity is governed by the binding of small lipophilic ligands, mainly fatty acids derived from nutrition or metabolism [21]. The naturally occurring 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 is one of the best known endogenous agonists of this nuclear factor, but other oxidatively modified fatty acids or oxidized alkyl phospholipids could act as PPARγ agonists. When activated, this transcription factor negatively regulates the expression of proinflammatory genes and plays a role in the resolution of the inflammatory processes [26]. For this reason, PPARγ agonists have been suggested to be useful as an anti-inflammatory therapy in different diseases, including acute pancreatitis [27].

In vitro experiments revealed that the inhibitory effect of 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 on the TNF-α induced nuclear factor κB activation was blocked by the presence of PAAF-derived lipids. This effect does not occurs with lipids obtained from other sources such as white adipose tissue, suggesting that modified lipids present in PAAF acts as PPARγ ligands, interfering the normal activation of this nuclear receptor [24]. The consequence of this interference could be the loss of an endogenous anti-inflammatory mechanism, thus resulting in an exacerbation of the inflammatory process triggered by acute pancreatitis (Fig. 1).

The effect of PAAF-derived lipids could be also observed when analyzing the interactions between DNA and PPARγ. Lipid concentrations equivalent to that present in ascitic fluid are able to reduce the PPARγ–DNA binding by 60%, while lipids obtained from white adipose tissue had no effect on this binding. It is of interest that the ability to interfere with the activation of PPARγ appears to correlate with the levels of lipoperoxidation. These facts illustrate the importance of the endogenous enzymatic mechanisms in regulating the levels of lipid oxidation during pancreatitis.

Antioxidant Mechanisms

Serum paraoxonase-1 (PON1) is an enzyme associated with high-density lipoprotein (HDL), the clinical interest in which resides in its ability to prevent or limit the oxidation of HDL [28]. This ability is of particular relevance since HDLs appear to be the primary transporters of oxidized lipids in plasma. Interestingly, it has been reported that PON1 activity and

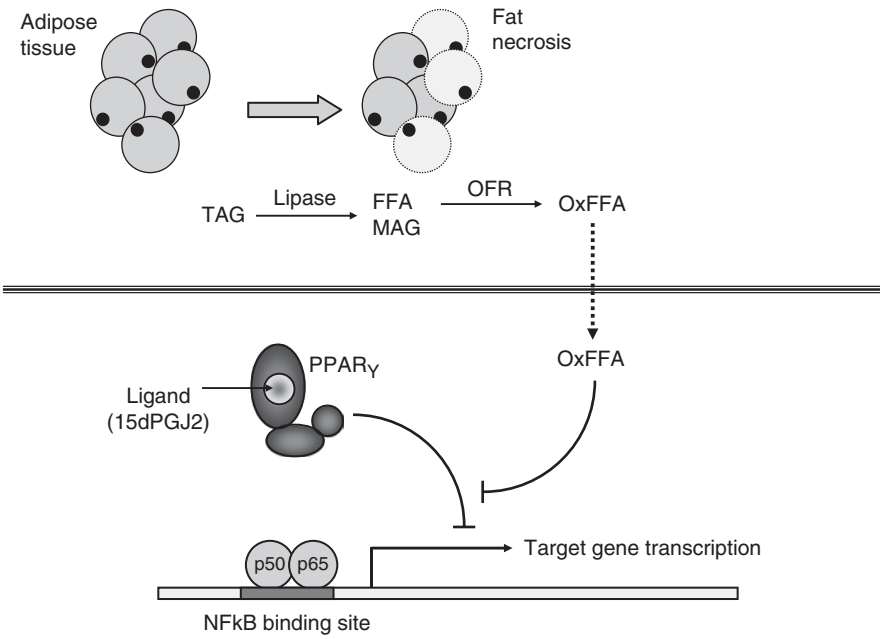

Fig. 1 The release of lipolytic enzymes during acute pancreatitis results in fat necrosis and the generation of free fatty acids. These lipid products are oxidized and interfere with the anti-inflammatory effect of other endogenous lipid mediators, such as 15-deoxy-prostaglandin J_2 (*15dPGJ2*). This interference could result in an exacerbation of the systemic inflammatory response triggered during severe acute pancreatitis. *TAG* triacylglycerols, *FFA* free fatty acids, *MAG* monoacylglycerols, *OFR* oxygen free radicals, *OxFFA* oxidatively modified free fatty acids, *PPARγ* peroxisome proliferator-activated receptor γ, *NFkB* nuclear factor κβ

HDL level were lower in acute pancreatitis, whereas the mean levels of malonyldialdehyde were significantly higher [29]. In our experimental model of taurocholate-induced pancreatitis, the loss in PON1 activity could be observed in the severe form of the disease, while in the mild form of pancreatitis the enzymatic activity remained unmodified. This decrease of PON1 activity could be explained by the proteolytic degradation of the protein, considering that during acute pancreatitis there is an important release of hydrolytic enzymes into the bloodstream. Western blot analysis revealed the presence of two different bands in the control group. The presence of these bands had been previously described and it was suggested that they correspond to two different oxidation states [30] or different glycosilation states of the enzyme [31]. Interestingly, after induction of acute pancreatitis a new band corresponding to low molecular weight appears. This increase paralleled a decrease in the intensity of the two bands corresponding to high molecular weight and the loss of enzymatic activity, suggesting a proteolytic degradation of PON1. This lack of PON1 activity results in increasing concentrations of circulating oxidized lipids that could interfere with PPARγ activation. This is of importance because it affects the control of the inflammatory response in the different macrophage populations involved in the systemic response associated with severe acute pancreatitis (Fig. 2).

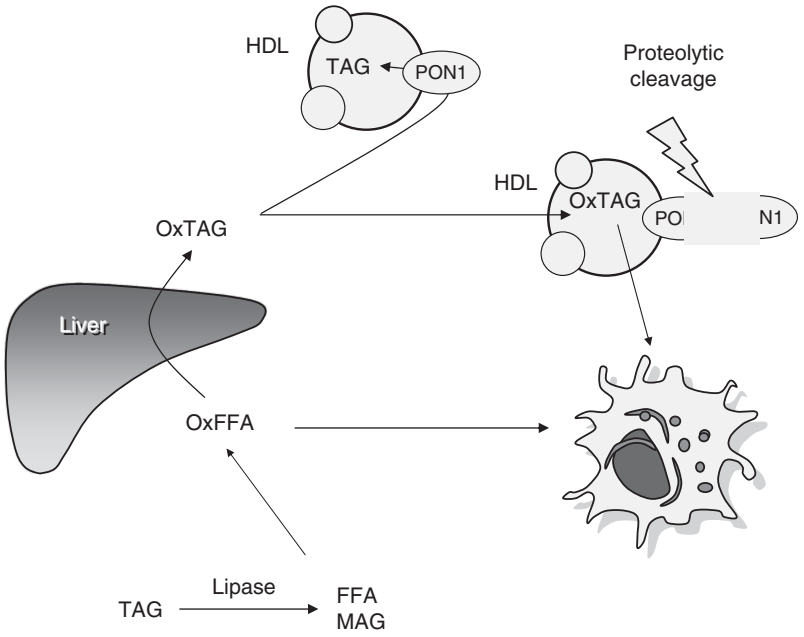

Fig. 2 During severe acute pancreatitis there is a significant decrease in serum paraoxonase-1 (*PON1*) activity. This decrease is related to inactivation of the enzyme by oxidized lipids, probably followed by proteolytic degradation of the enzyme. The lack of PON1 activity results in an increased effect of circulating oxidized lipids on macrophage function. *HDL* high-density lipoprotein, *OxTAG* oxidatively modified TAG

In the early stages of acute pancreatitis the generation of cytokines and other mediators by pancreatic acinar cells results in the sequential activation of different populations of macrophages and the induction of the systemic inflammatory response. This response remains under control in the mild forms of pancreatitis, probably owing to the effect of endogenous anti-inflammatory mediators, including soluble cytokine receptors, anti-inflammatory cytokines, as well as the activation of nuclear factors such as PPARγ that reduces the gene expression induced by proinflammatory cytokines. In contrast, in the severe forms of the disease, the regulatory mechanisms are overloaded and the inflammatory process is out of control. In this situation, the generation of oxidatively modified lipids by the action of lipolytic enzymes and the oxidative stress that occurs during pancreatitis, as well as the reduced levels of circulating PON1, contribute to the exacerbation of the inflammatory response by interfering in the control mechanism mediated by PPARγ.

Altogether these data indicate a new role for lipase in the pathogenesis of acute pancreatitis. In addition to the direct effect promoting the steatonecrosis characteristic for the severe forms of the disease, the fatty acids, released as products of the reaction catalyzed by lipase, act as active players in this process. Lipid products emerge as modulators of the inflammatory response associated with acute pancreatitis that contributes to the exacerbation of the systemic effects in the severe forms of the disease.

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Gemcitabine Changes the Gene Expression in Human Pancreatic Cancer Cells: Search for New Therapeutic Molecular Targets

4

Y. Motoo, T. Shimasaki, and T. Minamoto

Introduction

Gemcitabine (GEM) is the only effective anticancer drug for pancreatic cancer [1]. This implies the importance of developing modalities to enhance its antitumor effect. The basic action of GEM is the inhibition of DNA synthesis by DNA polymerase inhibition [2]. GEM is inactive until it enters the cells, and is activated by sequential phosphorylation with deoxycitidine kinase from difluorodeoxycitidine monophosphate to difluorodeoxycitidine diphosphate, and to difluorodeoxycitidine triphosphate. However, the precise molecular mechanism of the antitumor effect of GEM is yet to be elucidated.

Various genes which selectively change in cancer cells are candidates for molecular targeting. Especially, protein kinases involved in intracellular signal transduction have been extensively studied, and many kinase inhibitors are applied for cancer therapies [3]. Glycogen synthase kinase-3β (GSK-3β) is a multifunctional serine/threonine kinase, playing a key role in cellular signaling. We proposed that GSK-3β is a new molecular target in colon cancer [4, 5]. While GSK-3β inhibits Wnt signaling in normal cells, it promotes survival and proliferation in colon cancer cells [5]. We have also reported that GEM induces phosphorylation of the GSK-3β serine 9 residue, resulting in its inactivation, in pancreatic cancer cells [6].

In this study, we aimed to clarify the effect of GEM on the gene expression of apoptosis-related factors in PANC-1 human pancreatic cancer cells using a microarray analysis. We also examined the antitumor effect of the combination of GEM and GSK-3β inhibitor (GSKI) against PANC-1 cells *in vitro*.

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Materials and Methods

PANC-1 cells (American Type Culture Collection, Manassas, VA, USA) were cultured in Dulbecco's modified Eagle's medium with 10% fetal bovine serum. GEM (provided by Eli Lilly, Indianapolis, IN, USA) was added to the culture media. The cell growth was assayed with the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method. Apoptosis was detected using the DNA fragmentation ELISA (Roche Diagnostics, Indianapolis, IN, USA). Cells were collected at 24, 48, and 72 h, respectively, after treatment with the agents indicated, and total RNA was extracted. The gene expression profile was analyzed with the complementary DNA (cDNA) microarray method (Affymetrix Human Genome U133 Plus 2.0 array, Affymetrix, Santa Clara, CA, USA) and the Ingenuity pathways analysis (IPA) software program (Ingenuity Systems, Redwood City, CA, USA).

A small-molecule GSKI (AR-A014418) was purchased from Calbiochem/EMD Chemicals (San Diego, CA, USA). GEM (10-100 mg/L) and/or GSKI (5 mg/L) were added to the culture media of PANC-1 cells. Cell growth was analyzed with the MTT assay.

Results

In PANC-1 cells, the 50% inhibitory concentration (IC_{50}) of GEM was 16 mg/L (Fig. 1a). GEM inhibited the growth of PANC-1 cells dose- and time-dependently (Fig. 1b). DNA fragmentation ELISA showed a significant increase in apoptosis after GEM treatment (Fig. 1c). GEM increased phosphorylation of GSK-3β serine 9 (Fig. 2).

The cDNA microarray analysis revealed the gene expression changes after GEM treatment (Fig. 3). We selected a total of 372 genes which showed an increased expression of more than 1.5-fold on day 1 and more than threefold on day 3, or which showed a decreased expression of less than 0.7-fold on day 1 and less than 0.3-fold on day 3. The genes showing significant changes after GEM treatment were mostly those involved in gene expression, cell death, DNA replication, and the cell cycle. Three major groups of genes showed high scores in the IPA. Among these genes, the molecular networks of *p53* and *myc* were especially suggested to be involved in the GEM-induced gene expression changes.

The combination of GEM and GSKI resulted in a significant increase in the antitumor effect on PANC-1 cells dose-dependently, compared with GEM alone in vitro (Fig. 4). Microarray analysis revealed that the gene expression changes in the GEM plus GSKI group were smaller than those in GEM- or GSKI-alone groups. This result suggested that GSKI can modify GEM-induced gene expression. Therefore, we identified approximately 300 genes which were induced by GEM and modified by GSKI. In IPA for these genes, the molecular networks relevant to the GEM plus GSKI treatment were those of *p53* and *myc*. Western blot analysis showed that the expression of mutant p53 in PANC-1 cells was increased by GEM and decreased by GSKI. Among the genes modified by GSKI, *p8* (Fig. 5) was previously reported as an important gene in the antitumor effect of GEM [7].

