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Molecular Mechanisms of Parenchymal Injury and the Role of Duct Obstruction and Ductal Hypertension in the Pathogenesis of Chronic Pancreatitis

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Table of contents

List of Abbreviations……...5

1. Introduction…….....7

1. 1 Definition of chronic pancreatitis(CP) …….. ….7

1. 2 Etiology of chronic pancreatitis……..…….. 7

1. 3 Theories of chronic pancreatitis pathogenesis……..... 8

1. 4 New developments……... 9

1. 5 Pancreatic stellate cells……..……. 11

1. 6 Cellular immune response in chronic pancreatitis…….. 11

1. 7 Morphology of chronic pancreatitis……12

2. Parenchymal Regression in Chronic Pancreatitis Spares Islets Reprogrammed for Expression of NFκB and IAPs…… 13

2. 1 Introduction and background…….. 13

2. 2 TRAIL receptor profile of stimulated CM corresponds to islets in CP….. 14

2. 3 TRAIL modulates NFκB subunit expression in CM…… 15

2. 4 RelA Transcriptional Activity in CM……16

2. 5 General aspects in epithelia of normal pancreas and CP……..17

2. 6 Summary and conclusion…….. 20

3. Survivin expression in pancreatic intraepithelial neoplasia…….. 21

3. 1 Introduction and background……. 21

3. 2 Survivin transcripts increase in PanINs and PDA…….. 22

3. 3 Survivin expression in PanINs, PDA, and Nodal Metastases by IHC….22

3. 4 Summary and conclusion……... 23

4. Evidence of Notch activation in ectatic ducts of chronic pancreatitis…. 24

4. 1 Introduction and background…… 24

4. 2 Expression profiles indicate a differential regulation of Notch in ectatic ducts……... 26

4. 3 Validation of microarray results…….. 26

4. 4 Notch component alterations in pressure stimulated HPDE……27

4. 5 Immunohistochemistry…….. 27

4. 6 Summary and conclusion……. 28

General summary and conclusions……29

References……30

Publications……..37

Acknowledgements.....38

Curriculum vitae.....39

Published papers

Abbreviations

ADM	acino-ductal metaplasia
AP1	activator protein1
BIR	baculoviral IAP repeat
cDNA	complementary DNA
CIS	carcinoma-in-situ
CP	chronic pancreatitis
Dll	delta like
DNA	deoxyribonucleic acid
ECM	extracellular matrix
ELISA	enzyme linked immunosorbent assay
FACS	fluorescence activated cell sorting
HERP	HES-related repressor protein
HES	hairy/enhancer of split
HLH	helix loop helix
HPDE	human pancreatic ductal epithelial cells
IAP	inhibitor of apoptosis protein
IFN	interferon
IKK	I Kappa-B Kinase
IPMT	intraductal papillary mucinous tumor
MHC	major histocompatibility complex
min	minute(s)

mRNA	messenger RNA
NF-kB	nuclear factor kappa-B
NICD	Notch intracellular domain
NP	normal pancreas
PDAC	pancreatic ductal adenocarcinoma
PDH	pancreatic ductal hypertension
PSCs	pancreatic stellate cells
PanIN	pancreatic intraepithelial neoplasia
PCR	polymerase chain reaction
RNA	ribonucleic acid
TGF-β	tissue growth factor- beta
TNF	tumor necrosis factor
TRAIL	TNF related apoptosis inducing ligand
TRAIL- R	TRAIL-receptor

1. Introduction

1. 1 Definition of chronic pancreatitis (CP)

Chronic pancreatitis (CP), is a progressive, destructive, inflammatory process of multifactorial etiology that leads to irreversible obliteration of exocrine and endocrine pancreas and its replacement by fibrous tissue that results in the clinical manifestations typical of an „end-stage" disorder of pancreatic function. The disease has a bleak long term outlook overall.

1. 2 Etiology of CP

In developed countries, 60-70% of the patients with CP have a long history of heavy alcohol consumption. Less common etiology of chronic pancreatitis includes autoimmune disease, hypertriglyceridemia, hyperparathyroidism, tropical pancreatitis, pancreas divisum, obstruction of pancreatic duct by tumour, and genetic abnormalities [1]. However, none of these risk factors consistently lead to chronic pancreatitis. For example, alcohol is used by far greater number of people than those who actually go on to develop chronic pancreatitis thereby suggesting a variable genetic susceptibility. Chronic pancreatitis is now regarded as a multifactorial disorder where multiple risk factors operate together in the causation of the disease [2]. This is an important conceptual change in the understanding of chronic pancreatitis. In the TIGAR-O system, major predisposing risk factors for chronic pancreatitis have been categorized as toxic-metabolic(T), idiopathic(I), genetic(G), autoimmune(A), recurrent acute pancreatitis(R), or obstructive(0) [2,3].

1. 3 Theories of CP pathogenesis

In the past decades, four major theories have emerged to explain the pathogenesis of chronic pancreatitis [4] .

1. *Oxidative stress.* Alcohol-induced oxidative stress may generate free radicals in acinar cells, leading to membrane lipid oxidation and the activation of transcription factors, including AP1 and NFκB, which, in turn, induce the expression of chemokines that attract mononuclear cells. Oxidative stress thereby promotes the fusion of lysosomes and zymogen granules, acinar cell necrosis, inflammation, and fibrosis.

2. *Toxic-metabolic.* Toxins, including alcohol and its metabolites, can exert a direct toxic effect on acinar cells. This may lead to the accumulation of lipids in acinar cells, acinar cell loss, and eventually parenchymal fibrosis.

3. *Ductal obstruction by concretions.* Some of the inciting agents responsible for the development of chronic pancreatitis, such as alcohol, are believed to increase protein concentrations in the pancreatic juice. These proteins form ductal plugs that are observed in most forms of chronic pancreatitis but are particularly prominent in alcoholic chronic pancreatitis. The ductal plugs may calcify, forming calculi composed of calcium carbonate precipitates, and these calculi can further obstruct the pancreatic ducts and contribute to the development of chronic pancreatitis.

4. *Necrosis-fibrosis.* Acute pancreatitis results from autodigestion of pancreatic tissue by inappropriate activation of pancreatic enzymes and the subsequent immune responses as evidenced by the inflammatory infiltrates. It has been proposed that acute pancreatitis initiates a sequence of perilobular fibrosis, duct distortion, and altered pancreatic secretions. Over time and with multiple episodes, this can lead to loss of pancreatic parenchyma and fibrosis [4].

1. 4 New developments

New concepts integrating the cellular, genetic and molecular mechanisms have been put forward to explain pathogenesis of CP. One of them is, the Sentinal Acute Pancreatitis Event (SAPE), according to which unregulated trypsin activation initiates the first episode of acute pancreatitis (sentinal event). Trypsin activation and inactivation are regulated by $Ca2^{+}$. Stimulation with cholecystokinin evoked a sustained rise in $Ca2^{+}$, induced pronounced trypsin activation and extensive vacuole formation in the apical region of acinar cells [5]. Furthermore, bile acids and non-oxidative alcohol metabolites can elicit abnormal cytosolic $Ca2^{+}$ signals that result in necrosis and acute pancreatitis [6]. Cytokines liberated during the early inflammatory phase attract a distinct cellular infiltrate while profibrotic cells including stellate cells constitute the late phase of acute pancreatitis. The attraction and activation of pancreatic stellate cells (PSCs) sets the stage for the development of pancreatic fibrosis. The resultant scarring leads to the development of strictures that block the pancreatic ducts which in turn predisposes to recurrent attacks of acute pancreatitis. These observations along with the recently discovered genetic

mechanism of hereditary pancreatitis [7,8] and the pathologic case series [9], lend credence for a close relationship between acute pancreatitis, recurrent acute pancreatitis and the development of chronic pancreatitis. In a significant number (10-30%) of patients, chronic pancreatitis is not associated with any of the known processes and therefore termed idiopathic. There is accumulating evidence that many of these cases have a genetic basis. This led the idiopathic category to shrink in recent years. Genetic alterations associated with chronic pancreatitis are briefly summarized here.

PRSS1

Whitcomb and colleagues identified the third exon of the cationic trypsinogen gene on chromosome 7q35. Hereditary pancreatitis is caused by germ line mutations in the cationic trypsinogen gene also known as PRSS1 (protease, serine1). The most common PRSS1 mutation results in an arginine to histidine substitution, thereby eliminating the key site essential for the rapid self-destruction of trypsin in solutions. Therefore, trypsin becomes resistant to inactivation and the abnormally active trypsin results in development of acute pancreatitis and recurrence can lead to chronic pancreatitis [8].

SPINK-1

The serine protease inhibitor Kazal type1 (SPINK-1), as the name suggests, inhibits trypsin activity. The mutation of *SPINK1* gene causing loss of its function predisposes to increased risk of recurrent acute and chronic pancreatitis [10]. *CFTR*

Cystic fibrosis is caused by mutations in the cystic fibrosis transmembrane conductance regulator *(CFTR)* gene. CFTR is a key molecule expressed in pancreatic ducts. Some *CFTR* gene mutations may be pancreas specific. CFTR mutations associated with loss/decrease of bicarbonate secretion cause recurrent acute and chronic pancreatitis [11].

The concept of SAPE thus incorporates the molecular mechanism of pathogenesis while unifying previous theories [2] implying that CP is the results of recurrent and/or sustained immune activation that takes the cytotoxic injury to the acinar cells. This immune activation is to be followed by stimulation of anti-inflammatory responses that drive fibrosis where pancreatic stellate cells play a pivotal role.

1. 5 Pancreatic stellate cells (PSCs)

The identification and characterization of pancreatic stellate cells has initiated numerous studies in recent years focused on determination of their exact role in pancreatic fibrogenesis [12-15]. In their quiescent state, pancreatic stellate cells are periacinar in location and store vitamin A. On activation through pro- and antiinflammatory cytokines, they assume a myofibroblast-like phenotype [16]. This makes them capable of numerous biological functions like proliferation, migration, synthesis and secretion of extracellular matrix components as well as synthesis and secretion of matrix-degrading enzymes and their inhibitors. Therefore, pancreatic stellate cells possess the dual capacity of synthesizing as well as degrading ECM components thus implying their important role in the maintenance of the normal architecture of the healthy pancreas [17,18].

1.6 Cellular immune response in chronic pancreatitis

One area of major interest in chronic pancreatitis is the cellular response to injury. There are significant portions of two major T cell subsets as well as NK cells and macrophages in the cellular infiltrates of chronic pancreatitis [19]. In the male Wistar rat model of spontaneously developing chronic pancreatitis, CD8+ cytotoxic T cells invade pancreatic lobules and form close associations with acinar cells, some of which demonstrated apoptosis [20]. This provides circumstantial evidence for an involvement of autoreactive T cells in the parenchymal destruction in CP.

1. 7 Morphology of CP

Morphologically, CP is characterized by: 1. parenchymal fibrosis; 2. acinar atrophy with relative sparing of endocrine islets, atrophic acini undergo morphological alteration imparting them a tubular morphology described as acino-ductal metaplasia (ADM), and, 3. ductal changes are observed in the form of varying degrees of dilatation, distortion and presence of protein plugs in their lumina. The epithelial lining of dilated ducts exhibits atrophy or hyperplasia or squamous metaplasia. Chronic inflammatory infiltrates are seen around lobules and ducts. Recently described precursors of pancreatic cancer, the pancreatic intraepithelial neoplasia (PanIN) lesions are often found in CP tissues as incidental findings. Chronic pancreatitis is increasingly being perceived as a precancerous condition.

2. Parenchymal Regression in Chronic Pancreatitis Spares Islets Reprogrammed for Expression of NFκB and IAPs

2. 1 Introduction and background

Chronic pancreatitis (CP) is characterized by persistent inflammation of the pancreas leading to irreversible destruction of the exocrine parenchyma. Remarkably, the endocrine islets remain structurally and functionally intact for a prolonged period [21]. Eventually, in late stages of chronic pancreatitis, islets are destroyed as well, resulting in diabetes [22-24]. It is intriguing that the two, intimately assembled, epithelial compartments of pancreas - exocrine and exocrine, respond differently to the exact same injurious stimuli in their microenvironment, which eventually determine their fates during this inflammatory process viz. cell death or survival. There are no straightforward explanations for this phenomenon.

It is now well accepted that apoptosis contributes in part to parenchymal destruction during the course of CP. It was previously shown that, normal pancreatic islets express the CD95L(FasL) but do not express its receptor CD95(Fas) [23]. In chronic pancreatitis, this “immunoprivileged” status is preserved in endocrine cells, whereas the exocrine epithelia lose this status, neoexpress CD95 along with MHC-II [25] and are therefore rendered sensitive targets for activated T lymphocytes equipped with CD95L in the adjacent inflammatory infiltrate. Thus, the binding of CD95L on lymphocytes with CD95 induced on the surface of acinar cells under the influence of IFN-γ proves lethal for acinar cells while islet cells secure themselves by conserving their $CD95L^{+}$ status [23].

Further, Hasel et al, also found that, normal pancreas (NP) is devoid of TRAIL { tumor necrosis factor (TNF) related apoptosis inducing ligand} and TRAIL receptors(Rs). In CP, there is a strong induction of the death signal transducing TRAIL-R1, and -R2 in exocrine cells. In contrast, islet cells are essentially devoid of TRAIL-R1 and -R2 but are strongly TRAIL-R4 positive. TRAIL produced locally by activated pancreatic stellate cells binds to TRAIL-R1 and TRAIL-R2 on acinar cells inducing their apoptosis [24]. On the other hand TRAIL-R4 expressing islets continue to remain relatively intact. A logical question thus arises about the impact of TRAIL binding with TRAIL-R4. Recent studies carried out in T-lymphocytes hint at an alternative functional role of TRAIL-R4. TRAIL-R4 does not transmit a direct death signal but increasing evidence suggests that on binding with TRAIL, it activates NFκB [26]. We asked the question whether TRAIL-R4 expression activates NFκB in islets in the midst of progressive fibrosis and potentially offer them a survival advantage? In the present work, we attempted to define the mechanism by which endocrine islets manage to survive the hostile microenvironment of CP, which proves lethal for the exocrine pancreas. A clear understanding of the mechanism enabling islet cell survival in chronic pancreatitis could be exploited to improve the survival of islet cell grafts during transplantation for the treatment of diabetes. Vice versa, these approaches could also serve to retard acinar destruction thereby help preserve exocrine function.

2. 2 *TRAIL receptor profile of stimulated CM cells corresponds to islets in CP*

With the background that islet cells in CP are TRAIL-R1^{-}, TRAIL-R2^{-} , and TRAIL-R4^{+}, we tested human insulinoma cell line CM as a surrogate for islets, for TRAIL receptor surface expression. CM cells were constitutively devoid of TRAIL-R1, -R2, -R3, and -R4, which is a phenotype corresponding to that of islet cells of NP. Interestingly, the TRAIL-R profile of CM upon stimulation with TGFβ (24 hrs), IFNγ (18 hrs), and TRAIL (6 hrs) mimicked that of islets in CP, in being TRAIL-R1^{-}, TRAIL-R2^{-} , and TRAIL-R4^{+} in a minor but significant subset.

2. 3 *TRAIL modulates NFκB subunit expression in CM*

Next, CM cells were stimulated with TRAIL and IFNγ separately, and TRAIL+ IFNγ in combination for 48 hrs to determine the mRNA expression of NFκB subunits and NFκB targets IκBα and survivin.

The combination of TRAIL and IFNγ brought about a 4-fold rise in NFκB1 mRNA levels. On the other hand, mRNAs of transcriptionally active NFκB subunits RelA, RelB, and c-Rel were between 2 to 9.5 fold increased compared to untreated cells on TRAIL+IFNγ stimulation. Additionally, mRNA of NFκB target IκBα was down-modulated while survivin message increased considerably.Thus, these findings suggest that TRAIL-R4 acts as a signal transducer in modulating NFκB subunits in CM. Moreover, CM did not show sensitivity to TRAIL mediated apoptosis, as determined by quantifying the subG1-fraction in FACS, nor did the amount of insulin secretion change significantly under TRAIL treatment.

2. 4 *RelA Transcriptional Activity in CM*

The DNA binding activity of RelA of NFκB family of transcription factors in CM cells as determined by an ELISA based assay increased significantly following 48 hrs stimulation with IFNγ (20-fold rise) and TRAIL+IFNγ (10-fold) in comparison with untreated control.

2. 5 *General aspects in epithelia of NP and CP*

To be objective, we morphometrically estimated the relative proportion of stromal fibrosis and the epithelial compartment in each specimen and defined three grades of fibrosis in CP, i.e., mild fibrosis, 12.4 ± 1.8%; moderate, 41.2 ± 0.6%; and severe fibrosis, 73.9 ± 6%. Stroma constituted 6.3 ± 1.9% of NP.

Turning to analysis of pancreatic tissues, NP had low mRNA levels of NFκB subunits. These were increased in CP in parenchymal areas with fibrosis and most intensely in islets. Expression of NFκB regulated proteins IκBα, survivin, and cIAP1 was found in corresponding sites, again at their highest levels in islets surrounded by fibrosis. Specimens from patients with CP were markedly microheterogenous with respect to each aspect aimed at in this study. As observed in the pilot phase of this study, this heterogeneity seemed to be paralleled not by the degree of inflammatory lymphohistiocytic infiltration but by the degree of fibrosis. Even within the same section the relative amount of antigen detected was most pronounced in fibrotic areas, the intensity being markedly lower in relatively normal unaffected areas.

Cryosections were subjected to laser capture microdissection to purify components from each epithelial compartment of NP and CP with various grades of fibrosis in order to analyze and compare the mRNA expression levels of NFκB subunits by real time PCR. Corresponding tissue sections were subjected to immunohistochemistry (IHC) to detect proteins for which reliable antibodies were available. Next, we correlated the mRNA levels by real time PCR and the staining intensities by IHC with the extent of fibrosis. We found that NP was largely devoid of expression of NFκB constituents, and targets survivin, and cIAP1. Their message and/or protein levels in pancreatic epithelial compartments increased with the degree of fibrosis. At the extreme end of the disease spectrum i.e., CP with severe fibrosis, mRNA and/or protein was detected in high amounts, especially in islets spared in the midst of fibrosis.

RelA

In severe fibrosis, there was a sharp increase in RelA transcripts in islet cells but not in acini and ducts. Normal acini, ductal epithelium and islets showed no expression of RelA protein. In CP, RelA protein was detectable, the intensity of staining increased with progressive severity of fibrosis. Staining was most pronounced in the acini and islets in areas of severe fibrosis. In both, the acinar and islet cells the staining was cytoplasmic and nuclear. The ductal epithelium showed either no or focal, low expression of RelA. In CP with severe fibrosis characterized by exocrine parenchymal regression, the acini in fibrotic areas featuring a tubular morphology exhibited strong cytoplasmic and nuclear positivity. Pancreatic islets which were

intact within severely fibrotic areas stained most intensely for RelA, staining being cytoplasmic as well as nuclear.

NFκB1(p105)

There was a sharp, about 10-fold, increase in NFκB1 (p105) transcript numbers in islet cells in areas of severe fibrosis. As was the case for NFκB1(p100) no protein data are available for NFκB2(P100), RelB, and c-Rel due to the lack of a reliable antibody.

NFκB2(p100)

A similar pattern was observed in NFκB2(p100) mRNA levels with the exception of acinar cells showing 4.6 and 69-fold increase in moderate and severe fibrosis respectively. Islets showed nearly 24-fold amount of message in areas of severe fibrosis.

RelB

RelB mRNA was overexpressed in islet cells in mild, and even more so, in severe fibrosis.

c-Rel

In acini and ducts there was a slight increase in c-Rel mRNA with higher degrees of fibrosis. This enhancement was by far exceeded by the extensive, 58-fold overexpression of c-Rel in islets, occurring in conjunction with severe fibrosis.

IKKγ

In NP, IKKγ protein expression was restricted to some scattered acinar cells.

In CP, an induction of IKKγ was observed in the exocrine and endocrine epithelium paralleled by the degree of fibrosis. In specimens showing only mild fibrosis, IKKγ expression was restricted to islets; all pancreatic epithelia showed moderate to high immunoreactivity for IKKγ in areas with moderate fibrosis. In severe fibrosis, within the remaining endocrine epithelium IKKγ expression was at mild to moderate levels with lower levels in ducts and acini.

IκBα

The IKKγ target, IκBα, was undetectable in NP and in CP with mild and moderate fibrosis. In contrast, tissues affected with severe fibrosis express IκBα, this being more pronounced in islets and the surrounding acinar epithelium.

Against the constitutive paucity of RelA transcripts and low activators IKKγ and IκBα protein levels in NP, CP was characterised by fibrosis-related neoexpression and/or partial increase of mainly IKKγ and RelA, and less so of IκBα. The question thus arose as to whether RelA shown to be translocated to the nucleus by IHC, contributes to prolonged pancreatic islet cells survival in the context of CP by inducing anti-apoptotic factors, e.g., IAPs such as survivin and cIAP1.

Survivin

In NP, survivin protein was absent or at low levels in ductal, acinar, and islet cells. In

CP, the intensity of survivin expression increased with the extent of fibrosis. In CP cases with severe fibrosis, the intact islets exhibited intense cytoplasmic and nuclear staining. The residual acini and the tubular acini also stained strongly for survivin. This pattern closely resembles that of IKKγ, IκBα, and RelA immunoreactivity.

cIAP1

The staining pattern for cIAP1 mostly parallels that for RelA and survivin. While NP did not stain for cIAP1, the staining could be discerned in acini, ducts and pancreatic islets in a perinuclear cytoplasmic location in CP with mild fibrosis, becoming more pronounced with increasing fibrosis. In CP with severe fibrosis, the intact islets exhibit an intense cytoplasmic and nuclear pattern. The acini spared by the fibrotic process and the tubular acini also stained strongly for cIAP1.

2. 6 *Summary and conclusion*

To summarize, we have shown that normal pancreas is characterized by low expression levels of NFκB subunit transcripts in both exocrine and endocrine compartments. In CP, we found an induction of IKK-γ and RelA protein with maximum levels in islets. This increase in islets is strongly correlated with the degree of fibrosis. We further demonstrated a fibrosis-associated increase in mRNA for NFκB1, NFκB2, RelA, RelB, and c-Rel in islet cells while the only transcript that exhibits an increase in acini are those of NFκB2. TRAIL, via TRAIL-R4 broadly expressed in chronic pancreatitis, may be one, if not the major factor bringing about the above scenario as shown by functional experiments with CM cells

3. Survivin expression in pancreatic intraepithelial neoplasia (PanIN)

3. 1 Introduction and background

Chronic pancreatitis (CP) is believed to increase the risk of development of pancreatic cancer and in that respect considered a precancerous condition. Recently described pancreatic cancer precursors, the so called pancreatic intraepithelial neoplasia (PanINs) are often observed in the CP tissues. Tissue homeostasis is maintained through a balance between cell proliferation and apoptotic cell death. Recent evidence suggests that alterations in cell survival pathways contribute to tumor initiation and progression [27]. Aberrantly prolonged cell survival due to apoptosis suppression may contribute to carcinogenesis and carcinoma progression by providing a fertile soil for accumulation of genetic mutations and promoting resistance to immune-based cytotoxicity [27]. The inhibitor of apoptosis proteins (IAPs) are a family of proteins characterized by domain/s of ~70 aa termed the baculoviral IAP repeat (BIR). IAPs specifically inhibit caspases 3, 7, and 9 and thereby prevent apoptosis [28].**.** Survivin is a structurally unique member of the IAPs family because it contains only a single BIR domain and lacks a carboxy-terminal RING finger [29]. Most terminally differentiated normal adult tissues including normal pancreas either do not express survivin or express it at low levels [29, 30]. Survivin is dramatically overexpressed in most human cancers [29, 31] and is expressed in the majority of pancreatic adenocarcinomas [30,32-34]. Survivin immunoreactivity has been studied in the transition from intraductal papillary

mucinous tumor (IPMT) adenoma to IPMT CIS [35]. Therefore, we decided to investigate survivin along the PanIN progression to PDA.