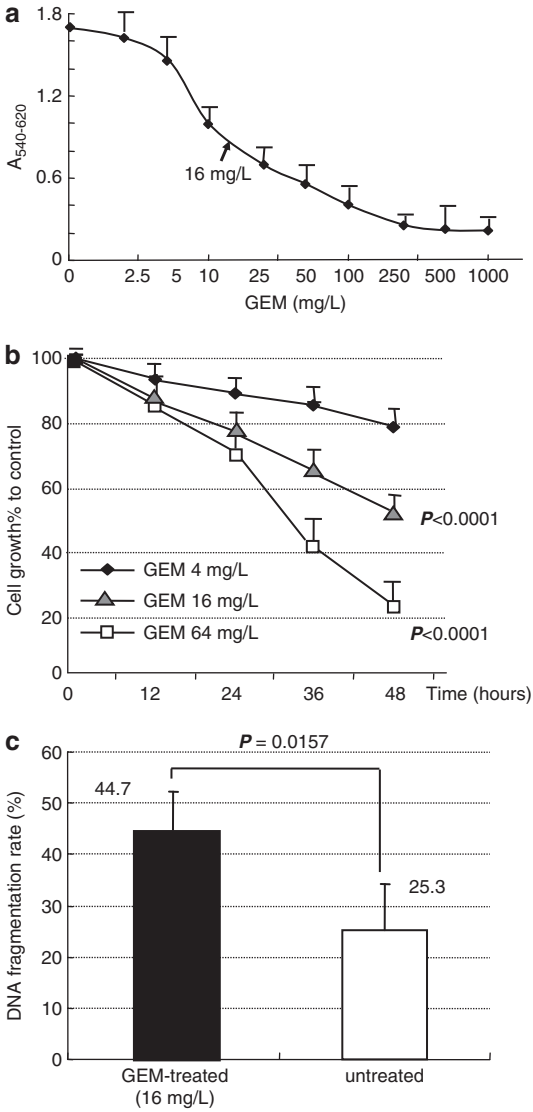


Fig. 1 Effects of GEM on cell growth and apoptosis in PANC-1 cells in vitro. **a** GEM inhibited the growth of PANC-1 cells dose-dependently. **b** GEM inhibited the growth of PANC-1 cells: a time course study. **c** Increase in apoptosis of PANC-1 cells after GEM treatment, detected with a DNA fragmentation ELISA. [From [6], with the author's permission]

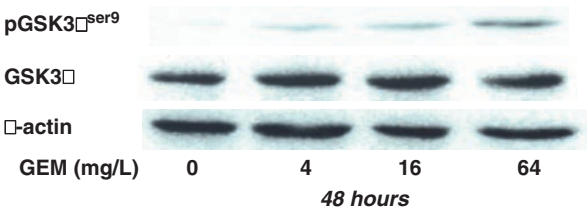


Fig. 2 Western blot analysis of the changes in expression of GSK3β and phosphorylation of its serine 9 residue after GEM treatment

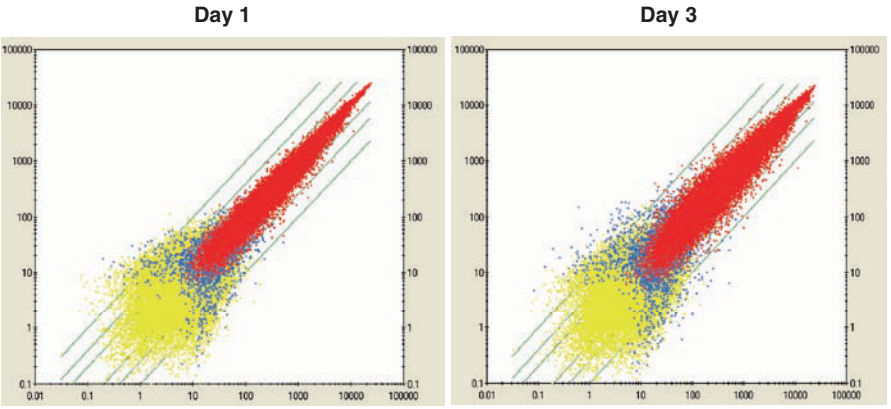


Fig. 3 Microarray analysis of the gene expression changes in GEM-treated PANC-1 cells on days 1 and 3

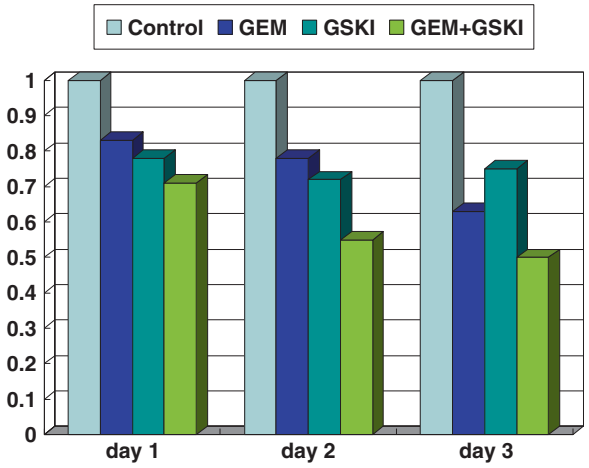
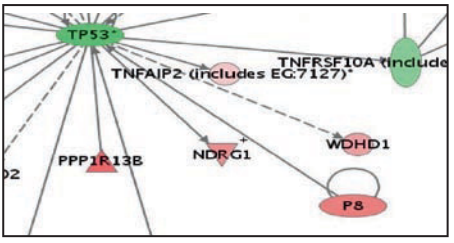
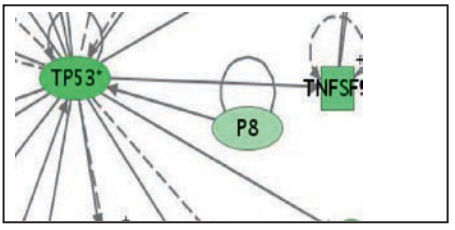


Fig. 4 Effects of GEM, GSKI, and their combination on the growth of PANC-1 cells, assessed with a MTT assay



GEM



GEM + GSKI

Fig. 5 Comparative pathway analysis for gene expression profiles of PANC-1 cells treated with GEM and treated with GEM and GSKI. It is noted that expression of *p8* was decreased by GSKI treatment

Discussion

Molecular mechanisms of the action of GEM include DNA synthesis inhibition in the S phase of the cell cycle, but there might be some other mechanisms. Microarray analysis can show the effect of some compounds on the messenger RNA (mRNA) expression, but it cannot evaluate protein expression, phosphorylation status, or protein-protein interaction in the cells. Therefore, the analysis of mRNA expression alone is not enough for monitoring intracellular events. As demonstrated in our study, it would be useful to utilize the IPA tool to identify genes which change in association with certain phenomena depending on mRNA expression levels and to analyze relevant gene pathways. In fact, we identified 372 genes which changed in expression on treatment with GEM in PANC-1 cells, and showed the involvement of the *p53* and *myc* pathways using the IPA tool.

As a molecular target to enhance the effect of GEM, we focused on GSK-3β. This enzyme regulates principal cellular functions such as the cell cycle, growth and differentiation, apoptosis and the cytoskeleton, and motility [8, 9]. In theory, GSK-3β is regarded as a tumor suppressor because it phosphorylates various transcription factors, such as c-jun and c-myc, or proto-oncoproteins such as β-catenin, thereby recruiting them for ubiquitin-mediated degradation [10]. This concept is largely based on the analysis of part of growth-signal-inhibiting functions of GSK-3β. However, GSK-3β is reported to be an essential signal molecule for cell survival mechanisms [11], and its functions were recently extensively studied in cancer cells [12, 13]. The present study suggests that GSK-3β is a molecular target in the treatment of pancreatic cancer and that the combination of GEM and GSKI could be applied to clinical practice.

The cDNA microarray analysis suggests that *p53* and *myc* pathways are involved in the GEM-induced gene expression changes in PANC-1 cells and that GSK-3β modifies these pathways. GSKI modified the expressions of genes such as *p8* and *TP53INP1* (tumor protein 53 induced nuclear protein 1) which are known genes relating to GEM sensitivity [7]. The modification of the expression of these genes by GSKI suggests that this is one of the mechanisms of enhancement of the antitumor effect of GEM by GSKI.

TP53INP1 is activated by p53, phosphorylates serine 46 of p53 in cooperation with homeodomain-interacting protein kinase 2, and induces apoptosis [14]. Recently, TP53INP1 was reported to be a critical tumor suppressor in pancreatic carcinogenesis [15]. The *p53* gene is mutated in the PANC-1 cells, and it is reported that the mutant p53 itself might have tumor-promoting activities [16]. The observation in our study of the decrease in mutant p53 protein expression by GSKI in PANC-1 cells suggests that the antitumor effect of GSKI partly depends on the level of mutant p53 expression. To test this hypothesis, we are working on the analysis of p53-related factors such as ataxia telangiectasia-mutated protein, ataxia telangiectasia and rad3-related protein, and Chk1/Chk2.

In conclusion, GSK-3β is a new molecular target in the therapy for pancreatic cancer, and GSK-3β inhibition might sensitize pancreatic cancer cells to GEM by modulating *p53* and *myc* pathways.

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Cannabinoids as Potential Antitumoral Agents in Pancreatic Cancer

5

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Pancreatic Cancer

Pancreatic cancer is one of the most malignant and aggressive forms of cancer [1]. With an incidence of ten per 10,000 men and seven per 10,000 women, it represents the fourth most common death-causing cancer in the USA [2] and the fifth in the Western world overall [3]. About 95% of pancreatic cancers cases are ductal adenocarcinomas. The anatomic localization of the pancreas and the nonspecific nature of the symptoms result in a complex and delayed diagnosis. Therefore, at the time of detection, 85% of patients show metastasic infiltrations in proximal lymphatic nodes, liver, or lungs, and only 15–20% of the tumors are typically found to be resectable [1]. In addition, less than 20% of the patients operated survive to 5 years. Treatment of unresectable tumors is currently based on the administration of fluorouracil chemoradiation for locally advanced tumors and gemcitabine chemotherapy for metastatic disease [1]. However, despite maximal optimization of these therapies, the median survival for the patients affected remains around 1 year. It is therefore of especial interest to set new therapeutic strategies aimed at improving the prognosis of this deadly disease.

Cannabinoids and Their Receptors

The hemp plant *Cannabis sativa* L. produces approximately 70 unique compounds known as cannabinoids, of which Δ^9-tetrahydrocannabinol (THC) is the most important owing to its high potency and abundance in cannabis [4, 5]. THC exerts a wide variety of biological effects by mimicking endogenous substances – the endocannabinoids anandamide and 2-arachidonoylglycerol – that bind to and activate specific cannabinoid receptors [5, 6].

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So far, two types of cannabinoid-specific $G_{i/o}$ protein coupled receptors, CB_1 and CB_2, have been cloned and characterized from mammalian tissues [7]. An additional cannabinoid receptor (GPR55, a putative "CB_3" receptor) has recently been identified [8, 9], although its participation in the several physiological functions of the endocannabinoid system remains to be elucidated. Most of the effects of cannabinoids rely on CB_1 receptor activation. This receptor is particularly abundant in discrete areas of the brain and peripheral nerve terminals, where it mediates endocannabinoid-dependent neuromodulation [10], but is also expressed in many extraneural sites. In contrast, CB_2 receptors were first described in cells and tissues of the immune system. Recent data, however, question this notion and support the existence of CB_2 receptors in the central nervous system [11] as well as in other peripheral locations [12]. Regarding pancreatic tissue, CB_1 and CB_2 receptors are present in Langerhans islets [13], whereas they are almost undetectable in the exocrine pancreas [14]. Remarkably, pancreatic tumors express both CB_1 and CB_2 receptors [14, 15] which, as discussed later, could be of relevance for selectively targeting pancreatic tumor cells with cannabinoids.

Extensive molecular and pharmacological studies have demonstrated that cannabinoids inhibit adenylyl cyclase through CB_1 and CB_2 receptors. The CB_1 receptor also modulates ion channels, inducing, for example, inhibition of N- and P/Q-type voltage-sensitive Ca^{2+} channels and activation of G protein activated inwardly rectifying K^+ channels [7]. Besides these well-established cannabinoid receptor coupled signaling events, cannabinoid receptors also modulate several pathways that are more directly involved in the control of cell proliferation and survival, including extracellular-signal-regulated kinase [16], c-Jun N-terminal kinase and p38 mitogen-activated protein kinase [17, 18], phosphatidylinositol 3-kinase/Akt [19], focal adhesion kinase [20], and the sphingomyelin cycle [21].

Antitumoral Activity of Cannabinoids

Cannabinoids have been known for several decades to exert palliative effects in cancer patients, and nowadays capsules of THC (Marinol™) and its synthetic analogue nabilone (Cesamet™) are approved to treat nausea and emesis associated with cancer chemotherapy [22]. In addition, several clinical trials are testing other potential palliative properties of cannabinoids in oncology such as appetite stimulation and analgesia [23, 24]. Besides these palliative actions, cannabinoids have been proposed as potential antitumoral agents on the basis of experiments performed both in cultured cells and in animal models of cancer. These antiproliferative properties of cannabis compounds were first reported 30 years ago, when it was shown that THC inhibits lung adenocarcinoma cell growth in vitro and after oral administration in mice [25]. Although these observations were promising, further studies in this area were not performed until the late 1990s, mostly by Di Marzo's group [reviewed in [26]] and Guzmán's group [reviewed in [23]]. A number of plant-derived (for example, THC and cannabidiol), synthetic (for example, WIN-55,212-2 and HU-210), and endogenous (for example, anandamide and 2-arachidonoylglycerol) cannabinoids are now known to exert antiproliferative actions on a wide spectrum of tumor cells in culture [23]. More importantly, cannabinoid administration to nude mice curbs the growth of various

types of tumor xenografts, including lung carcinoma [25], glioma [27], thyroid epithelioma [28], lymphoma [29], skin carcinoma [30], melanoma [31], and pancreatic carcinoma [14]. The requirement of cannabinoid receptors for this antitumoral activity has been revealed by various biochemical and pharmacological approaches, in particular by determining cannabinoid receptor expression in the tumors and by using selective cannabinoid receptor agonists and antagonists.

Antitumoral Activity of Cannabinoids in Pancreatic Cancer

It has recently been found that cannabinoids (both THC and the synthetic cannabinoid agonist WIN-55,212-2) promote apoptosis of several pancreatic cancer cell lines [14, 32]. This effect was also evident in vivo as cannabinoid administration reduced the growth of subcutaneous and intrapancreatic tumor xenografts generated in immunodeficient mice [14]. Moreover, results also showed that cannabinoids exert a strong inhibitory effect on the spreading of pancreatic tumor cells not only to adjacent locations such as the spleen but also to distal tissues such as the liver, diaphragm, stomach, and intestine, therefore suggesting that cannabinoids may also decrease the propagation of pancreatic tumor cells.

These antitumoral actions of cannabinoids in pancreatic cancer cells rely on CB_2 receptor activation [14]. As indicated earlier, both CB_1 and CB_2 receptors are expressed in pancreatic tumors [14, 15] but not in normal pancreatic tissue, in which the presence of these receptors is almost undetectable [14]. These findings suggest that engaging cannabinoid receptors (and in particular CB_2 receptors) may be used as a therapeutic strategy to specifically target cancer cells without affecting normal pancreas.

Mechanism of Cannabinoid Antitumoral Action

A number of studies performed during the last few years have shown that cannabinoid receptor activation on tumor cells modulates key signaling pathways involved in cell proliferation and survival. Although the downstream events by which cannabinoids exert their antitumoral action in tumor cells have not been completely unraveled, there is substantial evidence for the implication of at least two mechanisms: induction of apoptosis of tumor cells and inhibition of tumor angiogenesis (Fig. 1).

Induction of Apoptosis

Cannabinoids induce apoptosis of cultured tumor cells [reviewed in [23] and (Sarafaraz, 2008 [33]). Studies initially performed in glioma cells found that this effect relies on the activation of cannabinoid receptors and the accumulation of the proapoptotic sphingolipid ceramide [27, 34, 35]. However, the molecular mechanisms involved in the triggering of the apoptotic signal by cannabinoids have started to be understood only very recently.

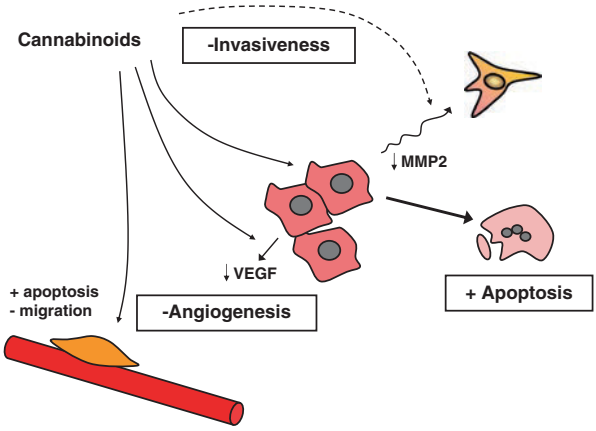

Fig. 1 Antitumoral effect of cannabinoids. Cannabinoid administration to mice decreases tumor growth by several mechanisms, including at least [1] reduction of tumor angiogenesis, [2] induction of tumor cell apoptosis, and perhaps [3] inhibition of tumor cell migration and invasiveness. *MMP2* matrix metalloproteinase-2, *VEGF* vascular endothelial growth factor

By using a DNA array approach, we have identified a series of genes that are selectively upregulated in cannabinoid-sensitive but not cannabinoid-resistant tumor cells upon THC treatment [36]. One of these genes was the stress-regulated protein p8 (also designated "candidate of metastasis 1" – Com-1), which belongs to the family of HMG-I/Y transcription factors and was originally described as a gene induced during the acute phase of pancreatitis [37]. Different experimental approaches confirmed that p8 upregulation is essential for the proapoptotic and antitumoral action of cannabinoids in gliomas and pancreatic tumors [14, 36].

The acute increase of p8 levels after cannabinoid treatment triggers a cascade of events that involves the upregulation of the activating transcription factor 4 (ATF-4) and the C/EBP-homologous protein (CHOP, also called "DDIT3" and "GADD153"). These two transcription factors cooperate in the induction of the tribbles homologue 3 (TRB3, also called "TRIB3"), a pseudokinase that has been implicated in the induction of apoptosis of tumor cells [38]. In line with this observation, selective knockdown of ATF-4 and TRB3 prevented cannabinoid-induced apoptosis, indicating that this signaling route also operates in glioma cells after treatment with cannabinoids [36] (Fig. 2). ATF-4, CHOP, and TRB3 (together with other genes selectively induced upon THC treatment of glioma cells) [36] participate in the endoplasmic reticulum (ER) stress response. A series of ER alterations such as calcium depletion, protein misfolding, and impairment of protein trafficking to the Golgi trigger this response, which involves attenuation of protein synthesis and selective transcription and translation of a series of genes, mainly involved in favoring correct protein folding [39]. When these ER alterations cannot be repaired by the ER stress response, the damaged cells undergo apoptosis. Several stimuli, including ischemia [40], viral infection [41, 42], and drugs such as tunicamycin [38] or cisplatin [43], induce apoptosis through this pathway. Of interest, cannabinoid-induced ceramide accumulation and ER stress induction seem to be closely linked. Thus, inhibition of ceramide synthesis de novo prevents THC-induced p8, ATF-4, CHOP, and TRB3 upregulation [36] as well as ER

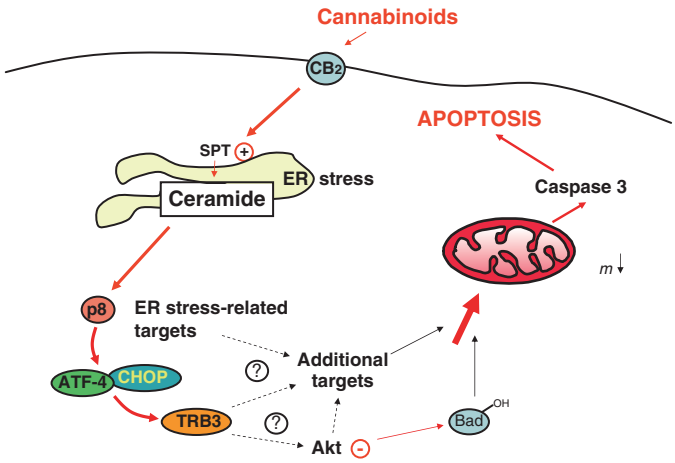

Fig. 2 Mechanism of cannabinoid proapoptotic action in pancreatic tumor cells. Cannabinoid treatment induces apoptosis of tumor cells via ceramide accumulation and activation of an endoplasmic reticulum (*ER*) stress-related pathway. The stress-regulated protein p8 plays a key role in this effect by controlling the expression of activating transcription factor 4 (*ATF-4*), C/EBP-homologous protein (*CHOP*), and tribbles homologue 3 (*TRB3*). This cascade of events triggers the activation of the mitochondrial intrinsic apoptotic pathway through mechanisms that have not been unraveled as yet

dilation (authors' unpublished observations), indicating that ceramide accumulation is an early event in cannabinoid-triggered ER stress and apoptosis in glioma cells.

Unlike this proapoptotic action of cannabinoids on transformed cells, treatment of nontransformed cells with these compounds does not trigger ceramide accumulation [44] or induction of the aforementioned ER stress-related genes [36]. Furthermore, cannabinoids promote the survival of nontransformed cells in different models of injury [45–47]. As discussed above, these observations suggest that the antiproliferative effect of cannabinoids is selective for tumor cells, the viability of normal cells being unaffected or even favored by cannabinoid challenge. In line with these observations, treatment of mice bearing orthotopic pancreatic tumor xenografts with cannabinoids induced apoptosis only in tumor tissue but not in normal pancreas [14].

The processes downstream of ER stress activation involved in the execution of cannabinoid-induced apoptosis are only partially understood. Decreased mitochondrial membrane potential and caspase 3 activation are observed in cannabinoid-treated tumor cells [36, 48–50], suggesting that execution of apoptosis occurs via activation of the mitochondrial intrinsic pathway (Fig. 2). Interestingly, cannabinoid treatment induces loss of mitochondrial membrane potential in $p8^{+/+}$ but not in p8-deficient mouse embryonic fibroblasts, suggesting that the p8-regulated pathway described above is required for the activation of the mitochondrial proapoptotic pathway. On the other hand, cannabinoids inhibit Akt in tumor cells, an effect that is prevented by pharmacological blockade of ceramide synthesis de novo [34]. In addition, cannabinoids lead to decreased phosphorylation of the BH3-only protein Bad [48], an Akt target whose phosphorylation inhibits apoptosis via the intrinsic pathway. These observations suggest that regulation of Akt could be involved in the connection between the ceramide/p8-regulated pathway and the activation of the mitochondrial proapoptotic route (Fig. 2).

Inhibition of Tumor Angiogenesis

To grow beyond the minimal size, tumors must generate a new vascular supply (angiogenesis) for purposes of cell nutrition, gas exchange, and waste disposal, and therefore blocking the angiogenic process constitutes one of the most promising antitumoral approaches currently available. Immunohistochemical analyses in mouse models of glioma [51], skin carcinoma [30], and melanoma [31] have shown that cannabinoid administration turns the vascular hyperplasia characteristic of actively growing tumors into a pattern of blood vessels characterized by small, differentiated and impermeable capillaries. This is associated with a reduced expression of vascular endothelial growth factor (VEGF) and other proangiogenic cytokines such as angiopoietin-2 and placental growth factor [30, 51, 52], as well as of type 1 [52] and type 2 [53] VEGF receptors, in cannabinoid-treated tumors. Pharmacological inhibition of ceramide synthesis de novo abrogates the antitumoral and antiangiogenic effect of cannabinoids in vivo and decreases VEGF production by glioma cells in vitro and by gliomas in vivo [53], indicating that ceramide plays a general role in cannabinoid antitumoral action.

Other reported effects of cannabinoids might be related to the inhibition of tumor angiogenesis and invasiveness by these compounds (Fig. 1). Thus, activation of cannabinoid receptors on vascular endothelial cells in culture inhibits cell migration and survival [51]. Endothelial cell apoptosis was also potently triggered by cannabinoid quinonoid derivatives, although this action seems to be cannabinoid-receptor-independent [54]. In addition, cannabinoid administration to glioma-bearing mice decreases the activity and expression of matrix metalloproteinase-2, a proteolytic enzyme that allows tissue breakdown and remodeling during angiogenesis and metastasis [51, 55]. In line with this notion, cannabinoid intraperitoneal injection reduces the number of metastatic nodes produced from paw injection in lung [52], breast [56], and melanoma [31] cancer cells in mice. Likewise, spreading of pancreatic tumor cells not only to adjacent locations such as the spleen but also to distal tissues such as the liver, diaphragm, stomach, and intestine is strongly inhibited upon cannabinoid administration.

Cannabinoids as Potential Anticancer Agents

On the basis of the aforementioned preclinical findings, the potential therapeutic application of cannabinoids as antitumoral agents has started to be tested. A pilot phase I clinical trial has recently been conducted involving nine patients with actively growing recurrent glioblastoma multiforme to whom THC was administered intratumorally [57]. Standard therapy for the patients had previously failed (surgery and radiotherapy) and they had clear evidence of tumor progression. The primary end point of the study was to determine the safety of intracranial THC administration. The effect of THC action on the length of survival and various tumor cell parameters was also evaluated. A dose escalation regime for THC administration was assessed. The initial dose of THC delivered to the patients was 20–40 μg at day 1, increasing progressively for 2–5 days up to 80–180 μg per day. The median duration of THC administration was 15 days. Under these conditions cannabinoid delivery was safe

and could be achieved without significant psychoactive effects. Median survival of the cohort from the beginning of cannabinoid administration was 24 weeks (95% confidence index 15–33). THC decreased tumor cell proliferation [as determined by Ki67 immunostaining; [31]] and increased p8 and TRB3 immunostaining [[36] and authors' unpublished observations] as well as tumor cell apoptosis [as determined by active caspase 3 immunostaining; [36]] when administered to two patients.

The fair safety profile observed for THC, together with its possible antiproliferative action on tumor cells, may set the basis for future trials aimed at evaluating the potential antitumoral activity of cannabinoids not only in gliomas but also in other malignancies, in particular pancreatic cancer. These possible new trials could involve one or more of the following modifications:

1. THC in combination with other chemotherapeutic agents. Pancreatic adenocarcinoma, like glioblastoma multiforme, is an extremely lethal disease. The success of potential treatments is usually hampered by the extreme resistance to chemotherapy displayed by these tumors. It is therefore conceivable that combined therapies could provide better results than single-agent therapies. For example, by synergizing via complementary signaling pathways, THC plus temozolomide or gemcitabine might exert a more potent clinical impact than either THC or any of these drugs alone.
2. Other cannabinoid ligands. Although the use of cannabinoids in medicine may be limited by their well-known psychotropic effects, it is generally believed that cannabinoids display a fair drug safety profile and that their potential adverse effects are within the range of those accepted for other medications, especially in cancer treatment [23, 24]. In line with this idea, THC delivery in the aforementioned clinical study was safe and could be achieved without overt psychoactive effects. As the possible antitumoral action of nabilone has never been evaluated preclinically, THC remains the unique cannabinoid receptor agonist currently available for cancer clinical trials. Nonetheless, most likely THC is not the most appropriate cannabinoid agonist for future antitumoral strategies owing to its high hydrophobicity, relatively weak agonistic potency, and ability to elicit CB_1-receptor-mediated psychoactivity. Unfortunately, the current synthetic cannabinoid agonists that have been reported to exert antitumoral actions in animal models and that could theoretically circumvent – at least in part – the pharmacokinetic and pharmacodynamic limitations of THC [e.g., WIN-55,212-2, a more potent and less hydrophobic CB_1/CB_2-mixed agonist [27], and JWH-133, a more potent CB_2-selective agonist [58]] are still very far from clinical application owing to the lack of thorough preclinical toxicology studies.