We determined survivin mRNA expression in microdissected, normal pancreatic ducts, PanIN lesions, and PDA. Further, we detected survivin protein in PanINs and compared it to that in pancreatic ducts of NP on the one hand and PDA on the other.

3. 2 *Survivin transcripts increase in PanINs and PDA*

Survivin mRNA expression in microdissected NP ducts, PanINs, and pancreatic ductal adenocarcinoma (PDA) by real-time PCR exhibited a close to exponential rise from very low levels in normal ducts to peak levels in PDA. Survivin transcript expression increased in low grade lesions to 2.7×(± 0.07) in PanIN1A and 4.8×(± 0.15) in PanIN1B. A further increase in survivin transcript levels was noted in high grade lesions, PanIN2 -10× (± 0.14) and PanIN3 -14×(± 0.25) compared to normal ducts. Microdissected PDA expressed the highest survivin mRNA levels with a remarkable 22×(± 0.30) increase compared to normal ducts.

3. 3 *Survivin expression in PanINs, PDA, and lymph node metastases by IHC*

The pancreatic ducts in NP exhibit no or weak expression of survivin protein. In PanINs-1A, survivin expression was detected at low levels. PanINs-1B exhibited diffuse cytoplasmic labelling at moderate levels. PanINs-2 and PanINs-3 on the other hand exhibited high antigen density. Nuclear labelling was conspicuous in some scattered cells in the high grade lesions (PanINs-2 and 3) beginning first at PanIN 2 stage. All the infiltrating PDA in our study exhibited very high survivin

immunolabeling but, in contrast, their associated normal pancreatic ducts were negative. Survivin immunolabeling was detected in the cytoplasm as well as nucleus in a variable number of tumor cells. Furthermore, mitotic figures in PDA were strongly survivin positive with a clear-cut delineation of the chromosomes. It was also evident that survivin immunolabeling was stronger in the tumor cells at the advancing front of the tumor in comparison to those within the tumor. Further, lymph node metastases of PDA revealed intense cytoplasmic labeling. Occasionally, nuclear staining was observed in our series. Remarkably, survivin staining was intense in lymphatic invasion.

3. 4 *Summary and conclusion*

In summary, the expression of survivin message and protein gradually increased from NP ducts to PanINs, and from PanINs to PDA. Survivin protein showed its peak expression in lymph node metastases, endolymphatic carcinoma cells, and tumor cells at the advancing tumor front. Nuclear localization was observed in high grade PanINs (emerging at PanIN2 stage), PDA, and metastatic deposits. Furthermore, mitotic figures exhibited survivin positivity. Thus, our findings indicate that nuclear translocation of survivin may be an early event in the progression of PanINs towards malignancy.

In conclusion, our results on survivin expression and its localization in PanINs suggest that survivin staining could possibly help identify lesions at risk for malignant transformation.

4. Notch activation in ectatic ducts of chronic pancreatitis

4. 1 Introduction and background

The theory of ductal obstruction by concretions is one of the four major theories that explain pathogenesis of chronic pancreatitis (CP) [4]. Some of the inciting agents, such as alcohol, are believed to increase protein concentrations in the pancreatic juice. The proteins form ductal plugs that are observed in most forms of CP and are particularly prominent in alcoholic CP. Duct obstruction due to any cause plays an important role in the pathogenesis of CP. Recently, Yamamoto et al., showed in their experimental rat model of CP, a persistent pancreatic ductal hypertension (PDH) for 2 wk induced morphologic changes similar to human CP [36]. Formation of strictures and ductal concretions in the early stages of the disease frequently cause duct obstruction leading to PDH and duct ectasia. Moreover, metaplastic and early neoplastic lesions are frequently observed during the course of CP. Acino-ductal metaplasia (ADM) was observed in male Wistar rats following pancreatic injury induced by duct ligation [36]. It is also thought that chronic inflammation produces alterations in the microenvironment of ductal epithelium that may increase the risk of neoplastic transformation by increased genomic damage and cellular proliferation [38, 39]. During repair, following parenchymal injury to the pancreas, genetic programs that operate during development of pancreas are reinstated and may also play a role in neoplastic transformation [40]. Notch is one such signaling pathway that is required for the proper development of pancreas and is reactivated during pancreatic injury and ensuing repair along the course of CP and pancreatic neoplasia [41,42].

An evolutionary conserved pathway, Notch regulates various aspects of cell differentiation, proliferation, and apoptosis in a tissue and context dependant manner. Four Notch receptors (Notch1 - Notch4) and five ligands [Jagged1,Jagged2, Delta-like1(Dll-1), Dll-3, and Dll-4] have been described in mammals [43,44]. Notch signaling is activated by interactions of adjacent cells via cell - to - cell contact of the membrane-associated Notch receptor and ligand. After ligand binding, two enzymatic cleavages occur to release the Notch intracellular domain (NICD) from the plasma membrane. The NICD translocates into the nucleus and binds to members of the CSL transcription factor family, which are thought to mediate most of the downstream effects of Notch signaling. Following NICD binding, the CSL family member CBF-1/RBP-Jκ, normally part of a co-repressor complex with histone deacetylase-1, becomes a transcriptional activator. Downstream targets of CBF-1 include a large family of β-helix loop helix (*βHLH*) transcription factors known as the hairy/enhancer of split (HES) and HES-related repressor protein (HERP). Alterations of these functions of Notch have been associated with different types of cancer with some exceptions [45]. Evidence is also emerging in support of an independent signaling function of activated intracellular domains of Jagged and Delta-like [46].

We aimed to study the molecular alterations in pancreatic duct ectasia resulting from long-standing obstruction and PDH. We laser microdissected the epithelial cells lining the ectatic ducts of chronic pancreatitis and normal pancreatic ducts. By using oligonucleotide microarrays, pathway analysis, and real time PCR, we found a differential regulation of Notch receptors, ligands, and targets in microdissected

normal pancreatic ducts versus ectatic ducts of CP. Furthermore, these alterations are reflected to an extent in vitro, in a human pancreatic duct epithelial (HPDE) cell line when subjected to a sustained elevated hydrostatic pressure.

4. 2 *Gene expression profiles indicate a differential regulation of Notch components in ectatic ducts versus normal pancreatic ducts*

Gene expression profiles of laser microdissected normal pancreatic ducts versus ectatic ducts of CP showed a differential regulation of Notch pathway components as revealed by pathway analysis (Ingenuity). In ectatic ducts, the expression of Notch ligands-Jagged/Delta was upregulated along with upregulation of a Notch target HERP while there was downregulation of another Notch target HES.

4. 3 Validation of microarray results

To validate that the microarray data accurately reflect mRNA levels, we used real time PCR to independently determine mRNA levels for representative genes of the Notch pathway in different samples of microdissected normal pancreatic ducts and ectatic ducts. Additionally, we analysed the mRNA levels of Notch receptors.

In microdissected ectatic ducts of CP, mRNA levels of Notch receptors - Notch1 was 1.3x (n.s.) and Notch3 increased 1.7x, ($p \leq 0.05$) while those of Notch2 decreased.

Further, in ectatic ducts, the Notch ligand - Delta-like1 mRNA was remarkably elevated by 3.7x ($p \leq 0.05$) while Jagged1 mRNA levels increased nearly 1.3x (n.s.).

The mRNA of the Notch target HERP1 increased by about 2.4x, ($p \leq 0.05$) in ectatic ducts while the HES1 message tended to be downregulated.

4. 4 *Notch component alterations in pressure stimulated HPDE corresponds to that in ectatic ducts of CP*

A static hydrostatic pressure of 200mm of Hg applied for 24h altered the expression of some Notch components including Notch receptors and their ligands, in HPDE. Some of these alterations resemble Notch alterations observed in laser microdissected ectatic ducts of CP compared with normal ducts.

In pressure stimulated HPDE, the transcript expression of Notch receptor – Notch1 increased by about 1.6x (n.s) compared to that of unstimulated control cells while Notch2 mRNA levels decreased and Notch3 remained unchanged. The transcript levels of Notch ligand, Jagged1, tended to increase and that of Delta-like1 tended to decrease. However, the mRNA expression of Notch targets HES1 and HERP1 remained unchanged.

4. 5 *Immunohistochemistry*

By immunohistochemistry, normal pancreatic ducts do not express Jagged. In contrast, the epithelial cells lining the ectatic ducts strongly expressed Jagged1 protein. The epithelial lining forming protrusions into the lumina of dilated and distorted ducts was also Jagged1 positive. Furthermore, the epithelial cells lining ectatic ducts flattened due to stretching resulting from raised intraluminal pressure also exhibit Jagged1. The tubulo-acinar complexes of CP highly expressed Jagged1. The pancreatic islets constitutively expressing Jagged1 served as an inbuilt standard.

4. 6 *Summary and conclusion*

The epithelium lining ectatic ducts of CP is made to withstand increased intraductal pressure over a prolonged period. Therefore, it is most likely that the Notch alterations we detected in microdissected ectatic ducts of CP represent a functional re-programming of epithelium effected through the physical stress of duct obstruction and PDH. Externally applied hydrostatic pressure over a day induced a set of Notch alterations in HPDE that closely mirror that in duct ectasia of CP pointing to PDH as an initiator of these events. Thus, in ectatic ducts of CP, Notch pathway activation induced by long standing, elevated intraductal pressure may lead to transformation, eventually, giving rise to metaplastic and early neoplastic lesions.

General summary and conclusion

In CP, we found a fibrosis- associated induction of IKK-γ and RelA protein along with an increase in mRNA of NFκB1, NFκB2, RelA, RelB, and c-Rel in islet cells. While, the only transcripts that exhibit an increase in acini are those of NFκB2. TRAIL, via TRAIL-R4 broadly expressed in chronic pancreatitis, may be partly responsible for bringing about this scenario as shown by functional experiments with CM cells.

The expression of survivin message and protein gradually increased along PanIN progression to PDA. Survivin protein showed peak expression in lymph node metastases, endolymphatic carcinoma cells, and tumor cells at the advancing tumor front. Nuclear localization was observed in high grade PanINs (first emerging at PanIN2 stage), PDA, and metastatic deposits. Nuclear translocation of survivin may be an early event in the progression of PanINs towards malignancy.

The Notch alterations in microdissected ectatic ducts of CP might represent a functional re-programming of epithelium induced by the physical stress of duct obstruction and PDH. External hydrostatic pressure exerted over the period of a day induced a set of Notch alterations in HPDE that closely mirror those in duct ectasia of CP pointing to PDH as an initiator of these events. The Notch pathway activation in ectatic ducts of CP may initiate transformation, eventually giving rise to metaplastic and early neoplastic lesions.

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I dedicate this work to my parents, my wife Litty and son Aaron.

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Parenchymal regression in chronic pancreatitis spares islets reprogrammed for the expression of NFκB and IAPs

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In advanced chronic pancreatitis (CP), islets are preserved even in the midst of scarring. We recently showed in CP local production of interferon (IFN)γ, transforming growth factor (TGF)β and death receptor ligand TRAIL (tumor necrosis factor-related apoptosis-inducing ligand), along with functional death receptor neoexpression and apoptosis in exocrine but not in endocrine cells. However, islets are strongly induced for TRAIL-receptor(R)-4 lacking the functional death domain. TRAIL-R4 signaling in T cells induces NFκB, which activates antiapoptotic programs. Here, we demonstrate that in insulinoma cells CM, TGFβ/IFNγ/TRAIL in combination induced TRAIL-R4 surface expression. TRAIL/IFNγ upregulated NFκB subunits and its target gene survivin while downmodulating IκBα mRNA. RelA transcriptional activity increased upon stimulation with IFNγ and IFNγ/TRAIL. *In situ*, normal pancreatic epithelia had low mRNA levels of NFκB subunits. These were higher in parenchymal areas of CP with severe fibrosis and highest in islets. NFκB-regulated proteins IκBα, survivin and another apoptosis inhibitor, cIAP1, were found in corresponding sites, again at highest levels in islets surrounded by fibrosis. In conclusion, islets in CP not only evade immune attack by nonexposure of functional death receptors in the presence of TRAIL-R4 but also additionally neoexpress NFκB and its target genes, survivin and cIAP1, to protect themselves from apoptosis.
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Keywords: chronic pancreatitis; fibrosis; NFκB; RelA; survivin; cIAPs

Chronic pancreatitis (CP) is characterized by a progressive loss and fibrosis of exocrine parenchyma, whereas the endocrine islets remain structurally and functionally intact for a prolonged period of time.[1,2] The mechanism/s by which islets survive in an adverse environment initially infiltrated by inflammatory cells and, at later stages, by expanding fibrous tissue is largely unknown. Immune evasion might be a major contributory mechanism. We have previously shown that islets express the death-inducing ligand CD95L (FasL) but do not express its receptor CD95 (Fas). In CP, this 'immunoprivileged' status is preserved in endocrine cells, whereas the exocrine epithelia neoexpress CD95 (Fas) along with HLA-DR.[3] We further showed that islet cells in normal pancreas (NP) are devoid of TRAIL (tumor necrosis factor (TNF)-related apoptosis-inducing ligand) receptors (R), which, like CD95, are members of the TNF receptor superfamily. In CP, there is an induction and high levels of expression of TRAIL-R1, -R2 and -R4 in exocrine cells. In contrast, islet cells are strongly TRAIL-R4 positive and are essentially devoid of the death signal-transducing TRAIL-R1 and -R2.[4] TRAIL-R4 lacks the cytoplasmic death domain and was therefore regarded as a decoy receptor. Recent studies carried out in T-lymphocytes, however, hint at an alternative functional role of TRAIL-R4. TRAIL-R4 has been shown to transduce a signal leading to induction of NFκB constituents, upon binding of TRAIL.[5] We identified pancreatic stellate cells (PSCs) producing the fibrogenic cytokine transforming growth factor (TGF)β[6,7] as the major source of TRAIL in CP.[4] Against this background, it is tempting to speculate that TRAIL, while contributing to exocrine parenchymal damage by TRAIL-R1- and -R2-mediated apoptosis, might at the same time signal endocrine cells via TRAIL-R4 in the sense of NFκB induction and action. Among the multitude of genes regulated by NFκB are antiapoptotic genes such as those for inhibitor of apoptosis proteins (IAPs).[8] IAPs prevent cell death by binding to and inhibiting active caspases.[9] This led us to investigate the role of NFκB constituents in the context of CP

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with particular emphasis on the mechanism/s and/or event/s responsible for islet preservation.

We show here that activated human insulinoma cells CM express TRAIL-R4 in the absence of TRAIL-R1 and -R2 and, upon TRAIL treatment, express increased amounts of mRNA of NFκB subunits and survivin. Treatment with interferon (IFN)γ and IFNγ plus TRAIL elevated RelA transcription in CM. Furthermore, in CP we found an induction of NFκB subunits in pancreatic epithelia, which, although at low levels in exocrine pancreas, is massive in pancreatic islet cells. The extent of local NFκB induction and protein expression correlates with the severity of fibrosis.

Additionally, survivin and cIAP1 were neoexpressed in islets, again most remarkably in the midst of scarring. Thus, our findings suggest that islet cells in CP, in addition to resorting to immune evasion, also activate antiapoptotic programs for survival.

Materials and methods

Cell Culture

The human insulinoma cell line CM was obtained from Professor P Pozzilli of St Bartholomew's Hospital, London, UK. The cells were grown in Iscove's modified Dulbecco's medium/RPMI 1640 (4:1) supplemented with 10% fetal bovine serum, 5 mmol/l L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin. Cells were maintained at 37°C in 5% CO_2.

Assessment of TRAIL-Induced Apoptosis

Sensitivity of CM cells to TRAIL-mediated apoptosis was determined using 100 ng/ml of human recombinant Killer™ TRAIL (Alexis, San Diego, CA, USA) for 6 h. To test for cytokine-enhanced sensitivity of CM cells towards TRAIL-mediated apoptosis, cells were pretreated with 1 ng/ml TGFβ (Roche) for 24 h followed by 500 U/ml IFNγ (Roche) for 18 h. Apoptotic death rates were determined by annexin V binding using an FITC-labeled mouse anti-human annexin V monoclonal antibody (mAb) (IgG1 isotype; PharMingen, San Diego, CA, USA) and 2 μl propidium iodide (50 mg/ml). After gating for propidium iodide negativity to exclude dead cells, 10^5 events were examined for each determination. Flow cytometry was performed on a FACScalibur with CellQuest software (Becton Dickinson, Heidelberg, Germany).

Flow Cytometry

Surface expression of TRAIL-R1, -R2 and -R4 on CM cells was determined by flow cytometric analysis. A total of 10^6 cells/sample were suspended in 50 μl FACS medium (phosphate-buffered saline containing 2% fetal bovine serum, 0.01 mol/l HEPES and 200 ng/ml sodium azide) and incubated for 1 h in an equal volume of the appropriate dilution of the mouse mAbs to TRAIL-R1 (clone M272, IgG1 isotype), TRAIL-R2 (clone M412, IgG1 isotype) and TRAIL-R4 (clone M440, IgG2a isotype) kindly provided by Amgen (Thousand Oaks, CA, USA). The mouse anti-human CD3 (clone Leu4, IgG1 isotype) (DAKO, Copenhagen, Denmark) was used as an irrelevant isotype-matched negative control. Cells were washed twice and incubated with 2 ng of FITC-labeled $F(ab')_2$ goat anti-mouse immunoglobulins (DAKO) for 30 min on ice. Finally, cells were washed twice and resuspended in 500 μl cold FACS medium containing 1 μg/ml propidium iodide (Sigma). Analysis was restricted to propidium-negative (viable) cells and was performed on a FACSCalibur cytometer with CellQuestPro software.

Pancreatic Tissues

In all, 18 patients, whose informed consent was obtained prior to surgery, were chosen for this study. A total of 14 patients underwent partial pancreatectomy for CP: 10 were suffering from alcoholic pancreatitis, two had chronic nonalcoholic pancreatitis associated with pancreas divisum, one developed a cyst after initial surgery and one had cryptogenic CP. Sections were subjected to morphometric assessment for the grading of fibrosis (see below). Four patients underwent partial pancreatectomy for pancreas divisum. The specimens of these patients were histopathologically diagnosed as NP and included in the study as normal controls. All pancreatectomy specimens were immediately transferred to the laboratory where representative tissue samples were snap frozen in liquid nitrogen and kept there until further processing. Other parts of the tissues were stored at −80°C, for later use in frozen section immunohistochemistry and laser capture microdissection (LCM).

Grading of Fibrosis

NP and CP cases for this study were selected and graded independently by two pathologists (UKB and CH) on hematoxylin and eosin (H&E)-stained cryosections based on the relative proportions of parenchyma and stromal fibrosis. There was generally good agreement in NP and CP cases with severe fibrosis. However, there was only partial agreement in grading of CP with mild and moderate fibrosis. Total agreement was achieved, however, on reviewing these cases together on a multiheaded discussion microscope. Further, stromal tissue in NP and the proportion of fibrosis in different cases of CP was accurately quantitated on H&E-stained cryosections using a light microscope with a video camera (Sony 3CCD) attached to an image capture card in a standard desktop computer with image analysis software (ImageTool, San Antonio, TX,

USA) to calculate the volume fraction of fibrosis. Per slide 10 different areas were assessed and the extent of fibrosis was then calculated as percentage of fibrosis in mild, $12.4 \pm 1.8\%$, moderate, $41.2 \pm 0.6\%\%$, severe fibrosis, $73.9 \pm 6\%$, and that of normal stromal tissue in NP, as $6.3 \pm 1.9\%$.

Tissue Processing for LCM

Frozen tissue specimens were cut at 8 μm thickness and serial sections transferred to PALM® MembraneSlides (PALM, Bernried, Germany). Four to 10 tissue sections were required to obtain sufficient number of cells from various cellular compartment of NP and CP with varying grades of fibrosis. Sections were stained with H&E as follows: slides were immersed in 70% ethanol for about 10 min followed by sequential dips in sterile deionized water, Mayer's hematoxylin solution, sterile deionized water, diluted alcoholic eosin, 95% ethanol and finally dehydrated with three changes of absolute ethanol for 3 min each. Slides were air-dried for 5 min before proceeding for LCM.

LCM

PALM® Laser Microbeam Microdissection System with Laser Pressure Catapulting and Robocut Software (PALM, Germany) was used for microdissection. Approximately 2000 cells from each epithelial compartments of NP and CP with various grades of fibrosis were microdissected and catapulted into LCM caps with their inner surface coated with a thin layer of inert mineral oil.

RNA Isolation from Microdissected Tissue

The material from two to three caps was pooled in a single purification column. Total RNA was isolated using Picopure™ RNA isolation Kit (Arcturus, Mountain View, CA, USA) according to the manufacturer's instructions. Total RNA was qualitatively and quantitatively assessed on RNA LabChip (Agilent, Wilmington, DE, USA) following the manufacturer's instructions. Only those samples with clearly visible ribosomal peaks and $OD_{280/260}$ ratios between 1.6 and 1.8 were further processed.

RNA Isolation from CM Cells

Human insulinoma cell line CM was used for stimulation experiments. CM (2×10^5) were stimulated with TRAIL, IFNγ and TRAIL + IFNγ in ideal conditions for 48 h. Exactly the same number of cells kept under identical culture conditions without addition of any substance was used as controls. Total cellular RNA was isolated using the RNeasy RNA isolation system (Qiagen, Valencia, CA, USA) following the manufacturer's recommendations. RNA was assessed by agarose gel electrophoresis and quantitated by UV absorbance.

RNA Amplification and Semiquantitative Real-Time PCR

The RiboAmp™ RNA Amlification Kit was used for amplifying the total RNA from microdissected tissues. The manufacturer's protocol for performing one round of amplification was used. The amount of RNA obtained after amplification was qualitatively and quantitatively assessed using the RNA LabChip (Agilent). RNA (2.5 μg) was reversely transcribed into cDNA using Superscript Reverse Transcriptase (Invitrogen), random hexamer primer and addition of an RNase inhibitor following the manufacturer's protocol for expression analysis of various NFκB subunits in microdissected tissues. Primers were designed using Primer Express Software (Perkin-Elmer, Foster City, CA, USA) to meet the following criteria: nonoverlapping primers as close as possible to each other, avoidance of sequences with propensity for dimer formation, low G–C content, avoiding run of nucleotides and a T_m at around 60°C. The sequences of the primer pairs are shown in Table 1. Primer pairs were purchased from MWG Biotech (Ebersberg, Germany). Cyclophilin expression was chosen as the endogenous control (forward primer: 5′-ATGGTCAACC CCACCGTGT-3′; reverse primer: 5′-TCTGCTGTCTTTGGGACCTTGTC-3′). First, the absence of nonspecific amplification was confirmed by analyzing the PCR amplification products by agarose gel electrophoresis. Amplicons generated from cDNA were also tested against no template control and RNA. In a further step, optimal primer concentrations were selected by mixing primer concentrations from 50 to 900 nmol of each forward and reverse primer. The curves were checked for low C_T, fast rising and for confirmation analyzed by agarose gel electrophoresis. Real-time PCR was performed using 4 μl cDNA (12.5 ng/μl), 4 μl primer mix (forward/reverse) at optimal concentrations, 12 μl sterile distilled water and 20 μl SYBR Green PCR Master Mix (PE Biosystems, Foster City, CA, USA) per reaction. The following cycling conditions were set: 50°C for 2 min, denaturation at 95°C for 10 min, followed by 45 cycles at 95°C for 20 s each, 60°C for 20 s and 72°C for 20 s. Gene expression of NFκB subunits of tissue affected by CP was measured relative to whole NP tissue as the calibrator sample. All quantitations were also normalized to cyclophilin as an endogenous control to account for variability in the initial concentration of total RNA. Analysis of quantitation was carried out by calculating as follows: 1. mean C_T value of three replicates per sample; 2. difference between mean C_T values of samples for each target (CP) and those of the endogenous controls (ΔC_T); 3. difference between mean C_T values of the samples for each target (CP) and the mean C_T value of the correspond-

Table 1 Sequence and concentration of primers

Gene	*Primer*	*Sequence*	*Concentration (nM)*
RelA	Forward	5′-TCA TGA AGA AGA GTC CTT TCA-3′	900
	Reverse	5′-TGA TCT GCC CAG AAG GAA ACA-3′	900
NFκB1	Forward	5′-ACA ACA TCT CAT TGG CAG GCT-3′	900
	Reverse	5′-GGT CAT AGA GAG GCT CAA AGT-3′	900
NFκB2	Forward	5′-GAT GGA TCT GAG TAT AGT GCG-3′	300
	Reverse	5′-CCG GAG ATT TGC TAT CAT GGA-3′	900
c-Rel	Forward	5′-CCT GTT GTC TCG AAC CCA AT-3′	300
	Reverse	5′-TCC CAA TCG TTC AAC ACA AA-3′	900
RelB	Forward	5′-ATC TGC TTC CAG GCC TCA TAT-3′	300
	Reverse	5′-ATC CGC AGC TCT GAT GTG TTT-3′	300
IκBα	Forward	5′-GCT GAT GTC AAT GCT CAG GA-3′	300
	Reverse	5′-CCC CAC ACT TCA ACA GGA GT-3′	900
Survivin	Forward	5′ TGC CCC GAC GTT GCC-3′	900
	Reverse	5′-CAG TTC TTG AAT GTA GAG ATG CGG-3′	900

ing calibrator (NP) ($\Delta\Delta C_T$). The quantitation is expressed as $2^{-\Delta\Delta C_T}$ to allow graphical presentation, and is shown as *x*-fold expression of the target gene in CP compared to NP set as 1.

Immunohistochemistry

Immunohistochemistry was performed on four NP and 14 pancreatic tissues affected by CP with different extents of fibrosis. Serial, 2-μm-thick cryosections were immediately fixed in ice-cold acetone for 10 min, air-dried and incubated for 1 h with the following mouse anti-human mAbs: anti-RelA (IgG1 isotype), IκBα/MAD-3 (IgG1 isotype), IKKγ/NEMO (IgG1 isotype) and cIAP1 (clone B75-1, anti-IgG1 isotype), all purchased from BD Biosciences (PharMingen, Belgium). For detection of survivin, a polyclonal rabbit anti-human antibody (R&D Systems, Germany) was used. Bound primary antibody was detected via goat anti-mouse and swine anti-rabbit immunoglobulins, respectively, conjugated to peroxidase-labeled dextran polymer in Tris-HCl buffer containing carrier protein (EnVision™, Dako). 3-Amino-9-ethyl-carbazole (Sigma, St Louis, MA, USA) was used as the substrate. Hematoxylin was used as a counterstain. The monoclonal mouse anti-human TTF1 (clone 8G7G3/1, anti-IgG1 isotype) purchased from Zymed Laboratories (South San Francisco, USA) and rabbit anti-human thyroglobulin obtained from DAKO (Copenhagen, Denmark) were used as irrelevant isotype-matched negative controls.

RelA Transcription Factor Assay

DNA-binding activity of RelA of the NFκB transcription factor family in CM treated with 500 U/ml IFNγ (Roche) and 100 ng/ml of human recombinant Killer™ TRAIL (Alexis, San Diego, CA, USA) separately and together for 48 h was determined using an ELISA-based assay (TransAM Kit™) obtained from Active Motif (Rixensart, Belgium). CM (2×10^5/well) in a six-well plate were used and each stimulation was performed in triplicate. An equal number of cells kept under identical conditions without the addition of any substance were used as controls. In brief, the nuclear extracts were prepared following the manufacturer's instructions and 5 μg of nuclear extract was added to microwells coated with a cold oligonucleotide containing the consensus-binding site for NFκB. After 1 h incubation at room temperature, the microwells were washed thrice with washing solution. Antibody directed against phosphorylated RelA was used to label the RelA bound to the oligonucleotide and this was followed by an incubation with a secondary antibody conjugated to horseradish peroxidase. Finally, the results were quantified by a chromogenic reaction. Results were analyzed using the Revelation software (Dynatech) and are given in pg/ml as means with standard deviations.

Results

TRAIL-R Surface Profile of Stimulated CM Cells Corresponds to that of Islets in CP

Against the background that islet cells are TRAIL-R1$^-$, TRAIL-R2$^-$ and TRAIL-R4$^+$,[4] we tested human insulinoma cell line CM for TRAIL receptor surface expression. CM cells were constitutively devoid of TRAIL-R1, -R2, -R3 (not shown) and -R4, which is a phenotype corresponding to that of islet cells of NP.[4] The TRAIL-R profile of CM upon stimulation with TGFβ (24 h), IFNγ (18 h) and TRAIL (6 h) mimicked

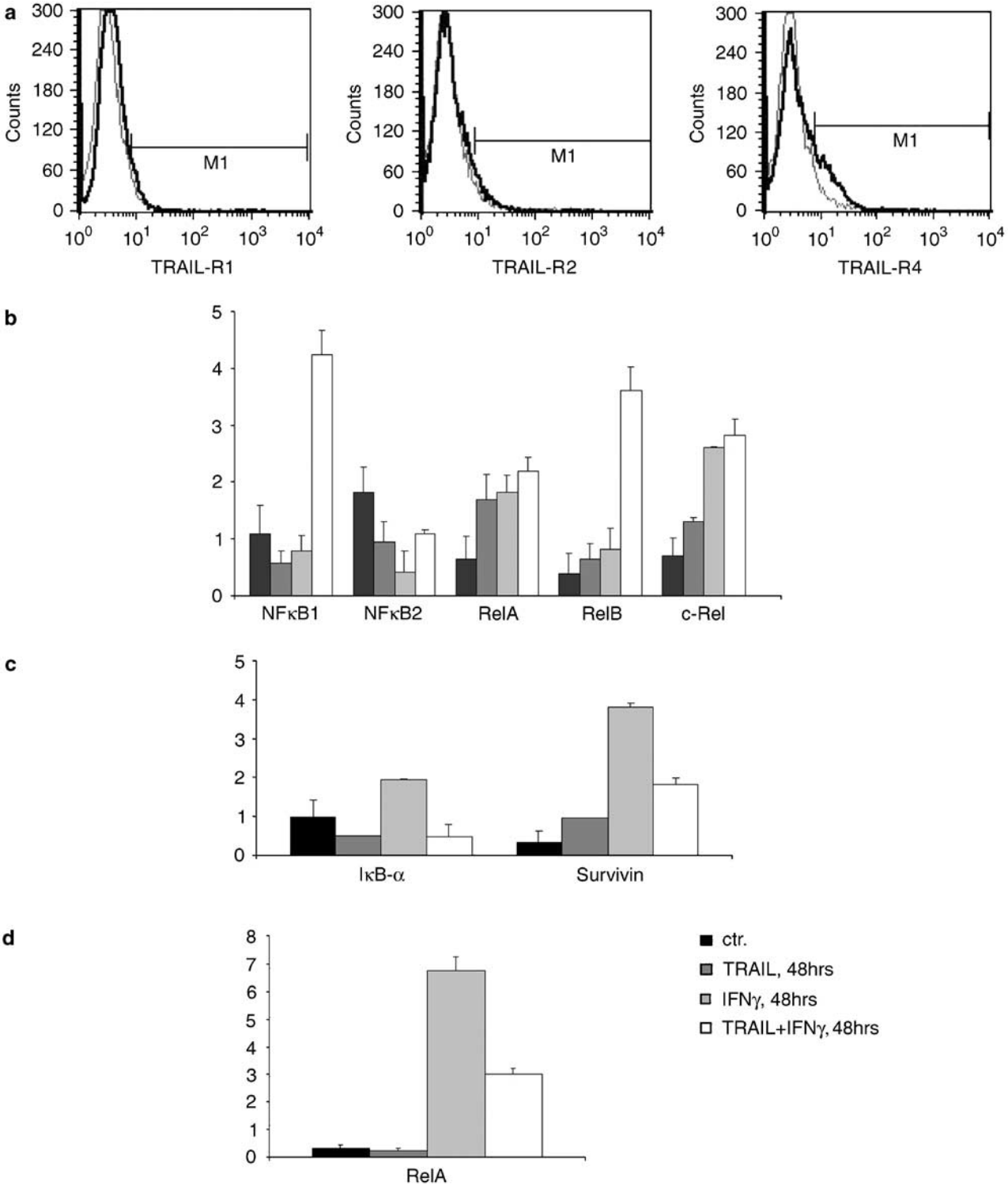


Figure 1 (**a**) FACS histograms showing surface expression of TRAIL-R1 (left panel), -R2 (middle panel), and -R4 (right panel) in black lines. M1 marks the subset of CM cells positive for TRAIL-Rs. Isotype-matched negative controls are shown as gray lines. (**b**) Semiquantitative real-time PCR for NFκB subunits in CM cells stimulated for 48 h with TRAIL, IFNγ and TRAIL + IFNγ compared with untreated cells. (**c**) Semiquantitative real-time PCR for NFκB targets IκBα and survivin in CM cells stimulated with cytokines for 48 h. IκBα mRNA levels increased with IFNγ, while a decrease was noted with TRAIL and TRAIL + IFNγ. Survivin mRNA increased considerably with TRAIL, IFNγ and TRAIL + IFNγ. (**d**) RelA transcriptional activity in CM activated by TRAIL, IFNγ and IFNγ + TRAIL determined by ELISA compared with untreated control. The bars indicating standard deviation are not always visible due to the minimal variations of values compared to the scale used.

that of islets in CP,[4] in being TRAIL-R1$^-$, TRAIL-R2$^-$, TRAIL-R3$^-$ (not shown) and TRAIL-R4$^+$ in a major subset of cells (Figure 1a).

TRAIL Modulates NFκB Subunit Expression in CM

Next, CM cells were stimulated with TRAIL, IFNγ and TRAIL + IFNγ for 48 h to determine the mRNA expression of NFκB subunits and NFκB targets IκBα and survivin.

The combination of TRAIL and IFNγ brought about a four-fold rise in NFκB1 mRNA levels. On the other hand, mRNAs of transcriptionally active NFκB subunits RelA, RelB and c-Rel were between two- to 9.5-fold increased compared to untreated cells on TRAIL + IFNγ stimulation (Figure 1b). Additionally, mRNA of NFκB target IκBα was downmodulated, while survivin message increased considerably (Figure 1c).

Thus, TRAIL-R4 acts as a signal transducer in modulating NFκB subunits in CM. Moreover, CM did not show sensitivity to TRAIL-mediated apoptosis, as determined by quantifying the sub-1 fraction in FACS, nor did the amount of insulin secretion change significantly under TRAIL treatment (data not shown).

RelA Transcriptional Activity in CM

The DNA-binding activity of RelA of NFκB family of transcription factors in CM cells as determined by an ELISA-based assay increased significantly following 48 h stimulation with IFNγ (20-fold rise) and TRAIL + IFNγ (10-fold) in comparison with untreated control (Figure 1d).

General Aspects in Epithelia of NP and CP

Turning to tissues, NP had low mRNA levels of NFκB subunits. These were increased in CP in parenchymal areas with fibrosis and most intensely in islets. The expression of NFκB-regulated proteins IκBα, survivin and cIAP1 was found in corresponding sites, again at their highest levels in islets surrounded by fibrosis. Specimens from patients with CP were markedly microheterogeneous with respect to each aspect aimed at in this study. As observed in the pilot phase of this study, this heterogeneity seemed to be paralleled not by the degree of inflammatory lymphohistiocytic infiltration but by the degree of fibrosis. Even within the same section, the relative amount of antigen detected was most pronounced in fibrotic areas, the intensity being markedly lower in relatively normal unaffected areas. Therefore, we morphometrically estimated the relative proportion of the stromal and the epithelial compartment in each specimen and defined three grades of fibrosis, that is, mild fibrosis, 12.4 ± 1.8%, moderate, 41.2 ± 0.6%, and severe fibrosis, 73.9 ± 6%; stroma constituted 6.3 ± 1.9% of NP (Figure 2).

Cryosections were subjected to LCM to obtain cells from each epithelial compartment of NP and CP in order to analyze the mRNA expression levels of NFκB subunits and stained for proteins for which reliable antibodies were available. Next, we correlated the mRNA levels (data given in Figure 3) and the staining intensities in IHC with the extent of fibrosis (data given in Table 2 and paradigmatic situations depicted in Figure 4). To summarize, NP was largely devoid of expression of NFκB constituents, survivin and cIAP1. Message and/or protein levels in pancreatic epithelial compartments increased with the degree of fibrosis. At the extreme, that is, in severe fibrosis, mRNA and/or protein was detected in high amounts, especially in islets spared in the midst of fibrosis.

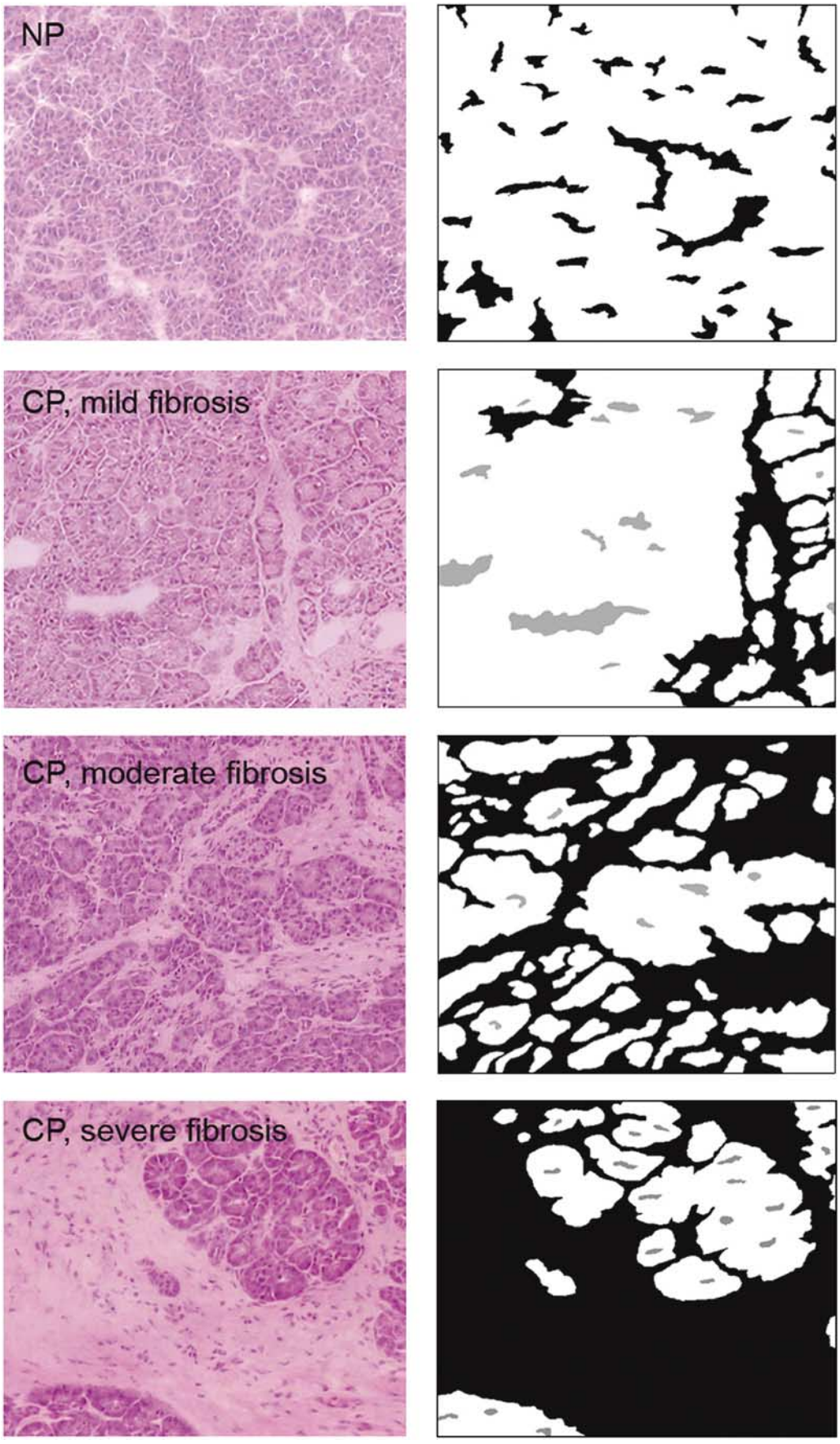
RelA

In severe fibrosis, there was a sharp increase in RelA transcripts in islet cells but not in acini and ducts (Figure 3). Normal acini, ductal epithelium and islets showed no expression of RelA protein. In CP, RelA protein was detectable and the intensity of staining increased with progressive severity of fibrosis. Staining was most pronounced in the acini and islets in areas of severe fibrosis. In both, the acinar and islet cells, the staining was cytoplasmic and nuclear. The ductal epithelium showed either no or, focally, very low expression of RelA. In CP with severe fibrosis associated with exocrine parenchymal regression, the acini in fibrotic areas featuring a tubular morphology exhibited strong cytoplasmic and nuclear positivity. Pancreatic islets that were intact within severely fibrotic areas stained most intensely for RelA, staining being cytoplasmic as well as nuclear (Figure 4).

NFκB1 (p105)

There was a sharp, about 10-fold, increase in NFκB1 (p105) transcript numbers in islet cells in areas of severe fibrosis (Figure 3). As was the case for NFκB1 (p100), no protein data are available for NFκB2 (P100), RelB and c-Rel due to the lack of a reliable antibody.

Figure 2 Morphometric analysis of the relative proportion of stromal and epithelial tissue in NP and CP. Three grades of fibrosis, that is, mild fibrosis, 12.4 ± 1.8%, moderate, 41.2 ± 0.6%, and severe fibrosis, 73.9 ± 6%, and NP, 6.3 ± 1.9% were calculated by binary analysis of H&E-stained cryosection (left panel). White areas depict the epithelial compartment, gray areas empty spaces (eg ductal and vessel lumina) and the black areas represent stromal tissue (right panel).


NP
CP, mild fibrosis
CP, moderate fibrosis
CP, severe fibrosis

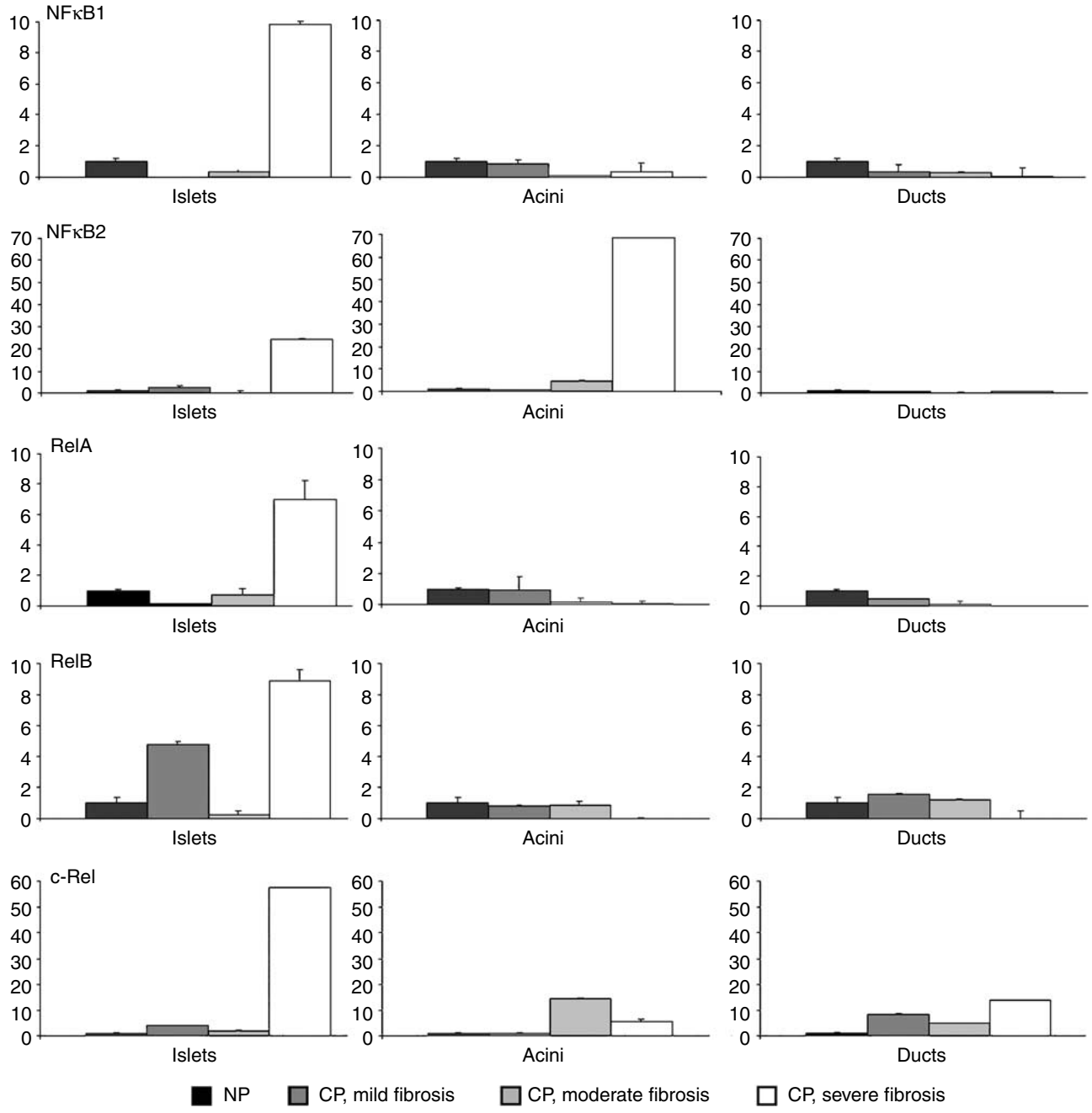


Figure 3 Semiquantitative real-time PCR for NFκB subunits. Cyclophilin was used as the internal standard. The diagram depicts the *x*-fold expression of NFκB1, NFκB2, RelA, RelB and c-Rel in different microdissected epithelial compartments of CP with mild, moderate and severe fibrosis compared with corresponding epithelial compartments of NP, set as 1, after normalization of the C_T values of each sample to its internal standard. The bars indicating standard deviation are not always visible due to the minimal variations of values compared to the scale used.