Future Perspectives

One of the most striking features of pancreatic adenocarcinomas is their high resistance to conventional chemotherapy. Nowadays it is widely believed that strategies aimed at reducing the mortality caused by these tumors should consist of targeted therapies capable of providing the most efficacious treatment for each individual patient and tumor. This new therapeutic approach would require not only the utilization of new cocktails of chemotherapeutic drugs

but, more importantly, the identification of the markers associated with the resistance of tumor cells to these new therapies. The significant antiproliferative action of cannabinoids in animal models of cancer, together with their low toxicity compared with other chemotherapeutic agents, might make these compounds promising new tools for the management of pancreatic adenocarcinoma. Studies performed in our laboratory suggest that resistance of glioma and pancreatic tumor cells to cannabinoid treatment correlates with the ability of these cells to block the activation of the ER stress pathway (authors' unpublished observations). In addition, we have observed that agents that induce ER stress exert a synergic action when administered with cannabinoids [36]. Likewise, overexpression of p8 or TRB3 sensitizes resistant cancer cells to a further treatment with cannabinoids [36]. These observations suggest that activation of this route may be investigated as a potential strategy to enhance the response of tumor cells to chemotherapy. Research to be performed during the next few years should help to clarify what the optimal conditions of cannabinoid utilization are by identifying the factors that confer resistance to cannabinoid treatment as well as the most efficient approaches for enhancing their antitumoral activity.

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Pathogenesis of Chronic Pancreatitis with Special Emphasis on Genes[1]

6

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Introduction

In industrialized countries up to 80% of all cases of chronic pancreatitis are due to excess alcohol abuse for many years prior to the clinical onset of the disease. However, most alcoholics never develop chronic pancreatitis. Thus, one has to discuss both protective and deleterious genetic factors. We reported recently that chymotrypsin C variants with diminishing activity or secretion are associated with chronic pancreatitis [3]. We postulate that an important role for intact chymotrypsin C is to destroy prematurely activated trypsin, thus preventing autodigestion.

The pathogenesis of alcohol-induced chronic pancreatitis is still not understood. One discusses alcohol-induced fatty degeneration of pancreatic acinar cells, disturbances in ethanol detoxification, reduced synthesis of lithostathines, changes in duct permeability, a pressure increase of the papilla of Vater, and an imbalance between free radicals and antioxidants [4, 5]. Chronic pancreatitis in adults is defined as a relapsing or continuing inflammatory disease of the pancreas characterized by irreversible morphological changes, upper abdominal pain, and, in some patients, permanent impairment of exocrine function, endocrine function, or both [6]. The resulting morphological changes can be summarized as irregular sclerosis with focal, segmental, or diffuse destruction of the parenchyma. Frequently dilatations, strictures, or intraductal plugs can be seen in the pancreatic duct system. Initially, chronic pancreatitis is characterized by a recurrent stage of acute pancreatitis (early-stage chronic pancreatitis) passing over to progressive pancreatic dysfunction and/or pancreatic calcifications (late-stage chronic pancreatitis).

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Epidemiology

There are no data regarding the incidence or prevalence of hereditary chronic pancreatitis (HCP) or of chronic pancreatitis in children. The incidence of chronic pancreatitis of any cause is expected to be about 3.5–10 per 100,000 inhabitants and year in Europe and the USA [7, 8].

PRSS1 Mutations in Chronic Pancreatitis

In 1952 Comfort and Steinberg [9] were the first to recognize that chronic pancreatitis may accumulate in selected families, suggesting a genetic background. HCP was defined as an autosomal dominant disease with a penetrance rate of approximately 80%. In 1996 several groups mapped a gene for HCP to chromosome 7 [10–12]. In the same year, Whitcomb et al. [13] identified A R122H mutation, in the cationic trypsinogen gene (*PRSS1*). Several other mutations were described subsequently (A16V, D22G, K23R, N29I, N29T, R122C) [14–20]. Until now, the R122H and N29I mutations of the *PRSS1* gene have been identified as the most common disease-associated mutations [13, 15, 19]. Subsequently, several authors identified associations of chronic pancreatitis (idiopathic and hereditary) with other genes, such as the anionic trypsinogen (*PRSS2*), the serine protease inhibitor, Kazal type 1 (*SPINK1*), and the cystic fibrosis transmembrane conductance regulator (*CFTR*) [21–24]. On the other hand, environmental factors such as smoking, alcohol consumption, or lack of antioxidants are assumed to be important manifestation factors especially in alcohol-induced chronic pancreatitis but also in HCP [25–28].

The definition of HCP as a classic autosomal dominant disorder represents current knowledge. In the recently published EUROPAC study [29], the diagnosis of hereditary pancreatitis was made on the basis of two first-degree relatives or three or more second-degree relatives, in two or more generations with recurrent acute pancreatitis, and/or chronic pancreatitis for which there were no precipitating factors. Cases in which these strict criteria were not met, but more than one affected family member was identified, mostly within the same generation, were classified as familial chronic pancreatitis. We define HCP if the patient has no other detectable cause of chronic pancreatitis and if he/she has one first- or second-degree relative with proven chronic pancreatitis.

In children the cardinal symptom is recurring, suddenly appearing epigastric pain. In contrast to adults, enduring pain is not a common clinical finding in children. Other symptoms are nausea, vomiting, and abdominal pressure pain. Children partially develop pancreatic insufficiency with steatorrhoea and insulin-dependent diabetes, but these complications normally occur later than in patients with chronic alcoholic pancreatitis.

Clinical Characteristics

Alcoholic chronic pancreatitis and HCP exhibit essentially identical clinical laboratory results and histopathological or morphological features. Remarkably, HCP manifests itself typically at an earlier age, and pancreatic calcifications and diabetes mellitus are less frequent

complications in comparison with chronic alcoholic pancreatitis. The fact that most subjects with the N29I or R122H *PRSS1* mutation investigated had mild disease or were even asymptomatic should certainly be emphasized [26, 30]. The median age of onset was 11 years in the N29I group and 10 years in the R122H group. Only 4% of our patients had severe chronic pancreatitis with exocrine and endocrine insufficiency, pancreatic calcifications and duct dilatations, as well as hospitalizations due to pancreatitis. In general, half of the mutation carriers had few or no complaints or complications [26]. An European study revealed a mean age of onset of 10 years and 14.5 years for affected carriers of the *PRSS1* mutations R122H and N29I, respectively, but showed no mutation-dependent differences in complications such as exocrine or endocrine insufficiency or increased pancreatic cancer risk [29]. Chronic pancreatitis presents with a wide range of pain from mild to severe and from intermittent to persistent. Interestingly, endocrine insufficiency can regress over time, which is in contrast to current belief that pancreatic diabetes is an irreversible sign of pancreatic failure [31].

Hereditary Chronic Pancreatitis and Pancreatic Cancer

As shown in an investigation of eight patients with pancreatic cancer in a cohort of 246 HCP patients, the lifetime risk of pancreatic cancer is about 50-fold higher than in the control population and corresponds to one per 1,066 person-years [32, 33]. It is only 20-fold elevated in patients with chronic alcoholic pancreatitis [34]. In our cohort of 101 HCP patients (25 N29I carriers, 76 R122H carriers), pancreatic cancer was diagnosed in three patients with the R122H mutation, with a median of 23 years after the onset of pancreatitis. This corresponds to a rate of about one per 1,200 person-years among affected R122H carriers [26]. The data basis for the estimation of the pancreatic cancer risk in patients with *PRSS1*-associated HCP is small. The largest clinical investigation in the pregenetic era, however, revealed no pancreatic cancer in 72 patients from seven families [27]. Affected mutation carriers should be strongly advised to stop smoking, as smoking is an additional risk factor for pancreatic cancer [33].

SPINK1 Mutations in Chronic Pancreatitis

Witt et al. [23] were the first to describe an association between mutations of the serine protease inhibitor, Kazal type 1 (*SPINK1*) and chronic pancreatitis. SPINK1 is a potent protease inhibitor thought to be a specific inactivation factor of intrapancreatic trypsin activity. During incubation of equimolar quantities of trypsin and SPINK1, the formation of a covalent bond between the catalytic serine residue of trypsin and the lysine carboxyl group of the reactive site of SPINK1 occurs. After prolonged incubation, trypsin activity reappears over time. This is explainable by the fact that SPINK1 is degraded by trypsin [35]. The most frequently found *SPINK1* mutation is N34S. This mutation was predominantly found in patients with idiopathic chronic pancreatitis. Further investigations showed an association of *SPINK1* mutations and alcoholic chronic pancreatitis as well as tropical chronic pancreatitis [36–41]. Since 1–2% of healthy controls carry the N34S mutation, this mutation alone seems to be insufficient to explain the pathogenesis of chronic pancreatitis in mutation

carriers. A recent study characterized and compared the functional defects of all nonsynonymous *SPINK1* mutations. The mutations N34S and P55S did not alter secretion of SPINK1, whereas mutation R65Q decreased secretion about twofold. Furthermore, mutations D50E, Y54H, and R67C abolished or markedly diminished secretion. These results identified intracellular folding defects as a novel mechanism of SPINK1 deficiency associated with chronic pancreatitis. However, the disease-causing biochemical defect in the N34S mutant is unrelated to secretion or trypsin inhibitory activity and still remains enigmatic [42].

CFTR Mutations in Chronic Pancreatitis

Cystic fibrosis (*OMIM 219700*) is an autosomal recessive disorder with an incidence in whites of approximately one in 2,500 live births. In 1989, *CFTR* (*OMIM 602421*) was identified as the underlying gene. In 1998 two independent groups reported an association of *CFTR* mutations with chronic pancreatitis [21, 22]. This association is pathophysiologically comprehensible since 1–2% of patients with cystic fibrosis suffer from chronic pancreatitis [43, 44]. The variety of pancreatic disorders in cystic fibrosis range from complete loss of exocrine and endocrine function to almost normal pancreatic function. So far, more than 1,500 mutations of the *CFTR* gene have been described [45]. According to their effect, the mutations are split up into five or six classes (I–V/VI) [46, 47]. In cystic fibrosis, the most common mutation is F508del, accounting for approximately 66% of all mutated alleles [48]. Interestingly, the clinical course of cystic fibrosis can be variable in patients carrying the same mutations, indicating the influence of environmental and other genetic factors.

Further studies confirmed the association of chronic pancreatitis and *CFTR* mutations, but the underlying mechanisms leading to the development of chronic pancreatitis are still poorly understood [49–54]. One main finding is the detection of mostly rare *CFTR* mutations showing a different spectrum of detected mutations than in cystic fibrosis and congenital bilateral aplasia of the vas deferens. Some authors state that compound heterozygous *CFTR* carriers have a distinct elevated risk for the development of chronic pancreatitis, which is even higher when an additional *SPINK1* mutation is present [52, 55]. The role of some *CFTR* mutations has to be reconsidered since the mutation I148T in exon 4, which was classified as a severe cystic fibrosis causing mutation, is not associated with cystic fibrosis. However, the complex allele 3199del6 and I148T seems to be the relevant factor [53]. Thus, *CFTR* mutations alone are not sufficient for the pathogenesis of chronic pancreatitis in most patients and further studies are needed to elucidate the role of CFTR in the pathogenesis of chronic pancreatitis.

PRSS2 Mutations in Chronic Pancreatitis

In a study of 2,466 patients with chronic pancreatitis (including 1,857 with hereditary or idiopathic pancreatitis) and 6,459 controls, the G191R variant of the anionic trypsinogen was overrepresented in controls (32 vs. 220, odds ratio 0.37; $P = 1.1 \times 10^{-8}$). The analysis of the recombinantly expressed G191R variant revealed a complete loss of trypsin activity due

to the introduction of a novel tryptic cleavage site that renders the enzyme hypersensitive to autocatalytic proteolysis. Thus, the G191R variant of PRSS2 seems to mitigate intrapancreatic trypsin activity and thereby plays a protective role against chronic pancreatitis [24].

Biochemical Analysis of Disease-Associated *PRSS1* Mutations

Classic HCP seems to follow an autosomal dominant inheritance with incomplete penetrance and highly variable disease expression. As stated already, the results of research done within the last decade implicate a more complex inheritance pattern. The *PRSS1* mutations are located in three clusters within the trypsinogen sequence: in the trypsinogen activation peptide, in the N-terminal part of trypsin, or in the longest peptide segment not stabilized by disulfide bonds between Cys64 and Cys139. All pancreatitis-associated *PRSS1* mutations discovered to date seem to cluster in the N-terminal half of the molecule encoded by exons 2 and 3. However, most investigations of the *PRSS1* gene in patients with suspected genetically determined chronic pancreatitis are restricted to these exons in most laboratories. Thus, possible C-terminal mutations may have been missed.

The discovery of pancreatitis-associated cationic trypsinogen mutations demonstrates that trypsinogen seems to play a central role in the pathogenesis of human pancreatitis. These mutations may disturb the balance of proteases and their inhibitors within the pancreas, leading to autodigestion of the organ.

The R122H and the N29I mutations are the most common *PRSS1* mutations worldwide. They have been frequently reported from Europe, North America, and Asia [56] and R122H was also recently found in a family of Aboriginal descent in Australia [57]. Neither mutation was detected in two hereditary pancreatitis families from Brazil [58] and no hereditary pancreatitis cases have been reported from Africa.

R122H and Increased Trypsin Stability

Whitcomb et al. [13] proposed that the Arg122-Val123 autolytic peptide bond in trypsin plays an important role in the degradation of prematurely activated trypsin in the pancreas. Disruption of this “fail-safe mechanism” by the R122H mutation would increase intrapancreatic trypsin activity and disturb the protease-antiprotease equilibrium and eventually provoke pancreatitis. Biochemical evidence supports the concept that Arg122 is important for autolysis of trypsin and mutations of this amino acid result in increased trypsin stability [59–62]. A study using caerulein-induced zymogen activation in isolated rat pancreatic acini demonstrated that autodegradation of trypsin mitigates cathepsin B mediated trypsinogen activation, suggesting that a fail-safe mechanism may be operational in the mammalian pancreas [63]. More detailed biochemical analysis indicated that the R122H mutation results not only in increased trypsin stability but also in increased autoactivation [61, 62]. A weak trypsin-inhibitory activity associated with the Arg122 site is also lost in the R122H mutant [61]. Thus, the pleiotropic biochemical effect of R122H raises the possibility

that the pathogenic alteration is unrelated to trypsin stability. More importantly, the model fails to explain how the other pancreatitis-associated *PRSS1* mutations might work, as the majority of these do not affect trypsin stability.