NFκB2 (p100)

A similar pattern was observed in NFκB2 (p100) mRNA levels with the exception of acinar cells showing 4.6- and 69-fold increase in moderate and severe fibrosis, respectively (Figure 3). Islets showed nearly 24-fold amount of message in areas of severe fibrosis.

RelB

RelB mRNA was overexpressed in islet cells in mild and even more so in severe fibrosis (Figure 3).

c-Rel

In acini and ducts, there was a slight increase in c-Rel mRNA with higher degrees of fibrosis. This

Table 2 RelA, survivin and cIAP1 expression in NP and CP

Compartment	*NP*			*CP, mild fibrosis*			*CP, moderate fibrosis*			*CP, severe fibrosis*		
	RelA	*Survivin*	*IAP1*	*RelA*	*Survivin*	*IAP1*	*RelA*	*Survivin*	*IAP1*	*RelA*	*Survivin*	*IAP1*
Acinar cells	–	–/(+)	–	+	(+)/+	+	++	++	++	+++	++	++
Tubular acini										+++	++	++/+++
Ductal epithelia	–	–/(+)	–	–	+	(+)/+	–/(+)	+/++	+/++	–/(+)	+/++	+/++
Islet cells	–	–/(+)	–	+	+	+	++	++	++	+++	+++	+++

Absent, –; low, (+); moderate, +; high, ++; very high, +++ antigen density.

enhancement was by far exceeded by the extensive, 58-fold overexpression of c-Rel in islets, occurring in conjunction with severe fibrosis (Figure 3).

IKKγ

In NP, IKKγ protein expression was restricted to some scattered acinar cells.

In CP, an induction of IKKγ was observed in the exocrine and endocrine epithelium paralleled by the degree of fibrosis. In specimens showing only mild fibrosis, IKKγ expression was restricted to islets; all pancreatic epithelia showed moderate to high immunoreactivity for IKKγ in areas with moderate fibrosis. In severe fibrosis, within the remaining endocrine epithelium, IKKγ expression was at mild to moderate levels with lower levels in ducts and acini (Figure 4).

IκBα

The IKKγ target, IκBα, was undetectable in NP and in CP with mild and moderate fibrosis. In contrast, tissues affected with severe fibrosis showed expression of IκBα, this being more pronounced in islets and the surrounding acinar epithelium (Figure 4).

Against the constitutive paucity of RelA transcripts and low activators IKKγ and IκBα protein levels in NP, CP was characterized by fibrosis-related neoexpression and/or partial increase of mainly IKKγ and RelA, and less so of IκBα. The question thus arose as to whether RelA immunohistochemically shown to be translocated to the nucleus (Figure 4) contributes to the remarkably prolonged survival in pancreatic islet cells in the context of CP by inducing antiapoptotic factors, for example, IAPs such as survivin and cIAP1.

Survivin

In NP, survivin protein expression was absent or at low levels in ductal, acinar and islet cells. In CP, there was a survivin expression, the intensity of which increased with the extent of fibrosis. In CP cases with severe fibrosis, the intact islets exhibited intense cytoplasmic and nuclear staining. The remaining acini and the tubular acini also stained strongly (Figure 4). This pattern closely resembles those of IKKγ, IκBα and RelA immunoreactivity.

cIAP1

The staining pattern mostly parallels the ones described above for RelA and survivin. While NP did not stain for cIAP1, the staining could be discerned in acini, ducts and pancreatic islets in a perinuclear cytoplasmic location in CP with mild fibrosis, becoming more pronounced with increasing fibrosis. In CP with severe fibrosis, the intact islets exhibited an intense cytoplasmic and nuclear pattern. The acini spared by the fibrotic process and the tubular acini also stained strongly (Figure 4).

Discussion

TRAIL-R4 does not transmit a direct death signal but increasing evidence suggests that it activates NFκB transcription factors, first demonstrated in T cells and transfectants.[5] We show here for the first time that this is also true for a human insulinoma cell line, CM. CM cells essentially lack surface expression of functional death receptors, TRAIL-R1 and -R2, and constitutively express TRAILR4 in a minority of cells. Pretreatment of CM cells with TGFβ and IFNγ upregulated R4, while R1 and R2 remained unchanged. Treatment of these *in vitro*-activated cells with TRAIL led to an increase in transcripts for transcriptionally active RelA, RelB and c-Rel subunits together with a slight decrease in mRNA of the generally transcriptionally inactive molecules NFκB1 (p105) and NFκB2 (p100). Furthermore, this resulted in a downmodulation of IκBα and remarkable increase in survivin transcripts. Since TRAIL-R4 is the only functionally expressed TRAIL receptor in CM cells, this TRAIL effect most probably is transmitted via TRAIL-R4. Untreated and IFNγ/TGFβ-stimulated CM cells were resistant to TRAIL-mediated apoptosis. Moreover, TRAIL modulated their NFκB repertoire in a way that activated antiapoptotic programs. Further, we found elevations in DNA-binding activity of RelA upon treatment with IFNγ and IFNγ + TRAIL.

With this, we turned to the *in situ* analysis of NP and CP. We show that NP is characterized by low expression levels of NFκB subunit transcripts in both exocrine and endocrine compartments. In CP, there is a massive induction of IKKγ and RelA protein, which is at its maximum in islets. Immu-

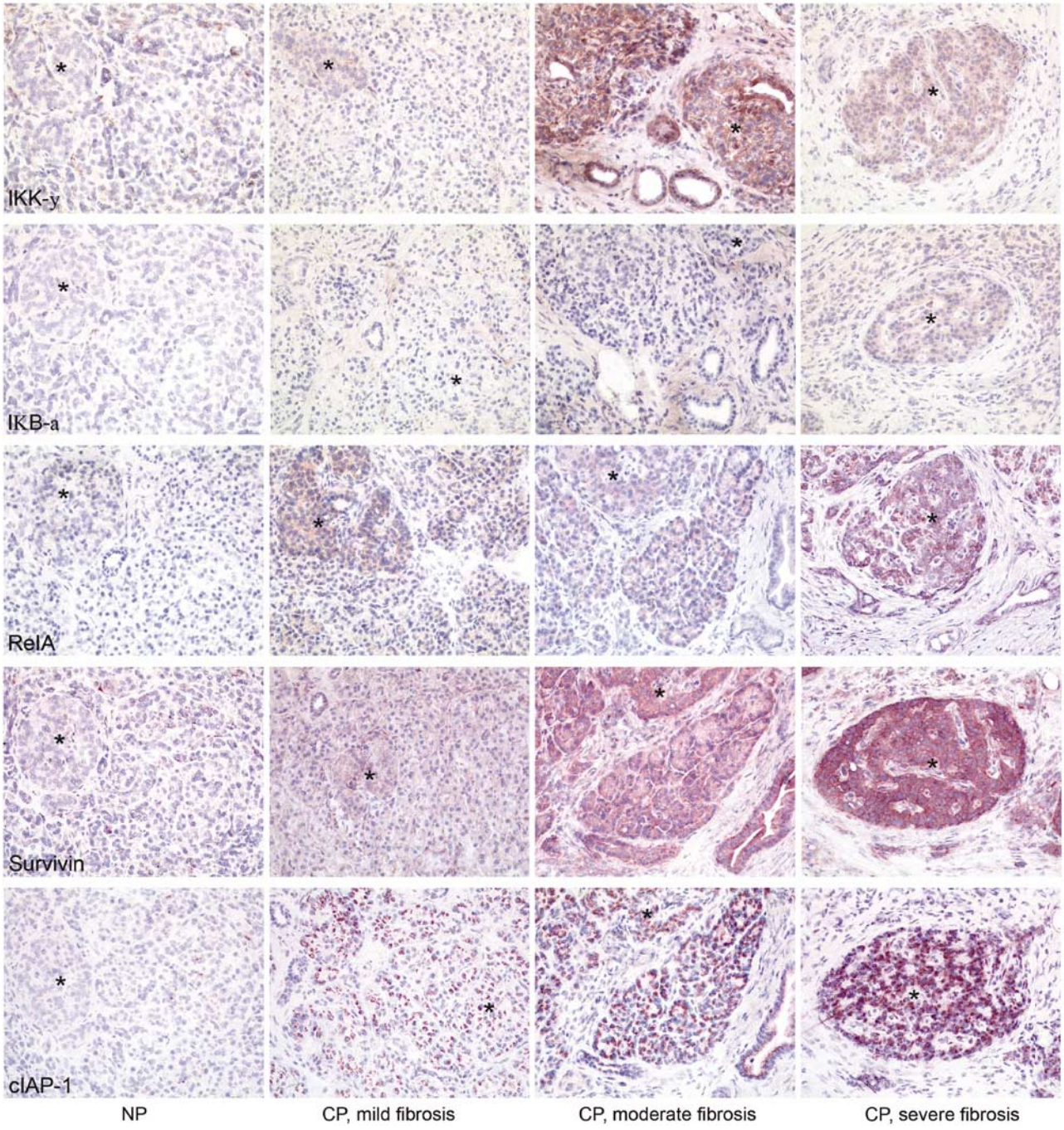


Figure 4 Immunohistochemistry for IKKγ, IκBα, RelA, survivin and cIAP1. IKKγ: NP tissues show no or only a very weak expression of IKKγ. Weak expression emerges in islets of mild fibrosis, whereas acini and ducts remain largely negative or exhibit only a weak staining. In CP with moderate fibrosis, there is a marked enhancement in staining intensity in the islets, acini and focally in the ducts; the islets are slightly positive in CP with severe fibrosis. IκBα: NP, CP with mild and moderate fibrosis are largely devoid of IκBα. In CP with severe fibrosis, there is a moderate expression in the islets and weakly so in the acini, but none in the ducts. RelA: normal acini, ductal epithelium and islets are devoid of RelA; in CP with mild fibrosis, staining is appreciable in acinar cells and much more intense in islets, while ducts show a very weak or no expression. In CP with moderate fibrosis, the acini and islets exhibit a stronger cytoplasmic positivity. In CP with severe fibrosis, the islets cells show a very strong cytoplasmic and nuclear staining; the remaining acini are also strongly positive. Survivin: acini, islets and ducts in NP express survivin only very weakly; staining is conspicuous in acini, islets and ducts in CP with mild fibrosis. With moderate fibrosis, there is an intense staining of all the epithelial compartments. In severe fibrosis, the islets exhibit further accentuation in the staining intensity in the cytoplasm and the nucleus. cIAP1: acini and ducts in NP do not stain for cIAP1. Staining emerges in the cytoplasm of acinar and islets cells with mild fibrosis; ducts are negative. In moderate fibrosis, there is further accentuation of staining in the acini, islets and staining emerges in ducts. In severe fibrosis, islets exhibit an even stronger reaction; acini and ducts are also stained intensely; staining is cytoplasmic as well as nuclear (*islets).

nohistochemistry further suggested that this extent of enhancement might be associated with the degree of fibrosis. Since commercially available antibodies to other NFκB subunits failed to meet our quality standards, we had to rely solely on the mRNA levels. Therefore, we first morphometrically assessed the degree of fibrosis and isolated mRNA of microdissected islets, acinar and ductal epithelial cells from corresponding serial sections. The results are clear-cut and reflect the pattern observed at the protein level. The second important and unprecedented finding is the fibrosis-associated increase in mRNA for NFκB1, NFκB2, RelA, RelB and c-Rel in islet cells. Together with our findings that transcriptionally active NFκB subunits are upregulated in CM cells upon TRAIL treatment, this interaction may also play a role in CP. Thus, TRAIL, via TRAIL-R4 broadly expressed in CP, may be one, if not a major, factor bringing about the above scenario.[10]

At present, there is a wealth of data on the defensive and protective role NFκB plays in the acute phase response in inflammation.[11,12] It was suggested that RelA and c-Rel are functional antagonists with respect to apoptosis, RelA overexpression inhibiting apoptosis, while c-Rel acts in a proapototic way by enhancing TRAIL-R1, -R2 or suppressing cIAP1, cIAP2 and survivin.[13] It was, however, also suggested that NFκB2 (p100) might be a direct activator of programmed cell death.[14,15] NFκB was shown to be involved in acute pancreatitis.[16] Activation of NFκB was detected as early as 10 min after supramaximal stimulation in the cerulein model of acute pancreatitis and seemed to limit the damage induced, while blocking NFκB activation had an adverse effect.[16] Conversely, intraductally administered *in vivo* gene transfer of RelA led to NFκB activation, acinar damage and acute pancreatitis in rats.[17] Thus, it remains unclear whether NFκB activation in pancreas is protective or deleterious.[18] Remarkably, in the acini of CP with severe fibrosis, we found a 69-fold NFκB2 mRNA expression, eventually tilting the balance in favor of apoptotic cell death in the exocrine compartment.

Our new findings collectively suggest that in CP, a progressive disease smoldering for years, there is a reprogramming of endocrine and, less so, exocrine cells for an altered state of NFκB-regulated transcriptional activity, the functional consequences of which, at the single-cell level, are largely unclear. One family of genes regulated by NFκB are IAPs, acting as suppressors of apoptosis by inhibiting caspase-3, -7 and -9.[9,19] It was suggested that cancer cells, for example, of pancreatic carcinoma,[20,21] can counteract apoptotic signals by the expression of IAPs. We found a pattern of fibrosis-associated enhancement of cIAP1 and survivin protein expression similar to RelA emerge in islets. Our finding of cIAP1 protein being undetectable in NP is at variance with published data suggesting that mRNA is present in all adult tissues.[22] The low levels or lack of survivin expression in NP is in line with data on the absence of mRNA in normal adult tissues[22] and the absence of protein in NP reported by Satoh *et al*.[21] However, survivin expression has been reported in human fetal islets.[23] We show for the first time a fibrosis-associated dramatic induction of both cIAP1 and survivin in CP, again, especially in islet cells.

Our findings suggest that sustained enhanced expression of NFκB subunits and, consequently, IAPs are lifeguards for endocrine cells in the adverse microenvironment of CP, while the pattern of NFκB activation is likely to be deleterious to exocrine parenchyma. One of the key inductors of this reprogramming might be TRAIL. Interestingly enough, TRAIL was shown in two independent experimental studies to protect islet cells in experimental type I diabetes in mice.[24] Mi *et al* reported TRAIL gene upregulation in islet cells during the development of diabetes of nonobese diabetic (NOD) mice. Further, treatment of freshly isolated islet cells with TRAIL did not result in apoptosis. Finally, TRAIL blockade exacerbated the onset of type I diabetes in NOD *Scid* recipients of transferred diabetogenic T cells. Lamhamedi-Cherradi *et al*[25] treated normal and TRAIL-deficient mice with multiple low-dose streptozotocin to induce diabetes. They went on to treat NOD mice with soluble TRAIL blocker. Both the incidence and the degree of insulitis in both disease models were enhanced in the absence, or blockade, of TRAIL. Although both groups of authors attribute the protective TRAIL effect to an inhibition of proliferation of diabetogenic T cells, it seems, against the background of our findings, worthwhile considering TRAIL-mediated protection of the islet cells as an alternative possibility.

The two major epithelial components of pancreas meet different fates in different chronic inflammatory diseases. In CP, where the exocrine parenchyma is progressively lost, the endocrine islets remain intact and functional, even in advanced stages of fibrous replacement. *Vice versa*, in type I diabetes, the islet cells vanish in the course of insulitis, while the exocrine parenchyma is unaffected. In both processes, apoptotic cell death is the key event.[26] Current understanding suggests that immune attacks are cell type specific, and there is little left to doubt this. Nevertheless, chronic inflammation functions via local release of chemokines, cytokines and soluble death receptor ligands, which lack the specificity of cognate T-cell–target cell interaction. These short-range acting molecules not only fuel the directed attack but are also likely to inflict collateral damage,[27] all the more if targets and innocent bystanders are so intimately assembled. Therefore, survival programs run by exocrine and endocrine cells regulating their fate in terms of apoptosis resistance or susceptibility must fundamentally differ in CP and type I diabetes, although the death pathways and their interfering inhibitors are very likely just the same in both cell types.

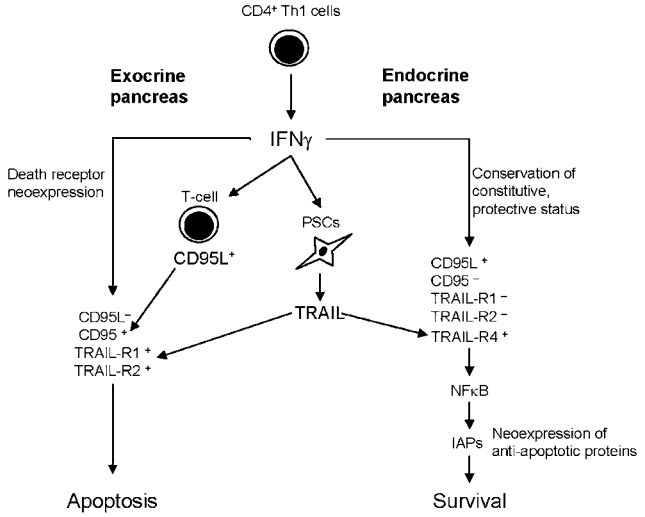


Figure 5 Hypothetical model of dichotomy of fates of pancreatic epithelia in CP.

Summarizing our previous findings[3,4] and integrating our new data, we propose the following model of CP leading to enforced death of exocrine cells and at the same time to armored survival of endocrine cells (Figure 5). Initiated by IFNγ locally released by CD4$^+$Th1 cells, exocrine pancreatic epithelia lose CD95L and neoexpress the functional death receptors CD95, TRAIL-R1 and TRAIL-R2. This renders them vulnerable to CD95L-mediated T-cell attack and/or to action of soluble TRAIL released by IFNγ-triggered PSCs. The effects of IFNγ and TRAIL on endocrine cells are completely opposite. In the setting of CP, islet cells retain their protective CD95L$^+$, death receptor$^-$ state but respond to IFNγ/TRAIL via TRAIL-R4 signalling with NFκB activation and subsequent neoexpression of IAPs. Thus, islet cells at risk of succumbing to collateral damage caused by local release of death-inducing molecules by the inflammatory infiltrate augment their functional repertoire ensuring survival in a locally adverse environment.

Acknowledgements

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Dichotomy of fates of pancreatic epithelia in chronic pancreatitis: apoptosis versus survival

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Chronic pancreatitis is now thought to have a multifactorial etiology. New concepts integrating cellular, molecular and genetic knowledge of the disease have been proposed to explain its pathogenesis. However, the mechanisms responsible for early exocrine parenchymal destruction and preservation of endocrine islets were unexplored until recently. In the course of chronic inflammation, pancreatic acini lose their 'immunoprotective' status by neo-expressing death receptors. Therefore, they become susceptible to apoptosis that is triggered by their respective ligands expressed on lymphocytes and released by pancreatic stellate cells. By contrast, islets retain their immunoprotective status and activate nuclear factor-κB (NF-κB)-induced anti-apoptotic factors, thus enabling survival. This knowledge might be exploited for devising therapeutic approaches to retard acinar loss and to prolong islet survival.

Etiopathogenesis of chronic pancreatitis

Chronic pancreatitis (CP) is characterized by persistent inflammation of the pancreas, which leads to irreversible destruction of the exocrine parenchyma and its replacement by fibrous tissue resulting in malabsorption, weight loss and severe, unrelenting pain. Remarkably, the endocrine islets remain structurally and functionally intact for a prolonged period [1] (Figure 1). Eventually, in late stages of CP, islets are also destroyed, resulting in development of diabetes [2–4]. It is intriguing that the endocrine and exocrine compartments of the pancreas, which are so intimately assembled, respond differently to injurious stimuli in their microenvironment. The etiological factors of CP are well defined. In developed countries, 60–70% of the patients with CP have a long history of heavy alcohol consumption. Less-common etiology of CP includes autoimmune disease, hypertriglyceridemia, hyperparathyroidism, tropical pancreatitis (see Glossary), pancreas divisum, obstruction of pancreatic duct by tumour and genetic abnormalities [5]. However, none of these risk factors consistently leads to CP. For example, alcohol is used by far greater numbers of people than those who develop CP, thereby suggesting a variable genetic susceptibility. CP is now regarded as a multifactorial disorder where multiple risk factors operate together in the causation of the disease [6]. This is an important conceptual change in the understanding of CP. Major predisposing risk factors for CP have been categorized as toxic–metabolic, idiopathic, genetic, autoimmune, recurrent acute pancreatitis or obstructive by the TIGAR-O system [2,6].

It is surprising that the pathogenesis of CP has remained enigmatic for several years. However, recent progress in cellular and molecular biology have shed new light on the pathogenesis of this complex disease. Here, we attempt to summarize theories and underline recent advances in the development of CP giving a particular emphasis on early acinar destruction versus islet-cell preservation during disease progression. A clear knowledge of mechanisms that enable prolonged islet-cell survival in CP might be used to prolong life of islet cells transplanted for the treatment of diabetes. These approaches might also prove useful for retarding acinar regression.

Theories of chronic pancreatitis

In the past decades, four major theories have emerged to explain the pathogenesis of CP [7].

- Oxidative-stress theory. Alcohol-induced oxidative stress might generate free radicals in acinar cells, leading to membrane-lipid oxidation and activation of transcription factors, including activator protein 1 (AP1) and nuclear factor-κB (NF-κB), that, in turn, induce the expression of chemokines that attract mononuclear cells. Thus, oxidative stress promotes the fusion of lysosomes and zymogen granules, acinar-cell necrosis, inflammation and fibrosis.
- Toxic–metabolic theory. Toxins, including alcohol and its metabolites, can be toxic to acinar cells. This toxicity might lead to the accumulation of lipids in acinar cells, acinar-cell loss and, eventually, parenchymal fibrosis.
- Theory of ductal obstruction by concretions. Some of the inciting agents that are responsible for the development of CP (e.g. alcohol) are believed to increase protein concentrations in the pancreatic juice. These proteins form ductal plugs that are observed in most forms of CP but are particularly prominent in alcoholic CP. These ductal plugs might calcify, forming calculi composed of calcium-carbonate precipitates, and these calculi can further obstruct the pancreatic ducts and contribute to the development of CP.
- Necrosis–fibrosis theory. Acute pancreatitis results from autodigestion of pancreatic tissue by inappropriate

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Glossary

CD95: a 48-kDa type I membrane protein and a member of the tumor necrosis factor (TNF) receptor family. Upon oligomerization by antibody or trimerization by its natural ligand CD95L, CD95 (also known as FAS) confers an apoptotic signal to apoptosis-sensitive cells.
CD95L: a 40-kDa type II membrane protein that belongs to the TNF family of cytokines. CD95 exists in a membrane-bound and in a soluble form. CD95L (also known as FASL) is expressed on activated T cells (preferentially, $CD4^+$ T helper 1 cells) and on plasma cells.
Perforin–granzyme: cytotoxic T lymphocytes granules contain potent serine proteases called granzymes and a pore-forming protein, known as perforin.
Hereditary pancreatitis: an autosomal dominant disease that is caused by germ-line mutation in the *PRSS1* or *SPINK1* genes. It is associated with recurrent attacks of acute pancreatitis in childhood and chronic pancreatitis in adolescence and increased risk of pancreatic cancer later in life.
Inhibitor of apoptosis proteins (IAPs): a family of caspase inhibitors that specifically inhibit caspases and, thereby, prevent apoptosis.
Interferon-γ (IFN-γ): a proinflammatory cytokine produced by activated lymphocytes. It is a potent activator of monocytic-cell functions.
NOD mice: non-obese diabetic mice are a model for type 1 diabetes, exhibit spontaneous autoimmunity that causes diabetes through destruction of pancreatic islets.
NOD–SCID mice: the severe combined immunodeficiency (SCID) mutation has been transferred onto diabetes-susceptible NOD mice.
Pancreatic stellate cells (PSCs): lipid and vitamin-A storing cells in the pancreas that, in response of injurious stimuli, undergo transformation from their resting or quiescent state to activated myofibroblast-like cells. They have a major role in extracellular-matrix synthesis and produce cytokines and chemokines.
TRAIL: tumor necrosis factor associated apoptosis-inducing ligand is a type II membrane bound TNF superfamily ligand. It is highly homologous to CD95L and exists in a membrane-bound and in a soluble form after proteolytic cleavage by cysteine proteases. TRAIL displays widespread expression and is upregulated upon lymphocyte activation. TRAIL receptors belong to TNF-receptor superfamily and five have been characterized. The first of the TRAIL receptors to be identified was TRAIL receptor 1 (also known as DR4), which bears an intracytoplasmic death domain and mediates apoptosis. TRAIL receptor 2 (also known as DR5) bears intracytoplasmic death domain and, similar to TRAIL receptor 1, mediates apoptosis. TRAIL receptor 3 (also known as DcR1) lacks the death domain and is anchored to the membrane via glycophosphatidyl inositol, whereas TRAIL receptor 4 (also known as DcR2) has a truncated and non-functional intracytoplasmic death domain. Therefore, TRAIL receptor 3 and TRAIL receptor 4 are thought to act as 'decoy' receptors. However, there are evidences for an alternative functional role of TRAIL receptor 4: it has been shown to signal to NF-κB upon TRAIL-binding in Jurkat T cells. The fifth TRAIL receptor, known as osteoprotegerin, exists in a dimeric, soluble secreted form. It has been shown to have a role in osteogenesis and inhibition of apoptosis in Jurkat T cells.
Tropical pancreatitis: a form of pancreatitis that occurs in children in Africa and Asia attributed to malnutrition.

activation of pancreatic enzymes and subsequent immune responses, as evidenced by inflammatory infiltrates. It has been proposed that acute pancreatitis initiates a sequence of perilobular fibrosis, duct distortion and altered pancreatic secretions. Over time and following multiple episodes, this can lead to loss of pancreatic parenchyma and fibrosis [7].

New developments in chronic pancreatitis

Irrespective of the underlying etiology, the morphological changes in CP follow an almost identical pattern. New concepts that integrate cellular, genetic and molecular mechanisms have been suggested to explain the pathogenesis of CP. The sentinal acute pancreatitis event (SAPE), according to which unregulated trypsin activation initiates the first episode of acute pancreatitis (sentinal event) is one of these concepts. Trypsin activation and inactivation are regulated by Ca^{2+}. Stimulation with cholecystokinin evokes a sustained rise in Ca^{2+} and induces pronounced trypsin activation and extensive vacuole formation in the apical region of acinar cells [8]. Furthermore, bile acids and non-oxidative alcohol metabolites can elicit abnormal cytosolic Ca^{2+} signals that result in necrosis and acute pancreatitis [9]. According to SAPE, cytokines that are liberated during the early phase of inflammation attract a distinct cellular infiltrate, whereas pro-fibrotic cells, including stellate cells, are characteristic of the late phase of acute pancreatitis. The attraction and activation of pancreatic stellate cells (PSCs) set the stage for the development of pancreatic fibrosis. The resulting scarring leads to the development of strictures that block the pancreatic ducts that, in turn, predisposes to recurrent attacks of acute pancreatitis. These observations, along with the recently discovered genetic mechanism of hereditary pancreatitis [10,11] and the pathological case series [12], indicate a close relationship between acute pancreatitis, recurrent acute pancreatitis and the development of CP. In up to 30% of patients, CP is not associated with any of the known processes and, therefore, it is known as idiopathic. There is accumulating evidence that many of these cases have a genetic basis.

Protease serine (trypsin) 1

Whitcomb *et al.* [11] identified the third exon of the cationic trypsinogen gene on chromosome 7q35. Hereditary pancreatitis is caused by germ-line mutations in the cationic trypsinogen gene also known as protease serine (trypsin) 1 (*PRSS1*). The most common *PRSS1* mutation results in an Arg to His substitution, thereby eliminating the site that is essential for the rapid self-destruction of trypsin in solutions. Therefore, trypsin becomes resistant to inactivation. The abnormally active trypsin results in development of acute pancreatitis, and recurrence can lead to CP [11].

Serine protease inhibitor Kazal type 1

The serine protease inhibitor Kazal type 1 (SPINK-1) inhibits trypsin activity. The mutation of *SPINK1* gene, causing loss of SPINK-1 function, predisposes to increased risk of recurrent acute and chronic pancreatitis [13].

Cystic fibrosis transmembrane conductance regulator

Cystic fibrosis is caused by mutations in the cystic fibrosis transmembrane conductance regulator gene *CFTR*. CFTR is a key molecule expressed in pancreatic ducts. Some mutations in the *CFTR* gene might be pancreas specific. *CFTR* mutations associated with loss or decrease of bicarbonate secretion cause recurrent acute and chronic pancreatitis [14].

Therefore, the concept of SAPE incorporates the molecular mechanism of pathogenesis and unifies previous theories [6], suggesting that CP is the result of recurrent and/or sustained immune activation that is cytotoxic to acinar cells. This immune activation is followed by stimulation of anti-inflammatory responses that cause fibrosis in which PSCs have a pivotal role.

Pancreatic stellate cells

In recent years, the identification and characterization of PSCs have initiated numerous studies focused on the determination of their exact role in pancreatic fibrogenesis [15–18]. In their quiescent state, PSCs are peri-acinar in

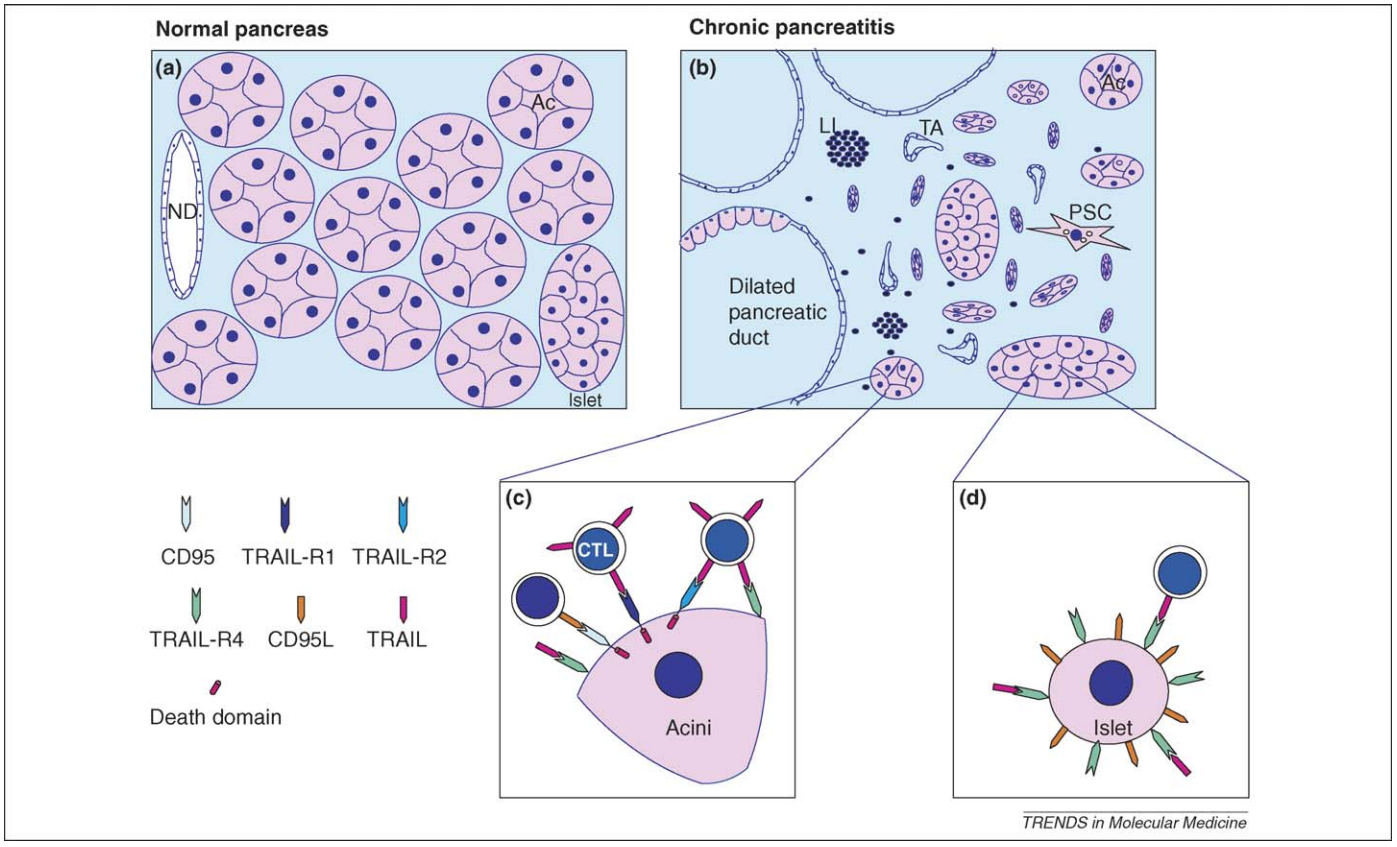

Figure 1. Histologically, normal pancreas is composed of an exocrine part that is made up of acini (Ac) and pancreatic ducts (ND), and an endocrine part that is made up of clusters of islet cells **(a)**. In CP, acini undergo atrophy and are progressively replaced by fibrous tissue. Some acini acquire a tubular morphology [tubular acini (TA)] and scattered lymphocytic infiltrates (LI) are observed, the pancreatic ducts become dilated owing to inspissated secretions and the islets remain intact **(b)**. Acini in CP express CD95, TRAIL receptor 1 and TRAIL receptor 2 with intracellular death domains, and, only weakly, TRAIL receptor 4 with a truncated intracellular death domain **(c)**. In contrast, islet cells retain their constitutive CD95L-positive status and abundantly neo-express TRAIL receptor 4 **(d)**. Abbreviations: CTL, cytotoxic T lymphocyte; TRAIL-R1, TRAIL receptor 1; TRAIL-R2, TRAIL receptor 2; TRAIL-R4, TRAIL receptor 4.

location and store vitamin A. Upon activation by pro- and anti-inflammatory cytokines, these cells assume a myofibroblast-like phenotype [19]. This makes them capable of numerous biological functions such as proliferation, migration, synthesis and secretion of extracellular matrix components and of matrix-degrading enzymes and their inhibitors. Therefore, PSCs possess the dual capacity of synthesizing and degrading extracellular matrix components, thus suggesting that they have an important role in the maintenance of the normal architecture of the healthy pancreas [20,21].

At the same time, several other studies have attempted to explain the molecular mechanisms that are responsible for acinar loss in CP. Therefore, it is appropriate to review current trends in understanding the development of CP, particularly the novel aspects of acinar destruction versus islet preservation.

Cellular immune response in chronic pancreatitis

One area of major interest in CP is the cellular response to injury. There are conspicuous amounts of T cell subsets, natural killer (NK) cells and macrophages in the cellular infiltrates of CP [22]. In the male Wistar rat model of spontaneously developing CP, $CD8^+$ cytotoxic T cells invade pancreatic lobules and form close associations with acinar cells, some of which undergo apoptosis [23]. This provides a circumstantial evidence for an involvement of autoreactive T cells in the parenchymal destruction in CP. This is further substantiated by the observation that mice that lack major histocompatibility complex class-II (MHC-II) develop an immune-based selective loss of exocrine pancreatic parenchyma that is caused by $CD8^+$ T cells [24]. $CD8^+$ cytotoxic T cells can cause the death of target cells by multiple mechanisms [25]. Activated $CD8^+$ cytotoxic T cells are capable of contact-dependant cytotoxicity and abundant cytokine production [26]. The cytotoxicity has been ascribed to two distinct but complementary pathways, each requiring close contact between the T cell and its target. The first is the perforin–granzyme pathway, which involves release of cytotoxic granules from the effector cell towards the target. This pathway is mainly used by $CD8^+$ T cells. The second pathway involves interaction between soluble or membrane-bound death ligands (FAS ligand CD95L and tumor necrosis factor-related apoptosis-inducing ligand TRAIL) and their signal-transducing receptors FAS (also known as CD95), TRAIL receptor 1 and TRAIL receptor 2 expressed on target cells and is mainly used by $CD4^+$ T cells. When CD95L and TRAIL on activated T cells bind to CD95, TRAIL receptor 1 or TRAIL receptor 2, respectively, on acinar cells, the interaction is sufficient to trigger apoptotic cell death. A minor but significant part of cell death is attributed to a MHC-independent destruction through NK cells. In addition, activated $CD4^+$ and $CD8^+$ T cells can produce high levels

of the proinflammatory cytokine interferon-γ (IFN-γ) [26], which was detected in significantly higher amounts in CP specimens compared with normal pancreas [27]. IFN-γ upregulates the expression of MHC-II molecules and enhances antigen expression on target cells, thus sustaining inflammation and stimulating non-specific effector cells such as macrophages and NK cells [28]. Intriguingly, endocrine islets remain intact even in later stages of CP, whereas the exocrine parenchyma is progressively destroyed [29].

Death receptors and their ligands

It has been shown that normal pancreatic islets express the CD95L but do not express its receptor CD95 [27]. In CP, this 'immunoprivileged' status is preserved in endocrine cells, whereas the exocrine epithelia lose this status and neo-express CD95 along with MHC-II [27] and become, therefore, sensitive targets for activated T cells equipped with CD95L in the adjacent inflammatory infiltrate. Thus, the binding of CD95L on lymphocytes to CD95 that is induced on the surface of acinar cells under the influence of IFN-γ is lethal for acinar cell, whereas islet cells secure themselves by conserving their CD95L positive status.

Furthermore, normal pancreas lacks TRAIL receptors. In CP, there is a strong induction of the death-signal-transducing TRAIL receptor 1 and TRAIL receptor 2 in exocrine cells (Figure 1). By contrast, islet cells abundantly express TRAIL receptor 4 and almost completely lack TRAIL receptor 1 and TRAIL receptor 2. TRAIL that is produced locally by PSCs binds to TRAIL receptor 1 and TRAIL receptor 2 on acinar cells inducing their apoptosis [30] (Figure 1). How does TRAIL receptor 4 expression promote survival of islet cells? TRAIL receptor 4 lacks the cytoplasmic death domain and has been therefore regarded as a decoy receptor. Moreover, recent studies that have been carried out in T cells propose an alternative functional role of TRAIL receptor 4. TRAIL receptor 4 does not transmit a direct death signal but increasing evidence suggests that, upon binding to TRAIL, it activates NF-κB [31], which might protect islets cells. In two independent experimental studies, TRAIL has been shown to protect islet cells in experimental type 1 diabetes in mice [32,33]. Mi *et al.* [33] reported upregulation of the gene encoding TRAIL in islet cells during the development of diabetes in non-obese diabetic (NOD) mice. The fact that freshly isolated islet cells are TRAIL resistant and that TRAIL blockade exacerbates the onset of type 1 diabetes in NOD–severe combined immunodeficiency (SCID) recipients of transferred diabetogenic T cells and in cyclophosphamide-treated NOD mice points to a protective effect of TRAIL during the onset of autoimmune diabetes. Furthermore, Lamhamedi-Cherradi *et al.* [32] treated normal and TRAIL-deficient mice with multiple low-dose of streptozotocin to induce diabetes. The authors observed that both the incidence and degree of inflammation were significantly enhanced in TRAIL-deficient animals. In CP, TRAIL might have comparable protective influence on islet cells by binding to TRAIL receptor 4 and, thereby, activating NF-κB. Another recently reported mechanism by which NF-κB might be activated in islet cells is the CD40 receptor that is expressed in mouse and human β cells. The expression of this receptor is regulated by proinflammatory stimuli [34]. The consequences of NF-κB activation on the two epithelial compartments of pancreas are completely converse.

Is NF-κB protective or detrimental?

NF-κB is an inducible dimeric transcription factor that comprises one or two of the five Rel family members: RelA, c-Rel, RelB, NF-κB1 and NF-κB2. NF-κB is expressed ubiquitously, recognizes a common sequence motif and regulates an exceptionally large number of genes in response to infections, inflammation and other stress-related stimuli [35]. It was observed by our group that normal pancreas is characterized by low expression levels of NF-κB subunit transcripts in both exocrine and endocrine compartments [3]. In CP, we found a maximal induction of inhibitor of κB kinase (IKK)-γ and RelA protein in islets. This increase in islets is strongly correlated with the degree of fibrosis. Furthermore, there is a fibrosis-associated increase in mRNA levels of NF-κB1, NF-κB2, RelA, RelB and c-Rel in islet cells, whereas the only transcripts that are increased in acini are those of NF-κB2. TRAIL, via TRAIL receptor 4, which is widely expressed in CP, might be one, if not the major, factor bringing about the scenario of acinar destruction and islet-cell preservation [3] (Figure 2).

At present, there is a wealth of data on the defensive and protective role that NF-κB has in the acute phase of inflammation [36,37]. NF-κB limited the tissue damage in the cerulein model of acute pancreatitis (where administration of cholecystokinin analogue cerulein is used to induce pancreatitis is Wistar rats), whereas blocking NF-κB activation had an adverse effect by causing more tissue damage by increasing apoptosis [38]. Conversely, intraductally administered *in vivo* gene transfer of RelA led to NF-κB activation, acinar damage and acute pancreatitis in rats [39]. It has also been shown that inhibition of NF-κB activation or translocation might be useful in preventing islet-cell dysfunction and death [40–43]. Thus, it remains unclear whether NF-κB activation in the pancreas is protective or detrimental [44]. It has been suggested that RelA and c-Rel are functional antagonists with respect to apoptosis induction. RelA overexpression inhibits apoptosis, whereas c-Rel acts in a pro-apoptotic way by enhancing the expression of TRAIL receptor 1 and TRAIL receptor 2, or by suppressing inhibitor of apoptosis proteins (IAPs) [45]. It has also been suggested that NF-κB2 might be a direct activator of programmed cell death [46,47]. Remarkably, in acini of CP with severe fibrosis, it seems that elevated NF-κB2 mRNA expression eventually tilts the balance in favour of apoptotic cell death in the exocrine compartment, whereas islets resist the insult [3]. It is likely that, in CP, there is a reprogramming of endocrine and, to a smaller degree, exocrine cells for an altered state of NF-κB-regulated transcriptional activity, the functional consequences of which, at the single-cell level, remain to be determined. One mechanism by which NF-κB inhibits apoptosis is the regulation of the family of genes encoding IAPs [48,49].