N29I and Enhanced Trypsinogen Autoactivation

Biochemical characterization of the N29I mutation using recombinant trypsinogen found no effect on the stability of this enzyme. Independent groups observed a moderately increased autoactivation [62, 64–66]. The N29T mutant exhibits a phenotype similar to that of R122H; both increased trypsin stability and enhanced autoactivation were documented [17, 63]. Since increased autoactivation was observed with the R122H, N29I, and N29T mutations, whereas N29I had no effect on trypsin stability, one may conclude that enhanced autoactivation is the common pathogenic mechanism of hereditary pancreatitis associated *PRSS1* mutations [62].

Animal Models

Transgenic expression of rat SPINK1 in mice reduced the severity of experimental secretagogue-induced pancreatitis [67]. These transgenic mice had 190% increased endogenous trypsin-inhibition capacity. Transgenic expression of SPINK1 did not hinder trypsinogen activation, but reduced trypsin activity after supramaximal stimulation with caerulein. These in vivo results underline the hypothesis that the enhanced inhibitory capacity of trypsin protects against pancreatitis. However, results from a mouse with targeted disruption of the pancreatic secretory trypsin inhibitor are puzzling [68]. A knockout (−/−) of the mouse homologue of human SPINK1, murine Spink3, is lethal within 2 weeks after birth. Spink3$^{-/-}$ embryos developed normally until day 15.5 after conception. Subsequently, autophagic degeneration of the pancreatic acinar cells started, interestingly without significant inflammatory cell infiltration. In this study, the authors were not able to detect enhanced trypsin activity in the acinar cells of the SPINK$^{-/-}$ animals. In a further study, enhanced tryptic activity was found in pancreatic acini prepared 1 day after birth using a more sensitive assay [69]. These data indicate that the total loss of SPINK3 function leads to a strong imbalance in favor of trypsin activity, resulting in acinar cell death and a lethal phenotype.

Two recent publications describe transgenic animals expressing R122H mutated trypsinogen [70, 71]. We developed a mouse expressing R122H human trypsinogen in the exocrine pancreas by using the rat elastase-2 promoter [71]. The animals showed only slightly elevated serum levels of lipase without any significant histological alteration, suggesting subtle acinar damage. After repetitive induction of experimental pancreatitis, pancreata of transgenic animals showed a higher inflammatory reaction than controls. The mild phenotype in these animals is probably caused by a low expression level of R122H mutated trypsinogen.

Transgenic expression of the R122H mutation of murine trypsin 4 in mouse pancreas led to progressive fibrosis and chronic inflammation of the pancreas [70]. Repetitive inductions

of experimental pancreatitis with supramaximal doses of caerulein resulted in extensive deposition of collagen in periacinar and perilobular spaces of the transgenic animals. This animal model with significant expression of R122H mutated trypsinogen seems to imitate the human disease.

The Trypsinogen and *SPINK1* Mutation Database

Since the first description of a trypsinogen mutation in hereditary pancreatitis, the experimental and clinical information on genetic alterations in chronic pancreatitis has been rapidly growing, resulting in a more and more complex data set. To address this issue, we implemented a continuously updated database in early 2001, which contains all genetic variants of the *PRSS* and *SPINK1* genes [72]. In addition to exact genetic data, this database contains links to the clinical characterization of patients with different mutations and to in vitro studies with mutant molecules.

Most laboratories have focused their studies on *PRSS1* exons 2 and 3, and until now no unambiguous disease-associated mutation has been identified in the other exons. It is still possible that new variants will be identified in exons 1, 4, and 5, or in the intronic and promoter regions. Interestingly, the triplication of a segment containing the *PRSS1* gene was found recently in patients with HCP. This triplication seems to result in a gain of trypsin through a gene dosage effect and represents a previously unknown mechanism causing HCP [73].

Further Pathogenetic Aspects of Chronic Pancreatitis

Well-recognized causative factors of chronic pancreatitis are anatomic anomalies, metabolic disorders, trauma, cystic fibrosis, and inflammatory bowel disease. Since HCP manifests itself predominantly in childhood or early adulthood, alcohol abuse as the most common predisposing condition can nearly be ruled out. Other rare aspects are hyperlipidemia type I, familiar (hypocalciuric) hypercalcemia, hereditary hyperparathyroidism, and autoimmune pancreatitis, the last of these usually manifesting itself in late adulthood [74–79].

Understanding of Pathogenesis: Consequences for Treatment?

Treatment of chronic pancreatitis is still only symptomatic and focuses on pain management, maldigestion, diabetes, pseudocysts, bile duct obstruction, duodenal obstruction, and pancreatic cancer [5, 80, 81]. To prevent disease progression, drinking of alcohol should be infrequent, whereas smoking should be avoided completely. Our knowledge of various genes involved in the pathogenesis of the disease has not led up to now to any consequences regarding treatment. Genetic testing should follow strict regulations [82].

Future Perspectives

From a genetic aspect, a most interesting quest in the near future will be for modifier genes that might explain the incomplete penetrance i.e., why some carriers of *PRSS1* mutations remain healthy, whereas their relatives with the same mutation exhibit severe disease. Despite intensive research, the disease mechanism remains poorly understood. Although the biochemical alterations caused by the mutations have been largely clarified in vitro, their phenotypic effect in vivo remains unclear in most instances.

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Bioecological Control of Disease, Especially Pancreatic Disease

7

S. Bengmark

Introduction

Patients with acute pancreatitis often suffer an uncontrolled superinflammation, and as a result of that a malfunctioning innate immune system, which frequently leads to complications: severe infections, systemic inflammatory syndrome, and sometimes multiple organ failure. Among the characteristics of the superinflammation are an exuberant, e.g., exaggerated and prolonged, inflammatory response, an aberrant cellular response, and extreme elevations in levels of cytokines and acute-phase proteins but also in levels of coagulation and growth factors. The cytokine storm, which occurs during the first few hours, will almost immediately reach the lungs and other distant organs via the lymphatics [1], and will condition them to be/make them susceptible to a subsequent infection. Overgrowth of potentially pathogenic microorganisms in the stomach and intestines, due to disease, inhibition of gastric and gastrointestinal (GI) secretions, and absence of a food stream, provides the source of bacteria for subsequent infection of the chest, pancreas, urinary tract, and other organs.

Instant Reaction: Narrow Therapeutic Window

The alarm reaction is instant: within minutes increases in the levels of various proinflammatory cytokines and within hours increases in the levels of various acute-phase proteins are observed, changes most often reaching their peaks within the first 24 h. It is increasingly recognized that the therapeutic window providing possibilities to control the exuberant phase response is narrow, most likely not much more than 24–36 h. Experience from liver transplantation suggests that a superinflammation observed in the late phase of

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the operation is intimately associated with subsequent sepsis. If at the end of the operation the endotoxin, the tumor necrosis factor α (TNF-α), and interleukin (IL)-6 levels were increased more than 6 times, the patients developed sepsis [2]. The conditions are similar in acute pancreatitis.

Efforts to control inflammation and subsequent complications, instituted later than 24–36 h after onset of disease, are prone to have limited preventive effects. Unfortunately patients with acute pancreatitis will often arrive at hospital 12–24 h after the onset of disease, which underlines the necessity of making efforts to control the inflammation the highest priority. It is strongly recommended that attempts are made immediately on arrival of the patient to restrict the superinflammation to the greatest extent possible. It is better to treat some patients unnecessarily than to miss the opportunity. The majority of immune cells (about 75%) are located in the GI tract, which is why enteral nutrition has proven to be a powerful tool to control the function of these cells. Studies in recent years have also demonstrated that immediate postoperative feeding is not only safe, but also prevents an increase in gut mucosal permeability, contributes to a positive nitrogen balance, and reduces the incidence of septic complications. It also reduces postoperative ileus and accelerates restitution of pulmonary performance, body composition, and physical performance [3, 4]. It has also been observed in a controlled study that delaying institution of enteral nutrition later than after 24 h leads to, compared with immediate supply, increased intestinal permeability and significantly higher incidence of multiple organ failure [5].

Choice of Treatment Strategy

Today's treatment strategy is to a large extent based on early enteral nutrition and use of antibiotics. It is increasingly observed that antibiotics have a limited if not no preventive effect on the course of disease [6]. It has long been known that the administration of antibiotics will suppress various immune functions, and especially macrophage activities such as the chemiluminescence response, chemotactic motility, and bactericidal and cytostatic ability [6, 7]. This is so with standard antibiotic administration and probably even worse with selective digestive tract decontamination. It is a historic landmark that routine antibiotic prophylaxis has been shown to be of no benefit in reducing the risk of developing infected pancreatic necrosis [8]. Enteral supply of nutrients must be done with care, and nutrition solutions which increase blood glucose levels should be avoided, as hyperglycemia is associated with impaired neutrophil dysfunction [9] and significantly increased infection and mortality rates, as demonstrated in trauma patients [10]. Standard commercial enteral nutrition solutions, rich in proinflammatory molecules containing dairy-derived proinflammatory molecules, should be avoided to the greatest extent possible. Total parenteral solutions and some commercial enteral diets have in animal experiments been shown to activate inducible nitric oxide synthase and disrupt the gut barrier function and the intestinal microflora and induce bacterial translocation [11].

Hospital-produced nutrition formulas, made of fresh fruits, vegetables, especially legumes, and fish/meat, and probably more suitable for enteral nutrition, have for questionable hygiene

and efficiency reasons been abandoned in hospitals in the developed world. Controlled clinical studies comparing the effects of standard nutrition solutions and hospital-made nutrition solutions on immunity and outcome are most highly desirable. Also, blood transfusions must be avoided to the greatest extent possible. A recent meta-analysis based on 20 peer-reviewed articles and more than 13,000 patients reported an average of 3.5 times increase in postoperative infections in surgical patients receiving allogenic blood transfusion [12].

Choice of Treatment

The enteral nutrition formulas used today are made mainly with the aim to provide calories and are to a large extent built on the concept used for parenteral nutrition, e.g., based on a mixture of various "chemicals." It is clear that such nutrition can never replace normal eating with its great variation in the supply of nutrients, antioxidants, plant fibers, and preventive nonpathogenic microorganisms. It is suggested that normal eating provides up to two million different molecules, which include several hundred different carotenoids and several thousand different flavonoids, some of which have an antioxidant effect 10 times or more powerful than that of vitamins such as C and E. Gastric release of nitric oxide is mandatory for maintenance of upper GI motility, for mucosal and splanchnic secretion, and for elimination of pathogens from the stomach. It is therefore of outmost importance that the nutrient solution supplied provides precursors for such production e.g., nitrate/nitrite. Eating natural food, rich in fresh fruits and vegetables, and hopefully also in fibers and probiotic bacteria, is optimal for the function of the innate system and for resistance to disease. Unfortunately, such nutrition is most often not possible in severely sick patients.

Choice of Feeding System

To improve the possibilities to provide immediate enteral nutrition, I developed a so-called self-propelling (autopositioning) and also regurgitation-resistant tube, based on a coil (spiral) in the end, which is made to maximally absorb the gastric motility for its transportation to an ideal position, just below the ligament of Trietz. It usually needs no assistance by endoscopy or X-ray. Nutrition is started as soon as the coil of the tube has been placed in the stomach without waiting for it to migrate into the intestine. Several studies have proven its superiority over other tubes, especially in patients with reduced or inhibited motility, such as patients with acute pancreatitis. A recent prospective study showed successful placement (within 24 h) in patients with normal gastric emptying in 78% of patients versus 14% of patients with a straight standard tube ($P = 0.041$), and in patients with impaired gastric emptying a 24-h success rate of 57% compared with 0% for patients with straight tubes ($P = 0.07$) [13].

Maintenance of Salivation and GI Secretion

The amount of GI secretion in an adult is as much as 10 L a day, of which 2.5 L is saliva and another 2.5 L is gastric secretion. These secretions are extremely rich in immunosupportive factors such as immunoglobulins, lactoferrin, lysozyme, fibronectin, and mucin, but are also an important source of healing factors such as epidermal growth factor. Removal of the salivary gland function results in gastric and intestinal ulcerations, poor wound healing, and poor regeneration of organs, especially the liver. From an immunological point of view, a drug that stimulates these secretions, instead of inhibiting them, should be preferred, and especially in the very sick and critically ill. Unfortunately, most drugs, especially those commonly used in intensive treatment units (ITUs), have strong antisecretory effects – for more information, including a list of drugs, see [14]. Low gastric pH is a prerequisite for gastric nitric oxide production, a function which is totally eliminated by supply of H_2 blockers and proton inhibitors [8]. Normal gastric acid production is also essential for absorption of several vitamins and antioxidants, including vitamin C and glutathione. Most important, in the absence of low pH, the stomach will become a reservoir for pathogens, which are often regurgitated into the lungs and are the cause of chest infections [15]. Use of H_2 blockers/proton inhibitors to prevent peptic ulcers was necessary in patients on total parenteral nutrition, but today with early and aggressive enteral feeding it is totally unnecessary [16–19].

Strict Blood Glucose Control

Elevated blood glucose level is deleterious to the function of the immune system and prevention of morbidity. Blood glucose levels below 8 mEq are also necessary for maintenance of normal GI motility [20] and for splanchnic and mucosal blood flow. Even though strict glucose control had been known for some time to reduce the incidence of wound infection after open heart surgery, it was not until recently that strict glucose control was adapted for modern ITU and postoperative care. For a long time, the state of the art was to tolerate blood glucose levels up to 12 mmol/L (220 mg/dL) in fed critically ill patients. However, strict glucose control to below 6.1 mmol/L has recently been shown to decrease blood stream infections by 46%, acute renal failure with need of hemofiltration by 41%, critical illness polyneuropathy by 44%, red cell transfusions by 50%, and mortality by 34% [21].