IAPs are a family of caspase inhibitors that specifically inhibit caspase 3, caspase 7 and caspase 9 and, thereby,

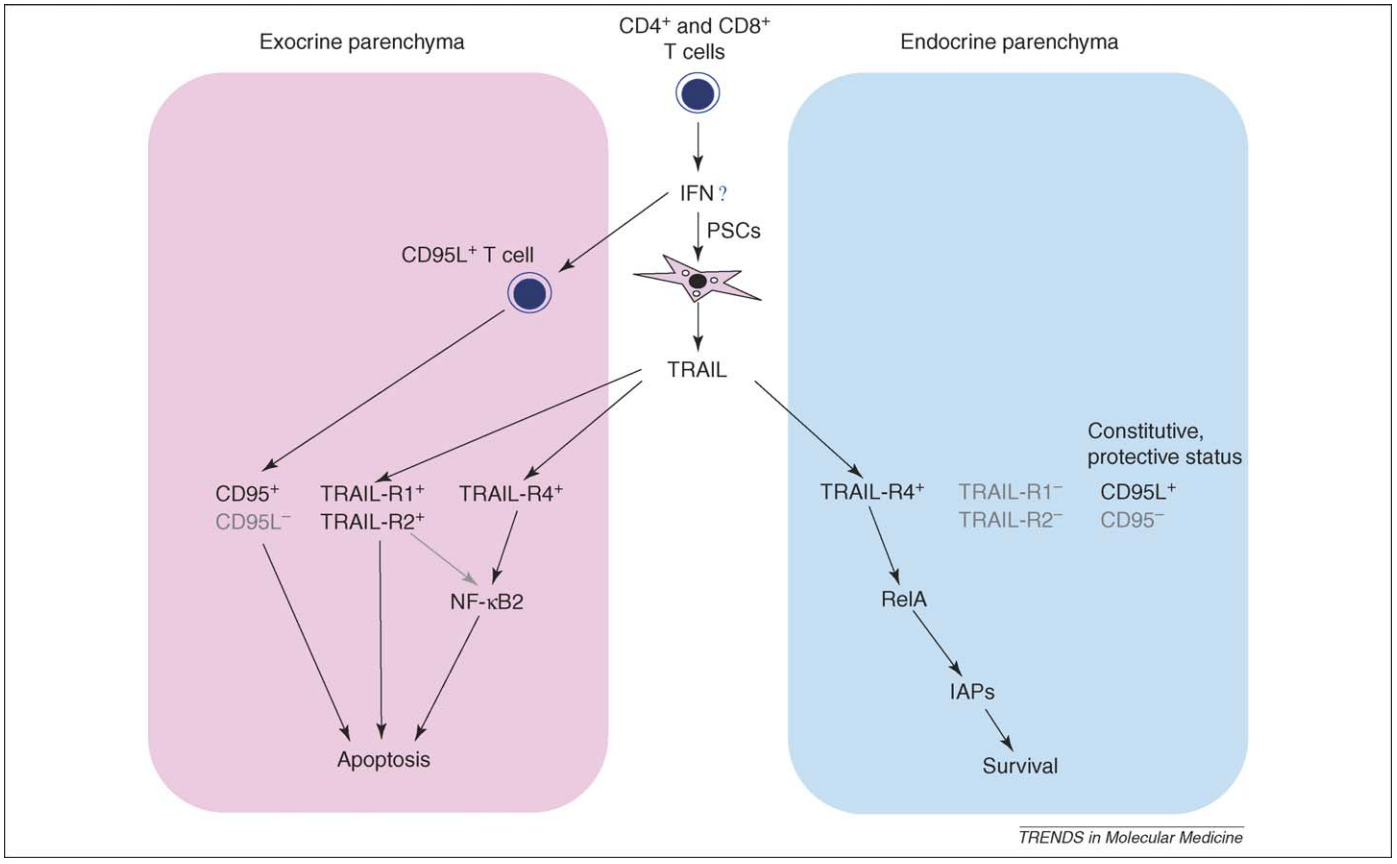

Figure 2. Molecular alterations in the two major epithelial compartments of the pancreas during CP. In a process initiated by IFN-γ, which is locally released by $CD4^+$ T helper 1 and $CD8^+$ T cells, exocrine pancreatic epithelia lose their 'immunoprivileged' CD95L-positive status and neo-express functional death receptors CD95, TRAIL receptor 1 and TRAIL receptor 2. This makes them vulnerable to apoptosis by $CD95L^+$ T cells and soluble TRAIL, which is produced by PSCs under the influence of IFN-γ. In addition, in acini there is a heavy induction of NF-κB2, which is a direct activator of programmed cell death. By contrast, IFN-γ and TRAIL have favourable consequences for the endocrine cells by activating RelA-induced anti-apoptotic program. In CP, islet cells not only retain their protective CD95L-positive, death receptor-negative state but also respond to IFN-γ–TRAIL via TRAIL receptor 4 signaling. TRAIL receptor 4 does not transmit a direct death signal but activates RelA, which in turn activates IAPs. Abbreviations: TRAIL-R1, TRAIL receptor 1; TRAIL-R2, TRAIL receptor 2; TRAIL-R4, TRAIL receptor 4.− indicates no expression, whereas + indicates expression.

prevent apoptosis [48,50]. A pattern of fibrosis-associated enhancement of cellular (c)IAP1 and survivin protein expression similar to that of RelA emerge in islets in CP [3]. This suggests that sustained enhanced expression of NF-κB subunits and, consequently, IAPs are lifeguards for endocrine cells in the adverse microenvironment of CP [3,48]. In a recent report, it has been shown that survivin induces global changes in β cells that might contribute to generalized escape from cell death through inhibition of multiple apoptotic pathways [51]. Conversely, the pattern of NF-κB activation is detrimental for the exocrine parenchyma. One of the key regulators of this reprogramming might be TRAIL. Therefore, the two major epithelial components of the pancreas have different fates in different chronic inflammatory diseases. In CP, where the exocrine parenchyma is progressively lost, the endocrine islets remain intact and functional, even in advanced stages of fibrous replacement. Vice versa, in type I diabetes the islet cells vanish in the course of insulitis, whereas the exocrine parenchyma is unaffected. In both processes, apoptotic cell death, which is prevented by CD95L and TRAIL, is the key event [52], supporting the current understanding that immune attacks are cell-type specific. Nevertheless, chronic inflammation functions via local release of chemokines, cytokines and soluble death-receptor ligands that lack the specificity of cognate T-cell–target-cell interaction. These short-range acting molecules not only fuel the directed attack but are also likely to inflict collateral damage [53], if targets and innocent bystanders are so intimately assembled. Therefore, survival programs that are run by exocrine and endocrine cells regulating their propensity to be resistant or sensitive to apoptosis differ in CP and type 1 diabetes. However, the death pathways and their interfering inhibitors are likely to be the same in both cell types. Against this background, we have proposed a model of CP that leads to enforced death of exocrine cells and, at the same time, to armoured survival of endocrine cells (Figure 2).

Dichotomy of fates of pancreatic epithelia in chronic pancreatitis

Exocrine pancreatic epithelia, in a process initiated by IFN-γ, which is locally released by $CD4^+$ T helper 1 and $CD8^+$ T cells, lose their immunoprivileged CD95L-positive status and neo-express functional death receptors CD95, TRAIL receptor 1 and TRAIL receptor 2. This renders them vulnerable not only to CD95L-mediated T-cell attack but also to the action of soluble TRAIL that is released by IFN-γ-triggered PSCs. For exocrine cells, the matters are further worsened by heavy induction of NF-kB2, which is a

Box 1. Outstanding questions

- Does inhibition of proinflammatory cytokines (e.g. IFN-γ) prevent inflammation?
- Can inhibition of CD95–CD95L, TRAIL receptor 1–TRAIL and TRAIL receptor 2–TRAIL prevent acinar apoptosis in CP?
- Is NF-κB, protective or detrimental? The exact role of its different subunits needs to be determined.
- Could survivin be used in gene therapy for improving survival of islet-cell transplants?
- Can inhibition of the perforin–granzyme pathway prevent acinar destruction?
- Can activation of PSCs be reversed or prevented?
- Is therapeutic apoptosis induction an option?

direct activator of programmed cells death. By contrast, IFN-γ and TRAIL have favourable consequences for endocrine cells. In CP, islet cells not only retain their protective CD95L-positive, death-receptor-negative state but also respond to IFN-γ–TRAIL via TRAIL receptor 4 signaling. TRAIL receptor 4, which lacks a functional intracellular death domain, does not transmit a direct death signal but activates RelA. Thus, RelA activation and subsequent expression of IAPs provides an additional mechanism through which islet cells that are at risk of succumbing to collateral damage caused by local release of death-inducing molecules that are produced by the inflammatory infiltrate and PSCs augment their functional repertoire ensuring survival in a hostile environment [3].

Concluding remarks

The characterization of signaling pathways that trigger apoptosis in the acinar compartment and armoured survival in the endocrine compartment during the course of CP is a major step forward in the understanding of its complex pathogenesis. This knowledge might promote efforts to diminish the destruction of the exocrine pancreas via specific targeted therapy (Box 1). The functional consequences of an altered state of NF-κB-regulated transcriptional activity, at the single cell level, remain to be determined. Concurrently, further insights might enable prevention of islet-cell destruction in type I diabetes and be useful for devising approaches to prolong the life of islet-cell grafts for its treatment. It has been recently reported that strategies to protect β cells from allogenic cytotoxic attack require the inhibition of the perforin–granzyme pathway [54]. Furthermore, transplantation of survivin transgenic islets in diabetic recipient mice caused long-term engraftment and correction of hyperglycemia [51]. Therefore, more research using isolated human acinar and islet cells will be needed. At present, research efforts are limited by the lack of or inaccessibility to human-derived cell lines and the ethical issues that are involved in the use of human β cells for research. Also, generation of suitable experimental models will be crucial for further research in the field. In the future, targeting PSCs might become a promising therapeutic approach either by interruption or reversal of their activation. The other approach that is envisaged is the elimination of PSCs by therapeutic apoptosis induction [21].

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Free journals for developing countries

The WHO and six medical journal publishers have launched the Health InterNetwork Access to Research Initiative, which enables nearly 70 of the world's poorest countries to gain free access to biomedical literature through the internet.

The science publishers, Blackwell, Elsevier, Harcourt Worldwide STM group, Wolters Kluwer International Health and Science, Springer-Verlag and John Wiley, were approached by the WHO and the *British Medical Journal* in 2001. Initially, more than 1500 journals were made available for free or at significantly reduced prices to universities, medical schools, and research and public institutions in developing countries. In 2002, 22 additional publishers joined, and more than 2000 journals are now available. Currently more than 70 publishers are participating in the program.

Gro Harlem Brundtland, the former director-general of the WHO, said that this initiative was "perhaps the biggest step ever taken towards reducing the health information gap between rich and poor countries".

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Survivin Expression in Pancreatic Intraepithelial Neoplasia (PanIN): Steady Increase Along the Developmental Stages of Pancreatic Ductal Adenocarcinoma

Umesh Bhanot, MD(Path), René Heydrich, Peter Möller, MD, and Cornelia Hasel, MD

Abstract: Pancreatic ductal adenocarcinoma (PDA) is one of the most aggressive gastrointestinal cancers and is thought to arise from noninvasive precursors-pancreatic intraepithelial neoplasia (PanIN). Aberrantly prolonged cell survival due to apoptosis suppression is likely to contribute to carcinogenesis and carcinoma progression where the inhibitor of apoptosis proteins (IAPs) may play an important role. IAPs specifically inhibit caspases 3, 7, and 9 and prevent apoptosis. Survivin is a unique member of the IAPs family that is expressed in most human cancers including PDA but is not expressed in most normal adult tissues. To measure survivin transcript levels in normal pancreatic ducts, PanINs, and PDA, we used laser capture microdissection and real-time polymerase chain reaction. Survivin protein expression in normal pancreatic ducts, PanINs, PDA, and its metastases to lymph nodes were evaluated by immunohistochemistry. In microdissected tissues, we found a steady and close to exponential increase in survivin transcript levels from low-grade lesions (PanINs-1) to high-grade lesions (PanINs-2 and 3) and further to PDA. This observation was strictly mirrored by survivin protein expression. In addition, survivin was localized to the nucleus in high-grade lesions (starting at PanIN-2 stage), PDA, and nodal metastases, suggesting that nuclear translocation of survivin may be an early event in transformation to malignancy.

Key Words: PanINs, survivin, pancreatic ductal adenocarcinoma

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Pancreatic ductal adenocarcinoma (PDA) has the poorest overall prognosis among gastrointestinal cancers and continues to exact extremely high-mortality rates. Most patients with PDA have a locally advanced or metastatic disease at the time of diagnosis. Once clinically evident, PDA progresses rapidly and is largely resistant to conventional chemotherapy and radiotherapy.[15] The fact that curative resection in early-stage PDA may improve survival rates indicates the significance of early detection of PDA.[27] Therefore, the detection and treatment of preinvasive ductal lesions of the pancreas gains paramount importance as an avenue for preventing invasive PDA.

In recent times, significant progress has been made in understanding the precursors that ultimately give rise to invasive pancreatic cancer. Although, these noninvasive epithelial lesions have been recognized in the smaller pancreatic ducts for almost a century, there was a lack of uniform terminology to designate them.

In 2001, an international consensus established the "pancreatic intraepithelial neoplasia (PanIN)" nomenclature and classification system for microscopic pancreatic duct lesions.[7,8] This has led to a series of molecular studies that have helped define a progression model for pancreatic neoplasia. PanINs are thought to progress from flat and papillary lesions without dysplasia (PanIN-1A and PanIN-1B), to papillary lesions with dysplasia (PanIN-2), to lesions showing carcinoma in situ (PanIN-3). PanINs have been examined extensively for alterations in genes such as *K-ras*, *p16*, *p53*, *DPC4*, and *BRCA2*.[5,16–18,25,26]

Aberrantly prolonged cell survival owing to apoptosis suppression may contribute to carcinogenesis and carcinoma progression by providing a fertile soil for the accumulation of genetic mutations and promoting resistance to immune-based cytotoxicity.[23] The inhibitor of apoptosis proteins (IAPs) are a family of proteins characterized by domain/s of ~70 aa termed the baculoviral IAP repeat. IAPs specifically inhibit caspases 3, 7, and 9 and thereby prevent apoptosis (Deveraux and Reed, 1999). Survivin is a structurally unique member of the IAPs family because it contains only a single baculoviral IAP repeat domain and lacks a carboxy-terminal RING finger.[2] Most terminally differentiated normal adult tissues including normal pancreas (NP) either do not express survivin or express it at low levels.[2,21] Survivin is dramatically overexpressed in most human cancers[1,2] and is a reliable marker of aggressive and unfavorable disease. Therefore, there is a recent surge in literature portraying survivin as a promising molecular target for cancer therapy. Survivin is expressed in the majority of pancreatic adenocarcinomas[10,20,21,24] and survivin immunoreactivity has been studied in the transition from intraductal papillary mucinous tumor

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(IPMT) adenoma to IPMT carcinoma in situ.[9] However, to the best of our knowledge, there are no studies to date elucidating survivin along the progression of PanINs to PDA.

We determined survivin mRNA expression in microdissected, normal pancreatic ducts, PanIN lesions, and PDA. By immunohistochemistry (IHC), we detected survivin protein in PanINs and compared the results to that in NP on the one hand and PDA on the other.

MATERIAL AND METHODS

Pancreatic Tissues

Tissues from NP, chronic pancreatitis (CP), and PDA were obtained at the time of surgery, snap frozen in liquid nitrogen, and stored at −80°C. In all, 36 pancreatic specimens were included in the study. Out of 17 Whipple resection (pancreaticoduodenectomy) specimens, 14 were performed for pancreatic carcinoma, 3 for CP, and out of 14 patients who underwent partial pancreatectomy for CP, 10 were suffering from alcoholic pancreatitis, 2 had chronic nonalcoholic pancreatitis associated with pancreas divisum, 1 developed a cyst after initial surgery and 1 had cryptogenic CP. The specimens of 5 patients who underwent partial pancreatectomy for pancreas divisum were histopathologically diagnosed as NP and included in the study as normal controls. Normal pancreatic ducts (n = 4), PanINs (n = 12), and PDA (n = 4), were subjected to laser capture microdissection (LCM) to determine their survivin transcript expression.

Tissue Processing for LCM

Frozen-tissue specimens were cut at 8 μm thickness and serial sections transferred to PALM MembraneSlides (PALM, Bernried, Germany). Between 10 and 20 tissue sections were required to obtain sufficient number of cells from normal ducts, PanINs, and PDA. Sections were stained with hematoxylin and eosin (H&E) as described previously.[6]

LCM

PALM LaserMicrobeamMicrodissection System with LaserPressureCatapulting and Robocut Software (PALM, Germany) was used for microdissection. Approximately 4000 cells each from normal ducts, PanINs, and PDA were microdissected and catapulted into LCM caps with a tiny drop of inert mineral oil evenly spread on their inner surface.

RNA Isolation From Microdissected Tissue

The material from 2 to 3 caps was pooled into a single purification column. Total RNA was isolated using a Picopure RNA isolation Kit (Arcturus, Mountain View, CA) according to the manufacturer's instructions. Total RNA was qualitatively and quantitatively assessed on RNA LabChip (Agilent, Wilmington, DE) following the manufacturer's instructions. Only those samples with clearly visible ribosomal peaks and $OD_{280/260}$ ratios between 1.6 and 1.8 were further processed.

RNA Amplification and Semiquantitative Real-time PCR

The RiboAmp RNA Amlification Kit was used for amplifying the total RNA from microdissected tissues. The manufacturer's protocol for performing one round of amplification was used. The amount of RNA obtained after amplification was qualitatively and quantitatively assessed using the RNA LabChip (Agilent). RNA (2.5 μg) was reversely transcribed into cDNA using Superscript Reverse Transcriptase (Invitrogen), random hexamer primer, and addition of a RNase-inhibitor following the manufacturer's protocol for expression analysis of survivin in microdissected tissues. Primer pairs were purchased from MWG Biotech (Ebersberg, Germany). Real-time polymerase chain reaction (PCR) was performed using survivin primers (F 5′-ATGGGTGCCCCGACGTTG-3′, R 5′-AGACATTGCTAAGGGGCCCAC-3′). Cyclophilin was used as the endogenous control (forward primer: 5′-ATGGTCAACC CCACCGTGT-3; reverse primer: 5′-TCTGCTGTCTTTGGGACCTTGTC-3′). First the absence of nonspecific amplification was confirmed by analyzing the PCR amplification products by agarose gel electrophoresis. Amplicons generated from cDNA were also tested against no template control and RNA. In a further step, optimal primer concentrations were selected by mixing primer concentrations from 50 to 900 nmol of each forward and reverse primer. The curves were checked for low C_T, fast rising, and for confirmation analyzed by agarose gel electrophoresis. Real-time PCR was performed using 4 μL cDNA (12.5 ng/μL), 4 μL primer mix (forward/reverse) at optimal concentrations, 12 μL sterile distilled water, and 20 μL SYBR Green PCR Master Mix (PE Biosystems, Foster City, CA) per reaction. The following cycling conditions were set: 50°C for 2 minutes, denaturation at 95°C for 10 minutes, followed by 45 cycles at 95°C for 20 seconds each, 60°C for 20 seconds, and 72°C for 20 seconds. Gene expression of survivin of samples was measured relative to cyclophilin as an endogenous control to account for variability in the initial concentration of total RNA. Analysis of quantitation was carried out by calculating: (1) mean C_T value of 3 replicates per sample; (2) difference between mean C_T values of samples for each target and those of the endogenous controls ($\wedge C_T$); (3) difference between mean C_T values of the samples for each target and the mean C_T value of the corresponding calibrator ($\wedge\wedge C_T$). The quantitation is expressed as $2^{-\wedge\wedge C}_T$, to allow graphical presentation, and shown as x-fold expression of the target gene compared with cyclophilin set as 1.

Identification of PanINs

H&E-stained slides of pancreatic tissues from each of the cases were screened by light microscopy for the presence of PanINs by 2 authors (U.K.B. and C.H.) with agreement in all the cases. Duct lesions were classified according to the WHO classification (Hruban et al[7]). In total, 216 H&E-stained slides of pancreatic tissues were evaluated to classify PanINs, therefore, on an average 6 slides per case were available for evaluation. In addition,

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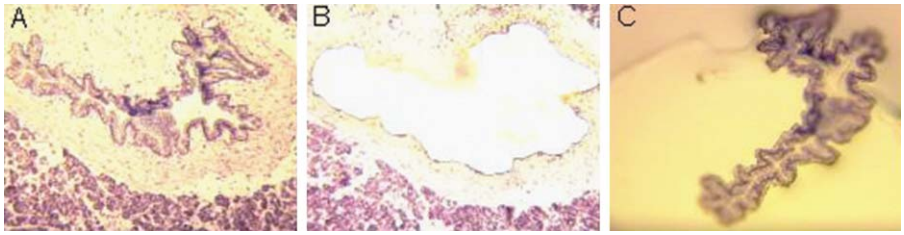

FIGURE 1. LCM of PanINs: A PanIN lesion, before (A); after (B); and in the LCM cap (C).

the slides of peripancreatic lymph nodes for metastatic deposits of pancreatic adenocarcinoma were examined. A total of 88 PanINs: PanIN-1A (n = 10), PanIN-1B (n = 30), 26 PanIN-2 (n = 26), and 22 PanIN-3 (n = 22) were identified.

IHC

For IHC, 3-μm sections were cut from formalin-fixed, paraffin-embedded surgical pathology specimen. Sections were deparaffinized in xylene and passed through descending grades of alcohol. Endogenous peroxidase activity was blocked with methanol supplemented by 3% H_2O_2 for 15 minutes. After washing with water, antigen retrieval was performed in 0.01 M citrate buffer (pH 6) using a microwave oven at 900 W for 20 minutes followed by cooling for 20 minutes. Slides were placed in phosphate buffered saline and then incubated for 1 hour with an affinity-purified rabbit antihuman survivin antibody (R&D Systems, Wiesbaden, Germany) diluted to 1:400. Bound primary antibody was detected via swine antirabbit immunoglobulins conjugated to peroxidase labeled-dextran polymer in Tris-HCL buffer containing carrier protein (EnVision, Dako, Denmark) for 30 minutes. 3-amino-9-ethyl-carbazole (Sigma, St. Louis, MA) was used as the substrate. Hematoxylin was used as counterstain. The rabbit antihuman thyroglobulin obtained from DAKO, (Copenhagen, Denmark) was used as an irrelevant isotype-matched negative control and yielded negative results.

Immunohistochemical Evaluation

Immunohistochemical evaluation of survivin immunolabeling was performed independently by 2 authors (U.K.B. and C.H.). Sections of NP in which the ductal epithelium showed no immunolabeling were taken as negative (−). Cytoplasmic staining in less than 20% of cells was considered low antigen density (+); whereas, cytoplasmic staining between 20% and 50% of cells was taken as moderate (++). Diffuse cytoplasmic staining in more than 50% of the cells was scored as high (+++); and the cytoplasmic staining in more than 50% of cells along with nuclear labeling in some was regarded very high antigen density (++++).

RESULTS

In all, 36 cases were included in the study: NP (n = 5), CP (n = 17), and PDA (n = 14). To determine survivin mRNA expression by semiquantitative real-time PCR, we microdissected NP ducts (4-cases), 12 PanIN lesions; PanINs1A (n = 4), PanINs1B (n = 3), PanINs2 (n = 3), and PanINs3 (n = 2), and PDA cells (4-cases) for comparision of survivin mRNA expression (Fig. 1).

Survivin protein expression by IHC was evaluated in 88 PanINs, PanINs-1A (n = 10), PanINs-1B (n = 30), PanINs-2 (n = 26), and PanINs-3 (n = 22), PDA (n = 14), and peripancreatic lymph nodes with metastases of PDA.

Survivin mRNA in Microdissected NP Ducts, PanINs, and PDA

Survivin mRNA expression in microdissected NP ducts, PanINs, and PDA by real-time PCR exhibited a close to exponential rise from very low levels in normal ducts to peak levels in PDA. Survivin transcript expression increased in low-grade lesions to $2.7 \times (\pm 0.07)$ in PanIN1A and $4.8 \times (\pm 0.15)$ in PanIN1B. A further increase in survivin transcript levels was observed in high-grade lesions, PanIN2-$10 \times (\pm 0.14)$, and PanIN3-$14 \times (\pm 0.25)$ compared with normal ducts. Microdissected PDA expressed the highest survivin mRNA levels

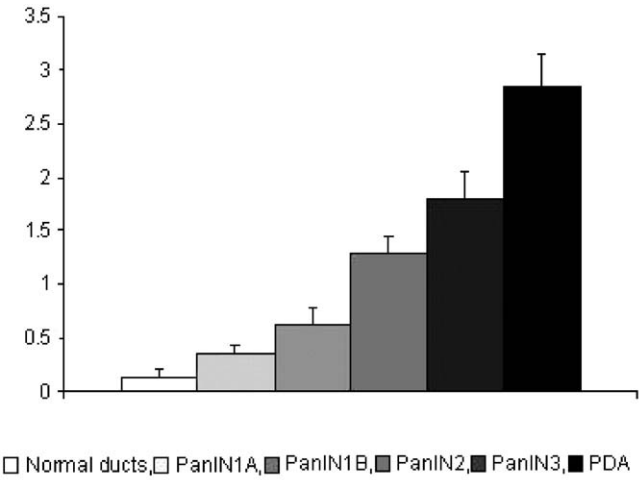

FIGURE 2. Survivin mRNA levels in laser capture microdissected NP ducts, PanINs, and PDA by real-time PCR. Survivin transcript levels depict a steady increase from low grade (PanIN1A and B) to high-grade lesions (PanIN-2 and 3) and further to PDA.

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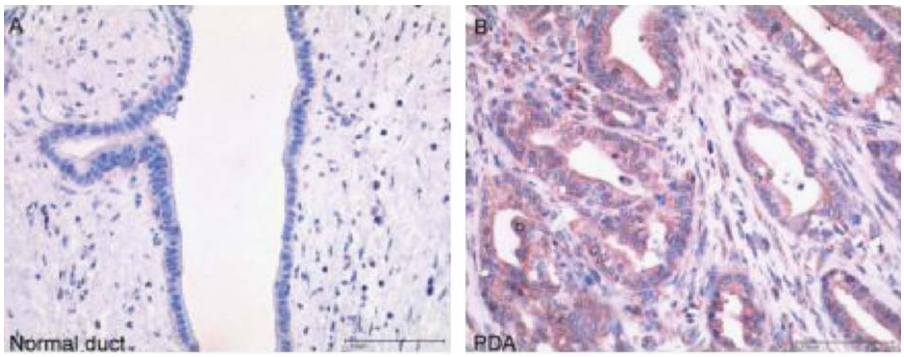

FIGURE 3. Survivin IHC in NP ducts and PDA. NP ducts do not express survivin (A), whereas in PDA there is a strong cytoplasmic expression along with nuclear expression of some cells.

with a remarkable $22 \times (\pm 0.30)$ expression compared with normal ducts (Fig. 2).

Survivin in NP Ducts, PanINs, PDA, and Lymph Node Metastases

The pancreatic ducts in NP showed no or weak expression of survivin protein (Fig. 3A). In PanINs-1A, survivin expression was detected at low levels. PanINs-1B exhibited diffuse cytoplasmic labeling at moderate levels. PanINs-2 and PanINs-3 on the other hand exhibited high antigen density (Figs. 4A–D). Nuclear labeling was conspicuous in some scattered cells in the high-grade lesions (PanINs-2 and 3) beginning first at PanIN 2 stage. All the infiltrating PDA in our study exhibited very high survivin immunolabeling (Fig. 3B) but in contrast, their associated normal pancreatic ducts were negative. Survivin immunolabeling was detected not only in the cytoplasm but also as nuclear pattern in a variable number of tumor cells. Furthermore, mitotic figures in PDA were strongly survivin positive with a clear-cut delineation of the chromosomes. It was evident that survivin immunolabeling was stronger in the tumor cells at the advancing front of the tumor in comparison to those within the tumor. Lymph node metastases of PDA revealed intense cytoplasmic labeling. Occasionally, nuclear staining was observed in our series (Fig. 4E). Further, very high survivin staining was also found in lymphatic invasion (Fig. 4F).

To summarize, the expression of survivin message and protein gradually increased from NP ducts to PanINs, and from PanINs to PDA. Survivin protein showed its peak expression in lymph node metastases, endolymphatic carcinoma cells, and tumor cells at the advancing tumor front. Nuclear localization was observed in high-grade PanINs (starting at PanIN2 stage), PDA, and metastatic deposits. Furthermore, mitotic figures exhibited survivin positivity. In conclusion, progression along PanINs and further progression from PanINs to invasive PDA and nodal dissemination of pancreatic carcinoma goes along with a steady increase in survivin expression.

DISCUSSION

Tissue homeostasis is maintained through a balance between cell proliferation and apoptotic cell death. Recent evidence suggests that alterations in cell survival pathways contribute to tumor initiation and progression.[23] The IAP family function as negative regulators counteracting apoptosis. Survivin is a recently described member of the IAP family that is expressed during fetal development in humans but rarely in adult tissues.[2] However, increased levels of survivin have been reported in most human tumors including PDA. It seems that survivin exists in 2 subcellular pools that is both cytoplasmic and nuclear[4] consistent with its function in regulation of both cell viability and cell division.[12] Moreover, changes in survivin mRNA levels are thought to correlate with increases in survivin protein and promoter activity.[22] Up-regulation of survivin has been suggested during an early step in tumorigenesis. Jinfeng et al,[9] reported cytoplasmic expression in 25% of IPMTs adenoma and 100% of IPMT CIS suggesting that survivin may play an important role in the transition from IPMT adenoma to IPMT CIS which is accompanied by a significant decrease in tumor cell apoptosis. Lo Muzio et al[14], reported increased survivin expression in 94% of precancerous oral lesions that progressed to squamous cell carcinoma whereas survivin positivity was found in only 33% of lesions that did not progress to malignancy. Furthermore, survivin expression has been reported to be markedly increased in the transition from adenoma with low-grade to high-grade dysplasia and in the transition from high-grade dysplasia to carcinoma during colorectal tumorigenesis.[11] To the best of our knowledge, survivin expression in noninvasive pancreatic duct lesions-PanINs has not been reported to date.

To determine survivin mRNA levels, we used laser capture microdissection to harvest pure cell populations from NP ducts, PanIN lesions, and PDA. Survivin transcript levels progressively increased from low-grade PanINs (PanIN1A—2.7x and PanIN1B—4.8x) to high-grade PanINs (PanIN2—10x and PanIN3—14x). Further, in microdissected PDA, survivin transcript levels were remarkably elevated that is, up to 22x compared with NP ducts. On the protein level, we found no or very

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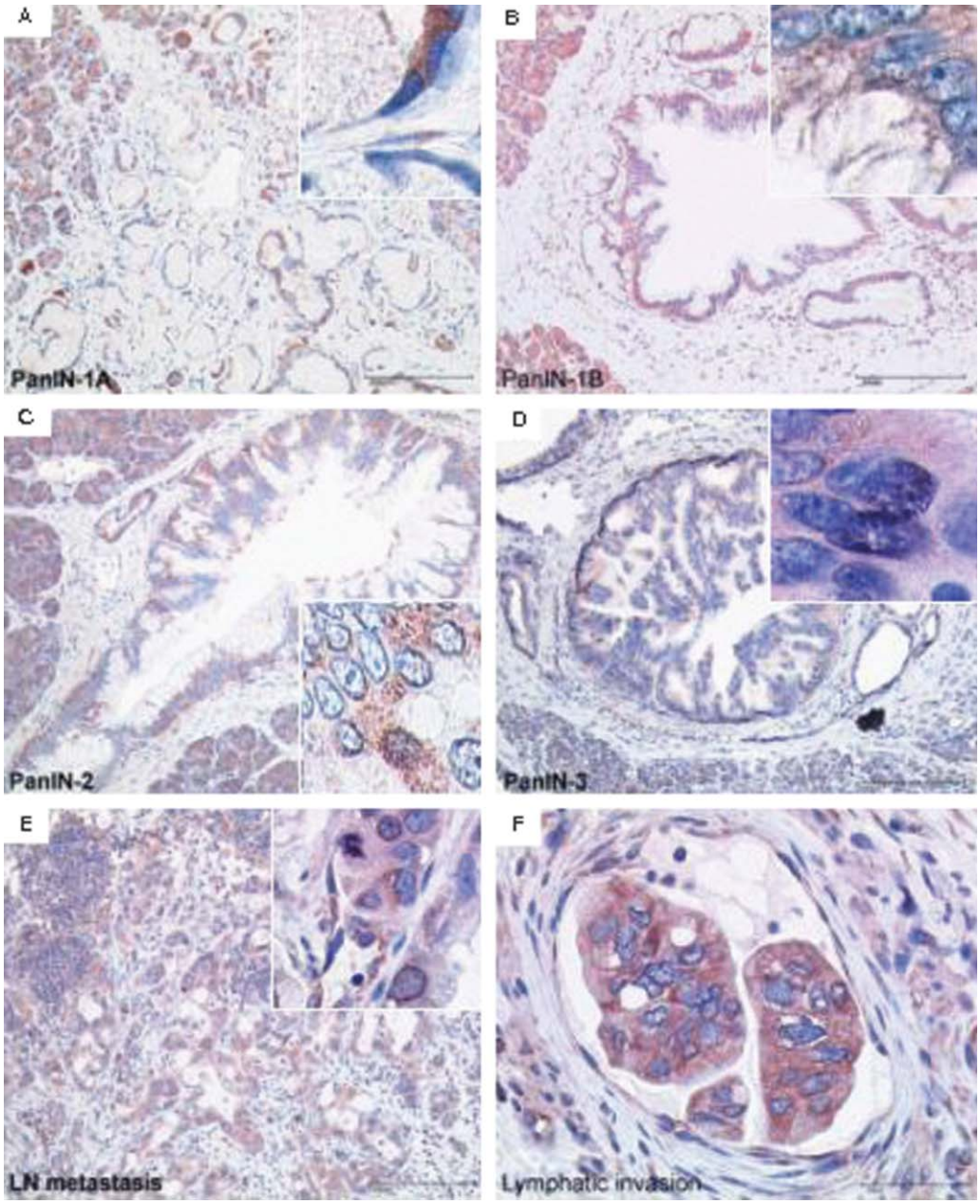

FIGURE 4. Survivin is expressed in a few scattered cells in PanIN1A, higher magnification is shown in the inset (A); whereas PanIN1B showing survivin expression in a majority of cells, higher magnification in the inset (A) (B); in PanIN2 a diffuse cytoplasmic immunolabeling is shown along with nuclear staining (see inset for higher magnification) (C); PanIN3 exhibiting a strong cytoplasmic labeling with some nuclei also labeled with survivin (inset) (D). Lymph node metastases of PDA show diffuse, strong cytoplasmic labeling along with nuclear staining in some cells (see inset), a mitotic figure labeled for survivin is shown (inset) (E); intense survivin immunolabeling is exhibited by lymphatic invasion of PDA (F).

low survivin expression in NP ductal epithelium. PanINs-1A showed low levels of cytoplasmic labeling , whereas PanINs-1B exhibited a moderate level of cytoplasmic expression. In PanINs-2, the immunolabeling was found to be at a high level with occasional nuclear staining. Survivin expression was further accentuated in PanINs-3 along with an increased number of nuclei showing positivity. Compared with Sarela et al[20] and Satoh et al[21] who reported survivin expression in 88% and 77% of their PDA cases, respectively, we detected moderate to intense survivin immunolabeling in all our PDA cases. Additionally, we observed strong immunolabeling at the invading front of PDA, with metastatic deposits of PDA in lymph nodes and lymphatic invasion exhibiting the most pronounced cytoplasmic immunolabeling. Scattered nuclei and mitotic figures in PDA also

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stained for survivin. In a recently published study on expression profiles in microdissected PanINs, authors suggested that PanIN-2 rather than PanIN-1B represents the earliest truly neoplastic lesion in the progression of pancreatic carcinogenesis.[3] Our observations gain even more ground because it has been suggested that nuclear survivin is involved in promoting cell proliferation.[13] Our finding of nuclear survivin labeling starting at PanIN-2 along with a significant 10x elevated survivin transcript levels in microdissected PanINs-2 supports these observations.

A number of studies have been carried out in various tumors to determine prognostic importance of survivin both at the level of message and protein. Increased survivin mRNA has been reported as a poor prognostic factor in a number of malignancies. However, Sarela et al[20] reported no association between survivin expression and survival in PDA whereas Kami et al[10] reported significantly shorter survival of patients with survivin positive versus survivin negative tumor. There are conflicting reports about the significance of nuclear translocation of survivin. Translocation of survivin from the cytoplasm to the nucleus is thought to constitute an important regulatory mechanism for cell proliferation and differentiation in hepatocellular carcinoma where nuclear localization of survivin correlated with tumor cell dedifferentiation.[19] In a recent study in pancreatic cancer patients, it has been reported that patients with high nuclear survivin staining in their tumors showed a longer survival whereas those with high cytoplasmic survivin staining had a shorter survival.[24]

We observed a gradual increase in cytoplasmic survivin expression which went along with the increasing grade of PanINs to PDA and was maximum in lymph node metastasis and lymphatic invasion. Nuclear translocation of survivin was evident in high-grade PanINs (PanINs 2 and 3), PDA, and metastases. Thus, our findings indicate that nuclear translocation of survivin may be an early event in the progression of PanINs toward malignancy.

In conclusion, our results on survivin expression and its localization in PanINs suggest that survivin staining could help identify lesions at risk for malignant transformation.

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Original Paper

Evidence of Notch pathway activation in the ectatic ducts of chronic pancreatitis

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Abstract

Ductal concretions in chronic pancreatitis (CP) are one of the causes of ductal obstruction, resulting in pancreatic ductal hypertension (PDH) and duct ectasia. Ductal epithelium subjected to chronic stress by PDH may undergo molecular alterations, thereby not only initiating and sustaining the inflammatory process but also activating molecules that have transforming potential. Acino-ductal metaplasia and pancreatic intraepithelial neoplasia (PanIN) are frequently seen in CP. Using laser capture microdissection, cDNA microarrays and Ingenuity Pathways Analysis, we found an altered Notch pathway in the ectatic ducts of CP. The microarray data was further validated by real-time PCR. We also found elevated transcripts of Notch receptors, Notch1 and Notch3 in microdissected ectatic ducts of CP. The Notch pathway ligands, Jagged/Delta-like and a Notch target, HES-related repressor protein (HERP), were up-regulated in ectatic compared to normal pancreatic ducts, while another target of Notch, hairy/enhancer of split (HES), was down-regulated. The transcripts of Delta-like1 and Jagged1 were increased 3.7-fold and 1.3-fold, respectively, while those of HERP1 were elevated 2.4-fold in the ectatic ducts of CP, compared to normal ducts. Immunohistochemistry showed that Jagged1 was not expressed in normal pancreatic ducts, while it was highly expressed in ectatic ducts. This pattern of Notch component alteration in ectatic ducts was mimicked to some extent *in vitro* in a human pancreatic duct epithelial (HPDE) cell line, when subjected to a pressure of 200 mmHg for 24 h. Therefore, we conclude that in the ectatic ducts of CP, PDH activates signalling pathways such as Notch, which have transforming potential.

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Keywords: Notch; chronic pancreatitis; duct ectasia; Jagged1; Delta-like1; HERP1

Introduction

Chronic pancreatitis (CP) is a destructive inflammatory process of multifactorial origin [1]. Irrespective of aetiology, the histopathological features of CP comprise inflammation, irregular glandular sclerosis, focal, segmental or diffuse destruction of exocrine parenchyma and eventual loss of endocrine parenchyma in advanced stages. The theory of ductal obstruction by concretions is one of the four major theories that explain the pathogenesis of CP [2]. Some of the inciting agents, such as alcohol, are believed to increase protein concentrations in the pancreatic juice. The proteins form ductal plugs that are observed in most forms of CP and are particularly prominent in alcoholic CP. Duct obstruction due to any cause plays an important role in the pathogenesis of CP. Recently, Yamamoto *et al* showed, in their experimental rat model of CP, that a persistent pancreatic ductal hypertension (PDH) for 2 weeks induced morphological changes similar to human CP [3]. Formation of strictures and ductal concretions in the early stages of the disease frequently cause duct obstruction, leading to PDH and duct ectasia. Moreover, metaplastic and early neoplastic lesions are frequently observed during the course of CP. Acino-ductal metaplasia was observed in male Wistar rats following pancreatic injury induced by duct ligation [4]. It is also thought that chronic inflammation produces alterations in the microenvironment of ductal epithelium that may increase the risk of neoplastic transformation by increased genomic damage and cellular proliferation [5,6]. During repair following parenchymal injury to the pancreas, genetic programmes that operate during development of pancreas are reinstated and may also play a role in neoplastic transformation [7]. Notch is one such signalling pathway that is required for the proper development of pancreas and is reactivated during pancreatic injury and ensuing repair along the course of CP and pancreatic neoplasia [8,9].

An evolutionarily conserved pathway, Notch regulates various aspects of cell differentiation, proliferation and apoptosis in a tissue- and context-dependent

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manner. Four Notch receptors (Notch1–Notch4) and five ligands [Jagged1, Jagged2, Delta-like1 (Dll-1), Dll-3, and Dll-4] have been described in mammals [10,11]. Notch signalling is activated by interactions of adjacent cells via cell–cell contact of the membrane-associated Notch receptor and ligand. After ligand binding, two enzymatic cleavages occur to release the Notch intracellular domain (NICD) from the plasma membrane. The NICD translocates into the nucleus and binds to members of the CSL transcription factor family, which are thought to mediate most of the downstream effects of Notch signalling. Following NICD binding, the CSL family member CBF-1/RBP-Jκ, normally part of a co-repressor complex with histone deacetylase-1, becomes a transcriptional activator. Downstream targets of CBF-1 include a large family of β-helix–loop–helix (βHLH) transcription factors known as the hairy/enhancer of split (HES) and HES-related repressor protein (HERP). Alterations of these functions of Notch have been associated with different types of cancer, with some exceptions [12]. Evidence is also emerging in support of an independent signalling function of activated intracellular domains of Jagged and Delta-like [13].

In the present study, we aimed to study the molecular alterations in pancreatic duct ectasia resulting from long-standing obstruction and PDH. Using oligonucleotide microarrays, pathway analysis and real-time PCR, we show a differential regulation of Notch receptors, ligands and targets in microdissected normal pancreatic ducts versus ectatic ducts of CP. Furthermore, these alterations are reflected to an extent *in vitro* in a human pancreatic duct epithelial (HPDE) cell line when subjected to a sustained elevated hydrostatic pressure.

Materials and methods

Pancreatic tissues

Ten patients, whose informed consent was obtained prior to surgery, were chosen for this study. Five patients who underwent partial pancreatectomy for CP were suffering from alcoholic pancreatitis. Five patients underwent partial pancreatectomy for pancreas divisum. The specimens of these patients were histopathologically diagnosed as normal pancreas (NP) and included in the study as normal controls. These CP specimens and normal controls were a part of the tissue panel used in our previous publication on the molecular mechanisms of CP [14]. All pancreatectomy specimens were immediately transferred to the laboratory, where representative tissue samples were snap-frozen in liquid nitrogen and kept there until further processing. Other parts of the tissues were stored at −80 °C for later use in immunohistochemistry and laser capture microdissection. This study was approved by the local ethics committee.

Tissue processing for laser capture microdissection (LCM)

Frozen-tissue specimens were cut at 8 μm thickness and serial sections transferred to PALM® Membrane Slides (PALM, Bernried, Germany); 4–10 tissue sections were required to obtain sufficient number of cells from normal pancreatic ducts and distended pancreatic ducts. Sections were stained with haematoxylin and eosin (H&E), as described previously [14].

LCM

A PALM® Laser Microbeam Microdissection System with Laser Pressure Catapulting and Robocut Software (PALM) was used for microdissection. Approximately 2000 cells from normal pancreatic ducts and distended ducts of CP were microdissected and catapulted into LCM caps.

RNA isolation from microdissected tissue and preparation of labelled cRNA

The material from two or three caps was pooled in a purification column. Total RNA was isolated using Picopure™ RNA isolation Kit (Arcturus, Mountain View, CA, USA) according to the manufacturer's instructions. Total RNA was qualitatively and quantitatively assessed on RNA LabChip (Agilent, Wilmington, DE, USA), following the manufacturer's instructions. Only those samples with clearly visible ribosomal peaks and $OD_{280/260}$ ratios of 1.6–1.8 were processed further. Preparation of cRNA was performed using the RiboAmp™ RNA Amplification Kit; the manufacturer's protocol for performing one round of amplification was used. Briefly, 5 μg total RNA was used to generate double-stranded cDNA incorporating a T7 promoter. A purification column was used for cDNA purification. Labelled cRNA was prepared from the double-stranded cDNA by *in vitro* transcription by T7 RNA polymerase in the presence of biotin-11-CTP and biotin-16-UTP (Enzo, Farmington, NY, USA).

Human pancreatic duct epithelial (HPDE) cells

HPDE cells were kindly provided by Professor MS Tsao, Princess Margaret Hospital and Ontario Cancer Institute, Toronto, Ontario, Canada. The HPDE cell line was established by transduction of *HPV16–E6E7* genes into primary cultures of normal pancreatic duct epithelial cells and demonstrated a near-normal genotype and phenotype [15,16]. HPDE cells were grown in a keratinocyte serum-free medium supplemented with bovine pituitary extract and epidermal growth factor (Gibco) [15,16] and maintained at 37 °C in 5% CO_2.

Pressure conditions

HPDE cells were placed in a previously described pressurized chamber based on the Flexcell Strain Unit

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and subjected to a sustained pressure of 200 mmHg for 24 h [17,18]. In short, the pressurized chamber enables the exertion of sustained pressure on cells *in vitro*, with a dynamic airflow and a defined membrane extension regulated by spacers. During operation up to 220 mmHg, O_2 partial pressures and pH in the cell culture medium do not change compared to control cultures kept at normal atmosphere [17,18]. HPDE cells kept under identical culture conditions without pressure were used as controls.

RNA isolation from HPDE cells

Total cellular RNA was isolated using the RNeasy RNA isolation system (Qiagen, Valencia, CA) and following manufacturer's recommendations. RNA was assessed by agarose gel electrophoresis, and quantitated by UV absorbance.

Microarray analysis

Microarray analysis was performed at the Microarray facility of the Medical Faculty of the University of Tübingen, Germany. Labelled, fragmented cRNA (15 μg) was hybridized for 16 h at 45 °C to a Human Genome U133 + 2.0 genome array (Affymetrix) containing more than 54 000 transcripts. After hybridization, the microarrays were automatically washed and stained with streptavidin–phycoerythrin, using a Fluidics Station 450. The probe arrays were scanned at 1.4 μm resolution, using a Genechip System 3000.

For downstream analysis, Affymetrix Cel files were imported into ArrayAssist 3.3 (Stratagene) and GC content (GC–RMA) normalized for further analysis. To filter for transcripts that were differentially expressed between two conditions, the signals were first filtered for an absolute change in signal level of two-fold. The remaining transcripts were subjected to statistical analysis, using a *t*-test with Benjamini–Hochberg false-discovery rate for multiple testing correction. Transcripts with a fold change of two and a corrected *p* value of 0.05 were considered statistically significant. Categorization was based on the NetAffx annotations (https://www.affymetrix.com/analysis/netaffx/index.affx).

Pathway analysis

A total of 672 genes, defined by the criteria described above, were used for pathway analysis with Ingenuity Pathways Analysis (IPA) software, version 3.1 (Ingenuity® Systems). The identified genes were mapped to genetic networks available in the Ingenuity database and were then ranked by score. The score was defined by the probability that a collection of genes equal to or greater than the number in the respective network could be achieved by chance alone. In this context, a value of 3 indicates that there is a 1/1000 chance that the focus genes are in a network due to random chance. Therefore, scores of 3 or higher have a 99.9% confidence of not being generated by random chance alone.