Generous Supply of Antioxidants

The tissue and blood concentrations of pro-oxidants are almost invariably high and the serum levels of various antioxidants and micronutrients low or extremely low in critically ill patients, including those with acute pancreatitis. As an example, total vitamin C and ascorbic

acid levels are reported to be less than 25% of normal values in such patients. A study performed in mainly trauma patients reported a 19% reduction in pulmonary morbidity and a 57% lower incidence of multiple organ failure in a group of patients receiving supplementation with α-tocopherol and ascorbate [22]. A recent controlled study in patients with acute pancreatitis reported significantly increased plasma levels of vitamin C in parallel with a significant reduction in levels of IL-2r, TNFα, IL-6, and IL-8 [23]. Furthermore, the ratio of CD4/CD8 and CD4 positive cells was significantly higher in the treated patients. In addition, a faster normalization of temperature and amylase level in serum and urine was observed. But more importantly the cure rate, the complication rate, and the length of hospital stay were significantly better. Thus far, most of the studies reported were done using conventional vitamins and intravenous supply. Antioxidants such as flavonoids have not been tried. As some of these are reported to have 10 times stronger antioxidant effects, it should be of the greatest interest to try these in severely sick patients such as those with acute pancreatitis. Healthy individuals receive most of their antioxidants, including exogenous glutathione, from fresh fruits and vegetables, and they are released and absorbed after fermentation by lactic acid bacteria (LAB) in the lower GI tract. As the mucosa in the lower gut almost entirely depends on nutrients from the lumen, it is likely that there is a premium in supplying antioxidants, which are delivered where they are much needed, e.g., at the mucosa of the lower GI tract.

Control of Microbial Flora

In addition to the digestion provided by saliva and GI secretion, does there exist an equally important system for digestion of food based on microbial enzymes in the lower GI tract? The colon is today recognized as an important immunological organ, and also as an important metabolic organ. Most likely it has more and certainly more complex functions than the rest of the GI tract. An indication of the complexity of the metabolic activities in the large intestine is the fact that the colonic "microbial organ" contains more than two million genes [24], to be compared with about 65,000 genes in the rest of the human body. Numerous substances, several hundred thousand, if not a million or two, are produced, released, and absorbed at the level of the lower small intestine and the large intestine. All depend on microbial fermentation for their release and absorption, which is why maintenance of flora is so important. Among these substances are various fatty acids, especially short chain fatty acids, carbohydrates, amino acids, polyamines, vitamins, antioxidants, phytoestrogens, and coagulation and growth factors. The main substrate for this production through microbial fermentation is plant fibers, fresh fruits, and vegetables. If such food cannot be provided to the sick patient, at least dried plant fibers, although much less effective, should be liberally supplied. However, one must keep in mind that many important antioxidants, such as glutathione, and amino acids such as glutamine do not sustain industrial processes such as drying or heating. High priority should, whenever possible, always be given to supplementing commercial feeding formulas with fresh fruits and vegetables, also in ITU patients, by any means possible.

The Concept of Synbiotics

All products released from consumed plant fibers (prebiotics) by the action of flora or supplemented bacteria (probiotics) are collectively called "synbiotics." Genetically, there is a large difference between different bacteria called "lactic acid bacteria" (LAB); often said to be greater than between man and a fish. Thus, it is of extreme importance that only LAB with strong and specific bioactivities are used. Most of the LAB used by the food industry have no or limited ability to ferment strong bioactive fibers such as inulin and phlein, no ability to adhere to human mucus, low antioxidant capacity, and most important do not survive the acidity of the stomach and bile acid. This is illustrated by a recent study. A standard commercial product containing *Lactobacillus acidophilus* LA5, *Bifidobacterium lactis* BP12, *Streptococcus thermophilus*, and *Lactobacillus bulgaricus* was mixed with 7.5 g oligofructose and in a controlled study was supplied to critically ill patients. Although significant reductions in the number of potentially pathogenic organisms could be observed in the stomach of the patients treated, no influence on intestinal permeability could be demonstrated nor could any clinical benefits be demonstrated when this particular formula was supplied to a mixed group of critically ill patients [25]. See also [26].

My personal experience during the last 15 years stems from studies using two different synbiotics – combinations of prebiotics and probiotics: [1] *a one LAB/one fiber composition*, produced by fermentation of oat meal with *L. plantarum* strain 299; [2] *a four LAB/four fiber composition* (Synbiotic 2000®), consisting of a mixture of 10^{10} (more recently also as Synbiotic 2000 FORTE® with 10^{11}) of each of four LAB – *Pediococcus pentosaceus* 5-33:3, *Leuconostoc mesenteroides* 32-77:1, *Lactobacillus paracasei* subsp. *paracasei* 19, and *Lactobacillus plantarum* 2362 – and 2.5 g of each of four fermentable fibers (prebiotics): β-glucan, inulin, pectin, and resistant starch.

Synbiotics in Chronic Liver Disease and Liver Transplantation

Most of the experience with synbiotic treatment stems from studies in patients with chronic liver disease. Synbiotics have the ability to reduce the production and absorption of endotoxin in the intestine, but they also downregulate production of proinflammatory cytokines. The in vitro production of TNF-α by peripheral blood mononuclear cells in response to stimulation by endotoxin or *Staphylococcus aureus* enterotoxin B was reduced by a median of 46% (range 8–67%) in eight of 11 (72.7%) cirrhotic patients supplied for 1 week with the four LAB/four fiber synbiotic composition [27]. Fifty-eight patients with so-called minimal encephalopathy were randomized into three groups and supplied for 1 month with [1] the four LAB/four fiber composition ($n = 20$), [2] only the fibers in the composition ($n = 20$), and [3] a placebo (nonfermentable, nonabsorbable fiber) ($n = 15$) [28]. Significant reductions in intestinal pH, serum endotoxin, and intestinal content of *Escherichia coli*, *Staphylococcus,* and *Fusobacterium* were observed in both treatment groups but not in *Pseudomonas* and *Enterococcus*, and were accompanied by significant improvements in ammonia/s, bil/s, liver enzymes, prothrombin time, and albumin/s. The levels of ALT

decreased significantly from 252 ± 182 to 84 ± 65 ($P < 0.01$) in the synbiotic-treated group and to 110 ± 86 ($P < 0.05$) in the fiber-only group, but not in the placebo group. Significant improvements were observed in psychometric tests and the degree of encephalopathy.

Both the one LAB/one fiber and the four LAB/four fiber compositions have been tried in human liver transplantation. Ninety-five liver transplant patients were divided into three groups: group 1, selective digestive tract decontamination (SDD) four times daily for 6 weeks ($n = 32$); group 2, live *L. plantarum* 299 (LLP) at a dose of 10^9 plus 10 g of oat and inulin fibers ($n = 31$) supplied for 12 days postoperatively; group 3, identical to group 2, but with heat-killed *L. plantarum* 299 (HLP) ($n = 32$) [29]. Signs of infections occurred in 48% of patients (15/32) receiving SSD, in 34% of patients (11/32) receiving HLP, and in 13% of patients (4/31) receiving LLP, $P = 0.017$. The most dominant infections were cholangitis, occurring in ten patients receiving SSD, in eight patients receiving HLP, and in two patients receiving LLP, and pneumonia, occurring in six patients receiving SDD, in four patients receiving HLP, and in one patient receiving LLP. This study was followed by a study in 66 patients supplied either with [1] the four LAB/four fiber composition (4×10^{10} LAB) or [2] only the four fibers in the synbiotic composition [30]. The treatment started on the day before surgery and continued until the 14th day after surgery. One of the 33 patients in the synbiotic-treated group (3%) showed signs of infection (a slight urinary infection) during the first month compared with 17 of the 33 patients (51%) in the group supplied with the four fibers only. The need for antibiotics was also significantly reduced in the synbiotic-treated group.

Synbiotics in General Surgery

Ninety patients were randomized into three groups: group 1, LLP at a dose of 10^9 plus 10 g of oat and inulin fibers ($n = 30$) for 12 days postoperatively; group 2, identical to group 1, but with HLP ($n = 30$); and group 3, standard enteral nutrition ($n = 30$) [31]. Each group consisted of 30 patients. The 30-day sepsis rate was 10% (three of 30 patients) in the two groups receiving either live or heat-inactivated LAB, compared with 30% (nine of 30 patients) in the group receiving standard enteral nutrition ($P = 0.01$). The largest differences were observed in the numbers of patients with pneumonia: enteral nutrition – six patients, LLP – two patients, HLP – one patient. The beneficial effects of synbiotic treatment seemed to be most pronounced in gastric and pancreatic resections with a sepsis rate of 7% for patients receiving LLP, 17% for patients receiving HLP, and 50% for patients receiving enteral nutrition. The LLP patients received significantly less antibiotics ($P = 0.04$); the mean length of antibiotic treatment was 4 ± 3.7 days for LLP, 7 ± 5.2 days for HLP, and 8 ± 6.5 days for enteral nutrition. The incidence of noninfectious complications were 30% (9/30) for enteral nutrition, 17% (5/30) for HLP, and 13% (4/30) for LLP. A second study has just been concluded in abdominal cancer patients. Forty-five patients were treated for 2 days before and for 7 days after operation with [1] the four LAB/four fiber composition (4×10^{10} LAB) (LEN), [2] only the four fibers in the synbiotic composition (FEN), or [3] standard parenteral nutrition [32]. The incidence of postoperative bacterial infections was 47% for parenteral nutrition, 20% for FEN, and 6.7% for LEN. Significant improvements in

prealbumin (LEN, FEN), C-reactive protein (LEN, FEN), serum cholesterol (LEN, FEN), serum endotoxin (LEN, FEN), white cell blood count (LEN), and IgA (LEN) were observed on the third and sixth postoperative days. At these time points no statistically significant differences could be observed in the levels of IgM, IgG, and complements or in the cytokines IL-1, Il-6, and TNFα.

Use of Synbiotics in Polytrauma

Two studies in polytrauma patients have been concluded with Synbiotic 2000 and Synbiotic 2000 Forte, but the results have not yet been published. One prospective randomized study in patients with acute extensive trauma compared treatment with Synbiotic 2000 (40 billion LAB/day) with treatment by supplementation of a soluble fiber, treatment with a peptide diet, and treatment with glutamine. Treatment with Synbiotic 2000 led to, compared with treatment with a peptide diet (11 of 26 patients – 42%, $P < 0.04$), treatment with glutamine (11 of 32 patients – 34%, $P < 0.03$), and treatment with only fibers (12 of 29 patients – 41%, $P < 0.002$), a highly significant decrease in the number of chest infections (four of 26 patients – 15%) [33]. The total number of infections was also significantly decreased: Synbiotic 2000, five of 26 patients (19%); only fibers, 17 of 29 patients (59%); peptide diet, 13 of 26 patients (50%); and glutamine, 16 of 32 patients. In another study, 65 polytrauma patients were randomized to receive once daily for 15 days Synbiotic 2000 Forte or maltodextrin (placebo). Significant reductions were observed in a number of parameters, such as the number of deaths (5/35 vs. 9/30, $P < 0.02$), severe sepsis (6/35 vs. 13/30, $P < 0.02$), chest infections (19/35 vs. 24/30, $P < 0.03$), central line infections (13/32 vs. 20/30, $P < 0.02$), and ventilation days (average 15 vs. 26 days) [34].

Use of Synbiotics in Acute Pancreatitis

Patients with severe acute pancreatitis were randomized into two groups to receive daily and administered through a nasojejunal tube for the first 7 days a freeze-dried preparation of either LLP at a dose of 10^9 plus 10 g of oat and inulin fibers (group 1) or the same as for group 1 but with HLP (group 2) [35]. The study was designed to be interrupted when on repeat statistical analysis significant differences in favor of one of the two group were obtained, which occurred when a total of 45 patients had entered the study. At that time 22 patients had received treatment with LLP and 23 patients had received treatment with the HLP. Infected pancreatic necrosis and abscesses were seen in one of the 22 patients (4.5%) in the LLP group compared with seven of the 23 patients (30%) in the HLP group ($P = 0.023$). The only patient in the LLP group who developed infection had signs of urinary infection on the 15th day, e.g., at a time when he had not received treatment during the previous 8 days. The length of stay was considerably shorter in the LLP group (13.7 days vs. 21.4 days), but the limited size of the material did not allow statistical significance to be achieved. In a second study by the same group, sixty-two patients with

severe acute pancreatitis (Apache II scores, synbiotic-treated group 11.7 ± 1.9, controls 10.4 ± 1.5) were supplemented for 14 days with either two sachets per day of Synbiotic 2000 (2 × 40 billion LAB/day and in total 20 g fibers) or only the same amounts of fibers in 20 g as in Synbiotic 2000. Nine of the 33 patients (27%) in the Synbiotic 2000 treated group and 15 of the 29 patients (52%) in the only-fiber-treated group developed subsequent infections. Eight of the 33 (24%) Synbiotic 2000 treated patients and 14 of the 29 (48%) only-fiber-treated patients developed systemic inflammatory syndrome, multiple organ failure, or both ($P < 0.005$) [36].

Use of Synbiotics in Pancreatic Cancer Operations

A prospective randomized double-blind trial was undertaken involving 80 patients following pylorus-preserving pancreatoduodenectomy [37]. All patients received enteral nutrition immediately postoperatively. Group A received a composition of Synbiotic 2000, and group B received a placebo (the fibers in Synbiotic 2000, but no LAB) starting the day before surgery and continuing for 8 days. The 30-day infection rate, the length of hospital stay, the duration of antibiotic therapy, noninfectious complications, and side effects were recorded. The infections were diagnosed at a mean of 9 days (group A) and 8 days (group B) following surgery. Five of the 40 patients in the Synbiotic 2000 treated group (12.5%) and 16 of the 40 patients in the control group (40%) developed postoperative infections ($P = 0.005$). No peritonitis (control group, six patients), pneumonia (control group, four patients), sepsis (control group, two patients), cholangitis (control group, one patient), and empyema (control group, one patient) were observed in the Synbiotic 2000 treated group. Four patients in the Synbiotic 2000 treated group and six patients in the control group developed wound infections. Urinary tract infection was seen in one patient in each group. *Enterobacter cloacae* grew in eight control patients (two treated patients), *Enterococcus faecalis/faecium* in seven patients (one treated patient), *E coli* in seven patients (no treated patients), and *S. aureus* in two patients (no treated patients). *Klebsiella pneumonia* grew in two patients in each group and *Proteus mirabilis* in one patient in each group.

Conclusions

As can be judged from the limited studies available today, symbiotic treatment seems to have a great potential as a tool to control inflammation and infection in connection with surgery and in acute diseases such as acute pancreatitis. Limited experience suggests that promising effects can also be obtained when it is used in pancreatic surgery, both resections and transplantation. The results of treatment in other chronic diseases such as chronic liver disease provide hope that positive effects might also be obtained when it is tried in chronic pancreatitis. Such treatment might also prove valuable in patients on a waiting list for an operation, particularly transplantation. This treatment should be an attractive option as it is relatively inexpensive, has no side effects, and does not induce antibiotic resistance.

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The Role of Endoscopic Sphincterotomy in the Management of Acute Biliary Pancreatitis

8

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Background

Gallstones are the commonest cause of acute pancreatitis in developed countries, accounting for approximately 40–60% of all cases [1]. Although it is nearly 100 years old, Opie's postulation that biliary obstruction is the initiating event in acute biliary pancreatitis remains to this day. Acute pancreatitis occurs when a migrating gallstone obstructs the pancreatic duct in patients who have a common biliary and pancreatic channel entering the ampulla of Vater. The common channel could be found in more than 70% of patients with acute pancreatitis, in contrast to only 20% of controls [2]. Eradication of biliary stones is mandatory to prevent a recurrent attack of acute pancreatitis, but the timing of definitive biliary tract surgery is controversial. Such timing has evolved from delayed intervention to the surgery being performed shortly after the resolution of acute pancreatitis, preferably during the same hospital admission. However, recurrent attacks of acute pancreatitis have been shown to be low after endoscopic sphincterotomy (ES) alone [3–6], rendering early cholecystoectomy for residual gallstones unnecessary. Controversies also exit regarding the role and timing of endoscopic retrograde cholangiopancreatography (ERCP) in the management of acute pancreatitis. In the past, ERCP was employed mainly as a diagnostic tool used electively at 2–6 weeks or even longer after the acute attack. Urgent ERCP and ES for identifiable common bile duct (CBD) and ampullary stones have been advocated by two randomized controlled trials [7, 8]. With this background, we evaluate a protocol of emergency ERCP for patients with predicted severe attack, early ERCP for patients with predicted mild attack, and interval laparoscopic cholecystectomy (LC) for the management of acute biliary pancreatitis.

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Patients and Methods

Acute biliary pancreatitis was diagnosed in patients who presented with acute abdominal pain with a serum amylase level greater than 1,000 U/L and the presence of stones in the biliary tract as determined by ultrasonographic or radiologic techniques. Emergency ERCP was performed within 24 h of admission for all patients predicted to have a severe attack. For patients predicted to have a mild attack, ERCP was performed during the next available elective endoscopy session within 72 h from admission. All ERCP were performed in our radiology department. Whenever CBD or ampullary stones were found, ES was performed in the usual manner at the same session. Active instrumental extraction, supplemented by mechanical lithotripsy when required, was used to clear the CBD of stones. Conservative management was continued after endoscopic intervention until resolution of the pancreatitis, defined as complete resolution of symptoms and normal amylase level and white blood cell count. Laparotomy and necrectomy were performed whenever clinically indicated. Depending on the availability of an operating session, elective LC was performed 2–12 weeks after initial presentation of the patient with acute pancreatitis and when clinical symptoms had completely subsided. Operative cholangiography was performed on a selective basis in patients whose preoperative ERCP failed and in some with a dilated CBD.

Results

We analyzed the results of 75 patients with acute biliary pancreatitis. They constituted 71% of 106 patients admitted with acute pancreatitis with various causes. There were 28 male and 47 female patients. A total of 57% of patients had an elevated serum alkaline phosphatase (ALP) level of more than 100 U/L and 63 patients had an elevated total bilirubin level of more than 20 mol/L on admission. The positive and negative predictive values of an elevated ALP level on admission as a predictive tool for a biliary cause of pancreatitis were 80 and 40%, respectively, whereas those for an elevated serum bilirubin level were 81 and 48%, respectively.

Bedside ultrasonographic examination of the hepatobiliary system and pancreas of all patients was performed within 24 h of admission. It diagnosed 60 of 64 patients with gallstones, with a sensitivity of 94% and a zero false-positive rate. However, the sensitivities of ultrasonography for predicting choledocholitiasis were low, being 36% for CBD dilatation and 15% for visualization of the stones, whereas the specificities for these parameters were 82 and 91%.

All 45 patients predicted to have a severe attack of pancreatitis underwent emergency ERCP within 24 h. The remaining 30 patients underwent elective ERCP 6–72 h from admission. CBD stones were detected in 52 patients, among whom 12 had stones impacted at the ampulla. ES was performed in all of these 52 patients, and 25 of them had a CBD clear of stones during the first session of ERCP. Twenty-seven patients underwent more then one session (two to four sessions) to confirm or achieve ductal clearance. ERCP failed in four patients for technical reasons (two patients) or anatomical reasons (two patients with previous Billroth

II gastrectomy). Postsphincterotomy bleeding occurred in two patients, which was noted immediately and treated successfully. No significant morbidity resulted from the bleeding, and neither of the patients received blood transfusion. No other complication related to the endoscopic procedure occurred, and there was no evidence of worsening pancreatitis after endoscopic intervention.

A total of 16 complications occurred in one patient. Although at least 16% of all patients admitted had clinical evidence of concomitant acute cholangitis, none had evidence of persistent biliary sepsis or recurrent cholangitis after endoscopic intervention. Five patients had clinical manifestation of a prolonged attack of acute pancreatitis. One of them deteriorated clinically with evidence of pancreatic and peripancreatic necrosis on CT scan. He underwent laparotomy and necrectomy, recovered from disease, and was discharged 23 days afterward. All other patients recovered uneventfully. The median time for complete resolution of acute biliary pancreatitis for all patients was 5 days.

Of the 75 patients with acute biliary pancreatitis, seven had undergone previous cholecystectomy and did not require further surgery after the CBD had been cleared of stones. Cholecytectomy was also not required in four patients in whom stones were not identified in the gallbladder on repeating ultrasonography, ERCP, and oral cholecystogram. Six patients with gallstones refused cholecystoectomy after the initial attack had subsided and defaulted on follow-up. Two patients underwent laparotomy and open cholecystectomy for management of complications of acute pancreatitis as mentioned above.

Forty-five patients underwent elective surgery for management of gallstone disease. Two patients who had a history of upper abdominal surgery underwent open cholecystectomy. Forty-six patients underwent elective LC 2–12 weeks from the initial attack of acute pancreatitis, and the procedure was completed in 44 of them. Conversion to open cholecystectomy was required in two patients owing to difficult dissection related to chronic cholecystitis. No major intraoperative or postoperative complication occurred. Minor postoperative complications occurred in two patients, including chest infection and wound infection, resulting in an overall morbidity of 4%.

Seven patients were considered to be poor surgical risks for cholecystectomy because of advanced age or concomitant medical problems. All seven underwent ERCP and ES for CBD stones during the initial attack of acute biliary pancreatitis. Cholecytectomy, however, was not performed for their gallstones. All except one remained well, with a median follow-up of 27 months. One patient presented again with abdominal pain and jaundice 18 months after the initial presentation with acute pancreatitis. Investigations revealed recurrent CBD stones, which were removed endoscopically. None of the seven patients had recurrent acute pancreatitis.

Discussion

Acute biliary pancreatitis is a serious complication of biliary calculus disease and is associated with significant morbidity and mortality. The incidence of biliary pancreatitis varies with place and the patient population. In Hong Kong, biliary causes constitute a common origin and have been reported to be responsible for 50% or more of all cases of acute

pancreatitis [9, 10]. The differentiation of biliary pancreatitis from other causes is considered important, as management strategies differ significantly. It appears that patients with acute biliary pancreatitis are older than patients with other causes. In our patient population, the patients with acute biliary pancreatitis belonged to an older age group (median 69 years) than those with pancreatitis due to nonbiliary causes (median 52 years) and only about 10% of the latter were older than 70. Although the age of patients (70 years) is not a sensitive test to differentiate a biliary cause from a nonbiliary cause (sensitivity 9%), it has a high specificity of 90%. Various biochemical data have been suggested as valuable predictive tools for acute biliary pancreatitis, including serum bilirubin levels [11], aspartate aminotransferase [12], and a combination of ALP, total bilirubin, and alanine aminotransferase [13]. In our patients the serum total bilirubin level appeared to be the most sensitive single biochemical parameter for acute biliary pancreatitis, with positive and negative predictive values of 81 and 48%, respectively (sensitivity 84%; specificity 42%). However, because there are conflicting data in the literature, it appears that no biochemical parameter can currently be considered a reliable indicator of acute biliary pancreatitis. Ultrasonography has frequently been used in patients with acute biliary pancreatitis and was found to be accurate in detecting gallstones in 92% of cases [14].

Repeating ultrasonographic examination at a later time may be more rewarding to improve visualization of the gallbladder. In our settings, early bedside ultrasonographic examination was performed soon after admission for all patients. This has been an important part of initial investigations, as prompt treatment, including emergency ERCP and ES, could be performed without delay. In the present study, ultrasonography had a sensitivity of 94% for detecting gallstones. It has not been accurate for predicting CBD stones though, so it cannot be relied on as the sole indicator for performing ERCP. The major difficulty with ultrasonography to predict biliary pancreatitis was in patients with previous cholecystectomy and patients with primary stones in the bile duct where gallbladder calculi could not be detected. Stratifying patients according to the severity of pancreatitis may be helpful when directing treatment, as those with severe disease could be selected and may benefit from intensive emergency treatment. Several clinical scoring systems are available to predict the severity of the attack, including those adopted by Ranson and Imrie [14 and 15]. However, the multiple laboratory criteria have the disadvantage that assessment of severity requires 48 h or longer for completion. Moreover, some of the parameters in the scoring system could be influenced by the treatment given during the 48-h period, including ERCP in patients with biliary pancreatitis. On the other hand, prediction of the severity of pancreatitis using glucose and urea levels on admission has the advantage of simplicity and ready availability soon after admission [16]. As indicated in this study, the morbidity and mortality of patients with severe disease predicted by either the Ranson scoring system or the glucose and urea criteria and who underwent emergency ERCP were minimal. The role and timing of ERCP in the management of acute biliary pancreatitis has been the focus of discussion in recent literature. In the absence of convincing evidence, ERCP was performed electively at 6 weeks or more after the acute attack to avoid aggravating the acute pancreatitis and introducing infection [17]. However, it has become clear that ERCP can be safely performed without adverse consequences soon after the onset of acute pancreatitis even within the first 12 h [18].

Stone et al. [19] showed a reduction in mortality rate of acute biliary pancreatitis from 6 to 3% with emergency transduodenal sphincteroplasty within 73 h of admission. Emergency ERCP with ES has a similar effect of relieving ampullary obstruction without inflicting major surgical trauma on these sick patients. In an animal model, the severity of acute biliary pancreatitis has been shown to depend on the duration of ductal obstruction, and that decompression of the ductal system can prevent progression of the disease [20]. These findings supported the contention that intervention with endoscopic removal of obstructing stones should be performed as early as possible. The first randomized controlled trial that clarified the role of urgent ERCP and ES came from Alexakis N. In that study, 121 patients with acute pancreatitis thought to be due to gallstones were randomized for treatment with urgent ERCP and ES within 72 h of presentation or for conventional treatment. Patients with predicted severe disease treated with urgent ERCP had a significantly shorter hospital stay (median 9.5 vs. 17.0 days) and fever complications (24 vs. 60%). However, in that study the role of ES in patients with ampullary or persistent CBD stones was not confirmed because they were not separately analyzed. It is also not certain if an even earlier intervention is more beneficial, or if a procedure deferred until patients deteriorate is efficacious. To evaluate the latter issues, Fan et al. [8], from Hong Kong, studied 195 consecutive patients with early pancreatitis who were randomized to undergo ERCP with or without ES within the first 24 h of presentation or initial conservative treatment followed by endoscopic examination if the patient's condition deteriorated. In the group of patients with CBD or ampullary stones the overall morbidity was indeed reduced (the major impaction improvement was the reduction of biliary sepsis), but the local and systemic complications were not significantly reduced. In the same study [8], selective ERCP was shown to be ineffective in salvaging patients who deteriorated while on conservative treatment. Some authors argue that early ERCP and ES result in unnecessary endoscopic intervention in some patients in whom the CBD stones will pass spontaneously. However, biliary sepsis may occur in these patients after admission, leading to rapid deterioration and significant morbidity and mortality. Therefore, early ERCP and ES is warranted to guard against possible acute cholangitis even for patients with mild pancreatitis. In the present study, no patient developed biliary sepsis after urgent or early endoscopic intervention, although at least 16% had evidence of acute cholangitis on admission. The procedure was found to be safe and effective. ERCP was successful in 95% of the patients, and stone clearance was successful in all of the 52 patients in whom CBD stones were demonstrated. The policy of early or emergency endoscopic intervention was also associated with a favorable outcome of our patients, with an overall morbidity of 10% and mortality of only 1%. To reduce the likelihood of recurrent acute pancreatitis, open cholecystectomy during the initial admission after the acute manifestation of acute pancreatitis had subsided was recommended [21, 22]. However, LC during the first week of admission has been shown to be associated with an increase in operative complications, an increased rate of conversion, and a longer postoperative stay [23and 24]. In the present study, partly related to the difficulty of arranging a early operative schedule, interval elective LC was performed within 2–12 weeks of the initial presentation. Because all patients had been proved to be free from CBD stones and 69% of all patients had ES, there was minimal risk of interim attacks of pancreatitis. Moreover, an interval of a few weeks to allow the acute inflammation to subside before LC may have beneficial effects on

the operative outcome. There is evidence that ES obviates the need for cholecystectomy in some patients with acute biliary pancreatitis [4, 6]. In the present study, cholecystectomy was not performed in seven patients because they were poor surgical risks. Although one of them had recurrent CBD stones, none had recurrent pancreatitis or other hepatobiliary problems leading to significant morbidity. However, larger patient populations and a longer follow-up are required before any significant conclusion can be reached.

Conclusion

Acute biliary pancreatitis can be managed safely and effectively with a combined endoscopic and laparoscopic approach. Emergency or early ERCP for all patients and ES for identifiable CBD stones are associated with a high success rate and low morbidity. Interval LC is a safe, effective approach with a low conversion rate and low preoperative morbidity. Our experience suggests that early ERCP and interval LC are the preferred approach for management of acute biliary pancreatitis.

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Indication for Surgery in Acute Necrotizing Pancreatitis

9

J. Werner

Introduction

The management of acute pancreatitis has been controversial for more than 100 years, varying between a conservative medical approach on the one hand and a surgical approach on the other. There has been great improvement in knowledge of the natural course of and the functional changes that accompany acute pancreatitis over the past 20 years. The clinical course of acute pancreatitis varies from a mild transitory form to a severe necrotizing disease. Severe pancreatitis is associated with organ failure and/or local complications such as necrosis, abscess formation, or pseudocysts [1].

Severe pancreatitis can be observed in 15–20% of all cases. The first 2 weeks after onset of symptoms are characterized by the systemic inflammatory response syndrome. In parallel, pancreatic necrosis develops to its full extent within the first 4 days after the onset of symptoms, while infection of necrotic pancreatic tissue develops most frequently in the second and third weeks [2]. Infection of necrotic pancreatic tissue is still the major risk factor of sepsis-related multiple organ failure and the main life-threatening complication in the later phase of severe acute pancreatitis [3–5]. Pancreatic infection correlates with the duration of the disease, and up to 70% of all patients with necrotizing disease present with infected pancreatic necrosis 4 weeks after the onset of the disease [2]. Moreover, the risk of infection increases with the extent of intrapancreatic and extrapancreatic necrosis [2, 3].

Treatment of acute pancreatitis in its early phase is solely supportive. Since development of infection of necrotic pancreatic tissue is the main determinant of morbidity and mortality in the late phase of severe pancreatitis, prophylactic antibiotic treatment should be applied. Evidence for the effectiveness of prophylactic antibiotics in the treatment of necrotizing pancreatitis has been given by various randomized controlled trials, and also by several meta-analyses [6].

Since surgery can only be effective when a septic focus can be identified and potentially removed, it is essential to differentiate between sterile and infected necrosis once pancreatic necrosis has developed. Infection of necrotic pancreatic tissue is usually suspected in patients

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who develop clinical signs of sepsis [7]. These patients should undergo CT- or ultrasonography-guided fine-needle aspiration (FNA) of necrotic pancreatic or peripancreatic tissue [5, 7]. FNA is an accurate, safe, and reliable approach to differentiate between sterile and infected necrosis [8]. It is important to note that only those patients who present with clinical signs of sepsis should undergo FNA, since FNA bears a potential risk of secondary infection.

Today, more patients survive the early phase of severe acute pancreatitis owing to improvements in intensive care medicine, thus increasing the risk of later sepsis [5, 9]. While surgical interventions should be applied only in selected cases within the first 2 weeks after the onset of the disease [10], surgery and minimally invasive interventional procedures are important in the treatment options in the later phase of the disease.

Indications for Surgery

Infected Necrosis

Proven infected necrosis as well as septic complications resulting from pancreatic infection are well-accepted indications for surgical treatment [5, 11, 12]. The mortality rate of patients with these indications is higher than 30%, and more than 80% of fatal outcomes in acute pancreatitis are due to septic complications [2, 5, 13]. When patients are treated nonsurgically, mortality rates of up to 100% have been reported for infected necrosis associated with multiple organ failure. With surgical treatment, the mortality rate for patients with infected pancreatic necrosis was decreased to about 10–20% in various specialized centers [5, 12–15].

Sterile Necrosis

A conservative approach is accepted in sterile necrosis as long as the patient responds to therapy [5, 7, 15]. However, when sterile necrosis is associated with organ failure, the role of surgery remains controversial. Some patients with sterile necrosis do not improve despite therapy in the intensive care unit (ICU). Thus, it is generally agreed that persistent or progressive organ complications despite maximal ICU treatment are an indication for surgery in patients with sterile necrosis [7]. However, there is no established uniform definition of when a patient should be considered a “nonresponder” to ICU therapy. In addition, surgery may be indicated in the rare event of rapidly progressive multiple organ failure in the first days of acute pancreatitis despite ICU therapy (“fulminant acute pancreatitis”) [7]. Nevertheless, given the poor outcome with both surgical and conservative therapy and the lack of published data, the optimal therapy for this subset of patients remains unclear.

Best Time Point of Surgery

Today, there is general agreement that surgery in severe pancreatitis should be performed as late as possible [7]. The rationale for late surgery is the ease of identifying well-demarcated necrotic tissue from the viable parenchyma, with the effect of limiting the extent of surgery

to pure debridement. This approach decreases the risk of bleeding and minimizes the surgery-related loss of vital tissue which leads to surgery-induced endocrine and exocrine pancreatic insufficiency.

Mortality rates of up to 65% have been described with early surgery in severe pancreatitis [2, 16], questioning the benefit of surgical intervention within the first few days after onset of symptoms. In the only prospective and randomized clinical trial comparing early (within 48–72 h of symptoms) and late (at least 12 days after onset) debridement in patients with severe pancreatitis, the mortality rates were 56 and 27%, respectively [16]. Although the difference did not reach statistical significance, the trial was terminated because of the evident risk of early surgery. Therefore, only in the case of proven infected necrosis or in the rare cases of severe complications such as massive bleeding or bowel perforation should early surgery be performed [5, 7].

Techniques of Open Pancreatic Necrosectomy

The aim is to control the focus of sepsis. By this, complications are avoided by stopping the progress of infection and the release of proinflammatory mediators. A generally agreed principle of surgical management includes the organ-preserving approach which combines debridement and postoperative removal of retroperitoneal debris and exudates. Four principal methods have been advocated: necrosectomy combined with [1] open packing [17], [2] planned, staged relaparotomies with repeated lavage [12], [3] closed continuous lavage of the lesser sac and retroperitoneum [2], and [4] closed packing [15].

Necrosectomy/Debridement

Necrosectomy has traditionally been undertaken by an open route. A longitudinal midline incision allows the assessment of the entire abdominal cavity, the irrigation of the entire abdomen, and a diverting ileostomy for patients in whom the necrotic process involves the retrocolic area. After the abdominal cavity has been opened, the gastrocolic and the duodenocolic ligaments are divided close to the greater curvature of the stomach, and the pancreas is exposed. Once the focus of necrosis has been exposed, debridement is carried out bluntly. This technique avoids removal of vital tissue and reduces bleeding complications. After all loose debris has been removed, the retroperitoneal cavity is irrigated with several liters of saline solution.

While necrosectomy is performed in a more or less identical fashion, the four techniques differ in the way they provide exit channels for further slough and infected debris. In the hands of experienced surgeons, mortality rates below 15% have been described for all four techniques: [1] open packing [17–20]; [2] repeated laparotomies [12, 14]; [3] closed packing [15]; [4] closed continuous lavage [2, 5, 20–22] (Table 1, open packing).

The cavity is lined with a nonadherent dressing and packed. The patient is returned to the operating room every 48 h for further debridement and repacking until no further necrosis is evident. After several reoperations, debridement may sometimes be performed in sedation in the ICU until healthy granulations appear. Then, the abdomen can be closed over drains, with or without lavage of the cavity [23].

Table 1 Outcome of different techniques for open necrosectomy

Technique	Patients (*n*)	Patients with infected necrotic tissue (%)	Mortality (%)	Relaparotomy (*n*)
Open packing				
Bradley 1993 [1, 11]	71	100	15	1–5/patient
Branum 1998 [18]	50	84	12	2–13/patient
Bosscha 1998 [19]	28	100	39	17 mean/patient
Nieuwenhuijs 2003 [20]	38	–	47	–
Planned relaparotomies				
Sarr 1991 [12]	23	75	17	2 to >5/patient
Tsiotos 1998 [14]	72	79	25	1–7/patient
Closed packing				
Fernandez-del Castillo 1998 [15]	64	56	6	11 (17%)
Closed continuous lavage				
Beger 1988 [21]	95	39	8	26 (27%)
Farkas 1996 [22]	123	100	7	–
Büchler 2000 [5]	29	93	24	6 (22%)
Nieuwenhuijs 2003 [20]	21	–	33	–

Planned, Staged Relaparotomies with Repeated Lavage

Following the primary necrosectomy, planned reoperations for repeated necrosectomies on an every other day basis are performed until all devitalized tissue has been removed, granulation tissue has started to form, and the surgeon is convinced that the necrotizing process is under control. For ease of repetitive surgical access, some surgeons incorporated zippers into the abdominal wall. Finally, the abdomen is closed in a delayed primary fashion over peripancreatic drains [12].

The other two techniques, necrosectomy and subsequent closed continuous lavage of the lesser sac [5, 22] and the closed packing [15], have implied a postoperative method to continuously remove residual necrotic pancreatic tissue.

Continuous Lavage of the Lesser Sac and Retroperitoneum

For closed postoperative local lavage, two or more double-lumen Salem Sump tubes (20–24 French) and single-lumen silicone rubber tubes (28–32 French) are inserted from each side, directed to the left and right, and placed with the tip at the tail of the pancreas, behind the descending colon, the head of the gland, and the ascending colon. The smaller lumen of the Salem drains is used for the inflow of the lavage and the larger lumen is used for the outflow. In addition, evacuation of smaller parts of the necrotic debris can be evacuated over silicone tubes. The gastrocolic and duodenocolic ligaments are reapproximated to

create a closed retroperitoneal space for postoperative lavage. Thirty-five to 40 L of lavage fluid (standard peritoneal dialysis fluid) is used in the first few days. Subsequently, the lavage volume can be reduced depending on the appearance of the effluent and the clinical course. Drains can be removed within the next 2–3 weeks [5, 21].

Closed Packing

This technique follows the same principle as the continuous postoperative lavage as it also ensures a continuing easy egress of residual necrotic material postoperatively. After the necrotic tissue has been removed and the cavity irrigated with saline, the residual cavity is filled with multiple, large, gauze-filled Penrose drains as well as closed-suction drains. This also packs the abscess cavity and by this controls minor bleedings. All drains must be brought out laterally to ease drainage. Drains can be removed successively after a minimum of 7 days of continuous drainage. At the same time, the gauze packing must be gradually removed, which results in a slow collapse of the cavity [15].

Discussion

Although the incidence of recurrent intra-abdominal sepsis decreased significantly compared with that in a single necrosectomy, the postoperative morbidity remained high with the open packing and the planned, staged relaparotomies with repeated lavage techniques (Tables 1, 2). Both methods have in common that they mandate several relaparotomies before final closure of the abdomen. There is a positive correlation between repeated surgical interventions and morbidity, including gastrointestinal fistula, stomach outlet stenosis, incisional hernia, and local bleeding. Especially the number of pancreatic and colonic fistulas was significantly higher compared with the number in necrosectomy with subsequent cleavage of the necrotic debris by closed continuous lavage or closed packing [5, 12, 15, 23] (Table 2). Thus, both techniques are particularly useful if the intervention has to be performed early in the course of necrotizing pancreatitis before full demarcation of the necrotic pancreatic tissue occurs. Thus, these two procedures should be considered in the rare case when early debridement is indicated [7].

In contrast to these two techniques, both closed continuous lavage and closed packing have implied a postoperative method to continuously remove residual necrotic pancreatic tissue [7]. Consequently, relaparotomies are frequently not necessary. By this, postoperative morbidity, especially the incidence of gastrointestinal fistulas and incisional hernias, is reduced (Tables 1, 2). The results of these two surgical strategies with regard to morbidity, relaparotomies, and mortality are comparable and thus dependent on the preference of the surgeon. At our institution, we routinely use the continuous lavage of the lesser sac and a single surgical approach was successful in 83% of cases. Today, necrosectomy and subsequent closed continuous lavage of the lesser sac is the most commonly applied approach [7, 10].

The differing success rates reported by groups using apparently similar approaches illustrate the difficulties in comparing these techniques (Tables 1, 2). Most techniques are associated

Table 2 Complications of different techniques for open necrosectomy

Technique	Patients (*n*)	Fistulas (%)	Hemorrhage (%)
Open packing			
Bradley 1993 [1, 11]	71	46	7
Branum 1998 [18]	50	88	–
Bosscha 1998 [19]	28	25	50
Planned relaparotomies			
Sarr 1991 [12]	23	79	26
Tsiotos 1998 [14]	72	46	18
Closed packing			
Fernandez-del Castillo 1998 [15]	64	69	3
Closed continuous lavage			
Farkas 1996 [22]	123	14	2
Büchler 2001 [5]	42	19	5

with an average mortality between 10 and 20%. However, the mortality in patients with established multiple organ failure is even higher [19]. In the absence of randomized trials, it is impossible to determine the hidden effects of factors such as referral pattern, patient selection, comorbidity of patients, presurgical percutaneous management, and indication for surgery within the literature.

The high mortality in patients with infected pancreatic necrosis despite surgery has led to the development of several minimally invasive techniques, including radiological, endoscopic, and minimally invasive surgery, as alternative procedures [10]. Proponents of using minimally invasive technologies in this clinical setting cite a desire to minimize the physiological insult in patients who are already critically ill [24, 25]. However, no data exist to clearly demonstrate that minimally invasive procedures are less prone to morbidity than open surgery. Safe retroperitoneal access and necrosectomy is possible in some but not all patients depending on the size and localization of the infectious foci. No randomized studies exist comparing one management technique with the other. All reports on minimally invasive surgery involved only small numbers of patients, involved retrospective analysis, and involved selected patients with an enormous variation of comorbidities and disease severity. In the absence of well-designed clinical trials, we must be cautious in the application of new technologies. Thus, today, outside of clinical trials, minimally invasive surgery should be limited to specific indications and to those patients who are critically ill and otherwise unfit for conventional surgery.

Today, open surgical debridement is the “gold standard” for treatment of infected pancreatic and peripancreatic necrosis. Necrosectomy and subsequent closed continuous lavage of the lesser sac is the technique with the lowest morbidity. Consequently, it is the most commonly adopted technique to continuously remove residual necrotic pancreatic tissue postoperatively.

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