RNA amplification and semiquantitative real-time PCR

The RiboAmp™ RNA Amplification Kit was used for amplifying the total RNA from microdissected tissues for real-time PCR. The manufacturer's protocol for performing one round of amplification was used. The amount of RNA obtained after amplification was qualitatively and quantitatively assessed using the RNA LabChip (Agilent). 2.5 μg RNA was reversely transcribed into cDNA, using Superscript Reverse Transcriptase (Invitrogen), random hexamer primer and addition of a RNase-inhibitor, following the manufacturer's protocol for expression analysis of various Notch components in microdissected tissues and HPDE cells. Primers were designed using Primer Express Software (Perkin-Elmer, Foster City, CA, USA) and purchased from MWG Biotech (Ebersberg, Germany). The sequences of the primer pairs are shown in Table 1. Cyclophilin expression was chosen as the endogenous control (forward primer, 5′-ATGGTCAACCCCACCGTGT-3′; reverse primer, 5′-TCTGCTGTCTTTGGGACCTTGTC-3′).

The method of primer testing, optimization and cycling conditions have been described previously [14]. All quantitations were also normalized to cyclophilin as an endogenous control to account for variability in the initial concentration of total RNA. Analysis of quantitation was carried out as described previously [14].

Immunohistochemistry

Immunohistochemistry was performed on five normal and five CP cases with dilated ducts. For IHC, 1 μm sections were cut from formalin-fixed, paraffin-embedded surgical pathology specimens. The sections were deparaffinized in xylene and passed through descending grades of alcohol. Endogenous peroxidase activity was blocked with methanol supplemented by

Table 1. Sequence of primers used for real-time PCR

Gene	Primer sequence	
Notch1	Forward	5′-agc ctc aac atc ccc tac aa-3′
	Reverse	5′-cca cga aga aca gaa gca ca-3′
Notch2	Forward	5′-tgt gac ata gca gcc tcc ag-3′
	Reverse	5′-cag ggg gca ctg aca gta at-3′
Notch3	Forward	5′-gtg tgt gtc aat ggc tgg ac -3′
	Reverse	5′-gtg aca cag gag gcc agt ct -3′
Jagged1	Forward	5′-cgg cct ctg aag aac aga ac-3′
	Reverse	5′-gtc acc aag caa cag atc ca-3′
Deltalike1	Forward	5′-gga atg caa gtg cag agt gg-3′
	Reverse	5′-tct tgc agg gct tat ggt gt-3′
HES1	Forward	5′-tct gag cca gct gaa aac ac-3′
	Reverse	5′-ggt act tcc cca gca cac tt-3′
HERP1	Forward	5′-ttg tgc caa ctg ctt ttg aa-3′
	Reverse	5′-aga gcg tgt gcg tca aag ta-3′

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3% H_2O_2 for 20 min. After washing with water, the slides were rinsed in Tris–buffer, pH 7.4, followed by antigen retrieval performed by pronase treatment for 5 min on each slide. The slides were rinsed in Tris–buffer and then incubated for 1 h with rabbit polyclonal Jagged1 antibody (C-114, Santa Cruz, USA), diluted to 1 : 70. Bound primary antibody was detected via anti-rabbit immunoglobulins conjugated to peroxidase labelled-dextran polymer in Tris–HCL buffer containing carrier protein (EnVision™, Dako, Copenhagen, Denmark) for 30 min. 3-Amino-9-ethylcarbazole (Sigma, St. Louis, MO, USA) was used as the substrate. Haematoxylin was used as counterstain. Omission of the primary antibody yielded negative results, excluding false-positive signals by the detection system.

Statistical analysis

The Mann–Whitney U-test was applied for statistical comparison of microdissected normal ducts and ectatic ducts as well as untreated controls and cells exposed to pressure. The results of this test were considered significant if $p \leq 0.05$.

Results

Gene expression profiles indicate differential regulation of Notch pathway components in microdissected ectatic ducts versus normal pancreatic ducts

Gene expression profiles of laser microdissected normal pancreatic ducts versus ectatic ducts in CP showed a differential regulation of Notch pathway components, as revealed by pathway analysis (Ingenuity). In dilated duct epithelium, the expression of Notch ligands Jagged/Delta was up-regulated, along with up-regulation of a Notch target, HERP, while there was down-regulation of another Notch target, HES (Figure 1).

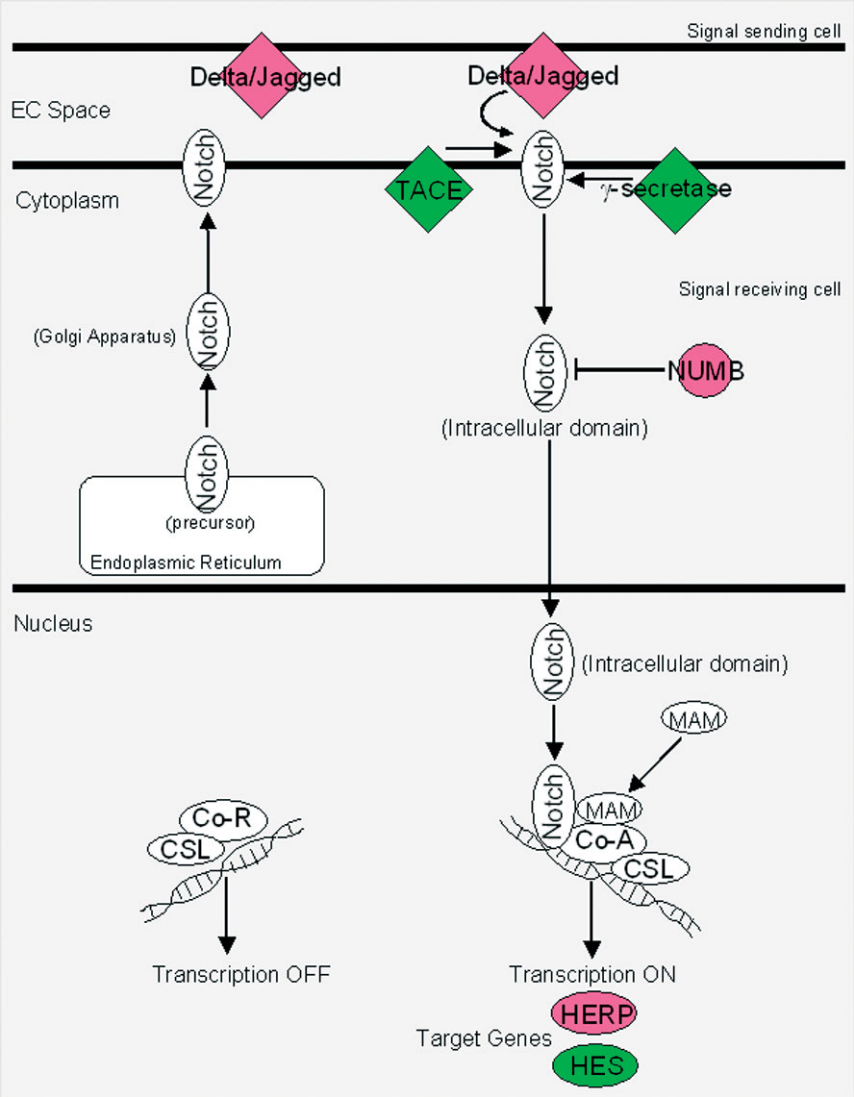

Figure 1. Ingenuity Pathways Analysis shows Notch pathway components regulated in normal pancreatic ducts versus ectatic ducts of chronic pancreatitis. Up-regulated and down-regulated Notch components are represented in red and green, respectively. Reproduced with permission from Ingenuity® Systems

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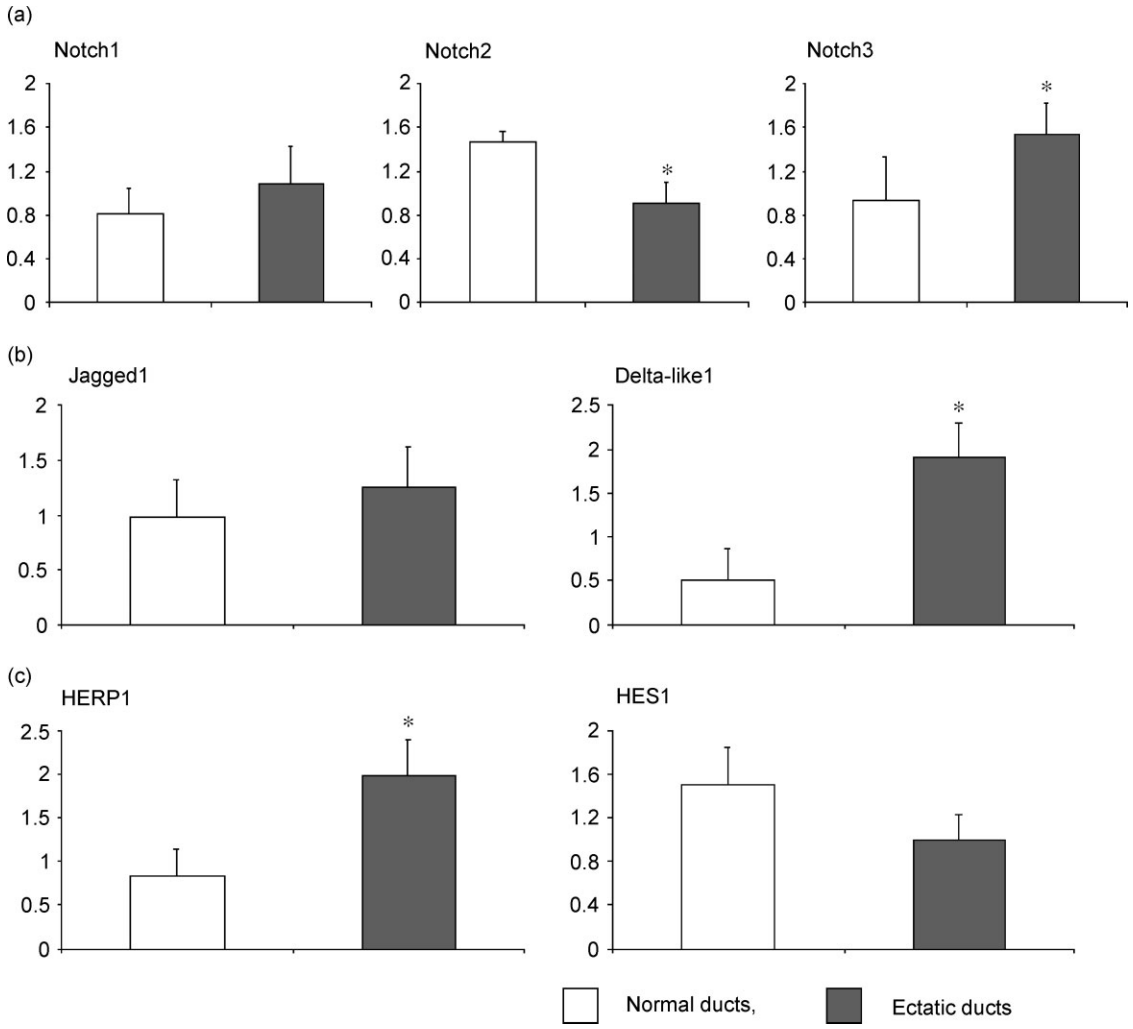

Figure 2. The relative expression of Notch (a) receptors, (b) ligands and (c) targets in laser microdissected ectatic ducts compared with normal pancreatic ducts, by real-time PCR (*$p \leq 0.05$)

To validate that the microarray data accurately reflected mRNA levels, we used real-time PCR to independantly determine mRNA levels for representative genes of the Notch pathway in different samples of microdissected normal pancreatic ducts and ectatic ducts (Figure 2). We also analysed the mRNA levels of Notch receptors. In microdissected ectatic ducts of CP, mRNA levels of Notch receptors were: Notch1, 1.3-fold increase (n.s.); Notch3, 1.7-fold increase ($p \leq 0.05$); Notch2, decrease (Figure 2a). Further, in ectatic ducts, the Notch ligand–*Delta-like1* mRNA was remarkably elevated by 3.7-fold ($p \leq 0.05$), while *Jagged1* mRNA levels increased nearly 1.3-fold (n.s.) (Figure 2b). The mRNA of the Notch target *HERP1* increased by about 2.4-fold ($p \leq 0.05$) in ectatic ducts, while the *HES1* message tended to be down-regulated (Figure 2c).

Notch component alteration in pressure-stimulated HPDE cells corresponds to that in ectatic ducts of CP

A static hydrostatic pressure of 200 mmHg applied for 24 h altered the expression of some Notch components, including Notch receptors and their ligands, in HPDE cells. Some of these alterations resemble Notch alterations observed in laser-microdissected ectatic ducts of CP compared with normal ducts.

In pressure-stimulated HPDE cells, the transcript expression of *Notch receptor–Notch1* increased by about 1.6-fold (n.s) compared to that of unstimulated control cells, while *Notch2* mRNA levels decreased and *Notch3* remained unchanged (Figure 3a). The transcript levels of the Notch ligand Jagged1 tended to increase, and those of Delta-like1 tended to decrease (Figure 3b). No changes in the mRNA expression of the Notch targets *HES1* and *HERP1* were found (Figure 3c).

Immunohistochemistry

Immunohistochemistry showed that the normal pancreatic ducts did not express Jagged1 (Figure 4a). In contrast, the epithelial cells lining the ectatic ducts strongly expressed Jagged1 protein (Figure 4b). The epithelial lining forming protrusions into the lumina of dilated ducts was also Jagged1-positive. Furthermore, the epithelial cells lining ectatic ducts that were

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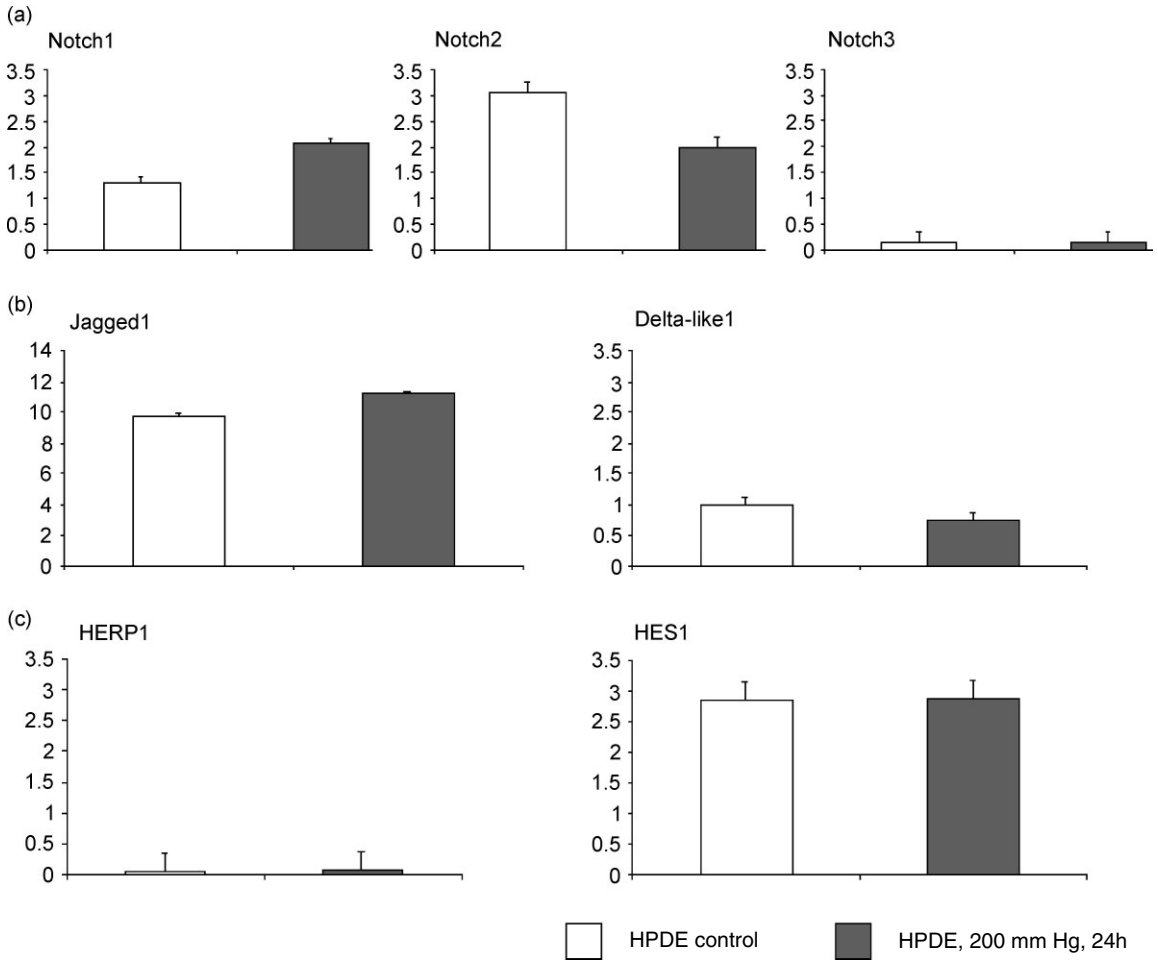

Figure 3. The relative expression of Notch (a) receptors, (b) ligands and (c) targets in HPDE of subjected to pressure versus control, by real-time PCR

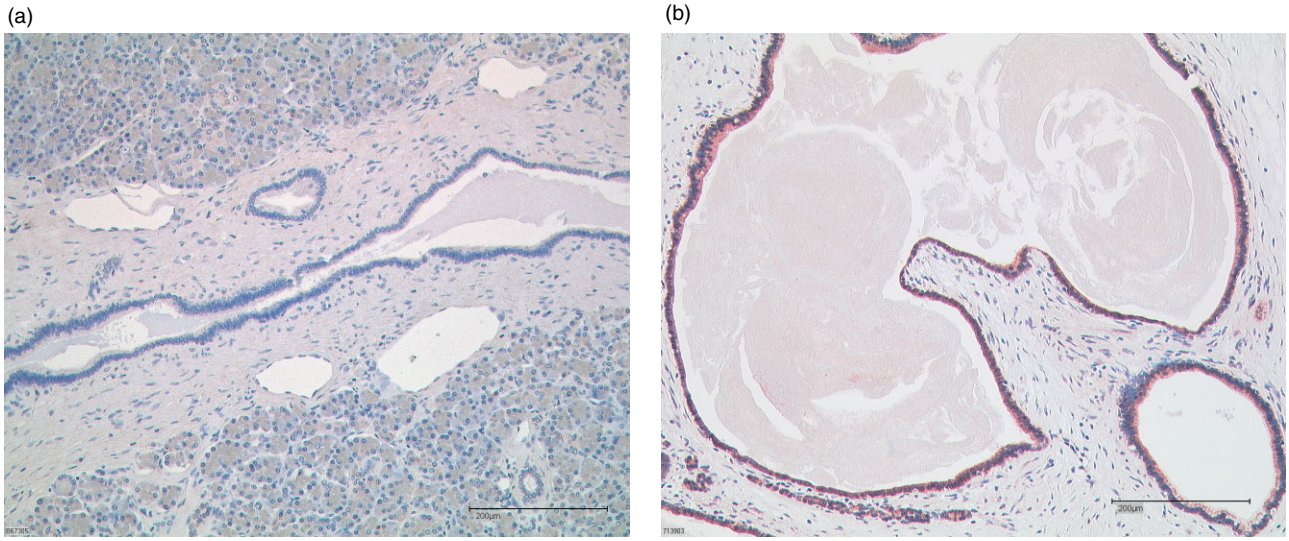

Figure 4. (a) Section of normal pancreas, showing no Jagged1 staining in normal pancreatic ducts. (b) An example of positive immunolabelling for Jagged1 in the epithelium of the ectatic ducts of CP

flattened due to stretching, resulting from raised intraluminal pressure, also exhibited Jagged1. The tubuloacinar complexes of CP highly expressed Jagged1. The pancreatic islets constitutively expressing Jagged1 served as an internal control.

Discussion

We showed, for the first time, evidence of Notch pathway activation in pancreatic ducts dilated due to long-standing obstruction in chronic pancreatitis (CP).

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Oligonucleotide microarrays and pathway analysis revealed a differential regulation of the Notch pathway in microdissected normal pancreatic ducts versus ectatic ducts of CP. The Notch ligand, Jagged/Delta-like, and Notch target, HERP, were up-regulated in ectatic ducts of CP. Further, we validated the microarray data by real-time PCR, and in addition found the mRNA of Notch receptors Notch1 and Notch3 to be increased by 1.3- and 1.7-fold, respectively, in ectatic ducts. Notch signalling is required for pancreas development and regulates pancreatic endocrine and exocrine cell fate [19]. It is reactivated during pancreatic injury and ensuing repair along the course of CP. Recently, it was shown, during the course of caerulein-induced pancreatitis, that Notch pathway components are induced during exocrine pancreas regeneration [20]. Pancreatic injury resulting from duct obstruction causes morphological changes, such as acino-ductal metaplasia, seen in the pancreatic duct ligation model of tissue damage where the Notch pathway is activated [4]. Re-expression of Notch is reported in metaplastic or dedifferentiated acinar cells [4]. Metaplastic lesions are known to give rise to early neoplastic lesions, in which Notch activation plays a role. Ectopic Notch activation is thought to be an early event in pancreatic carcinogenesis and Notch is dysregulated in pancreatic cancer [7,9]. HES1, a downstream target of Notch, is frequently expressed in metaplastic duct lesions and PanIN epithelium [9,4]. Chronic inflammation produces alterations in the microenvironment of ductal epithelium that may increase the risk of neoplastic transformation by increased genomic damage and cellular proliferation [5,6].

Increased Notch signalling was sufficient to transform normal breast epithelial cells through suppression of apoptosis [21]. There are numerous reports implicating Notch activation as a proto-oncogene. Conversely, there are also reports of Notch acting as a tumour suppressor in epidermis [22]. Notch signalling causes G_1 cell-cycle arrest in cultured small cell lung cancer (SCLC) cells [23]. In another report, Notch signalling inhibited growth of hepato-cellular carcinoma (HCC) cells [24]. These apparently contradictory data suggest a pleiotropism of Notch, which might be tissue- and context-dependent [12].

In ectatic ducts, the transcripts of *Notch ligand–Delta-like1* were 3.7-fold elevated. *Jagged1* mRNA was increased 1.3-fold (Figure 2b), and Jagged1 protein was expressed at high levels in ectatic ducts compared to normal pancreatic ducts (Figure 4). This could be due to post-transcriptional changes. Delta-like1 and Jagged1 activate Notch signalling by binding to Notch receptors. New data supporting an independent signalling function for activated intracellular domains of Delta-like, as well as Jagged, imply the existence of a bidirectional signalling mechanism [13]. It is therefore thought that Delta-like1 and Jagged1 are by themselves important in development and carcinogenesis, independent of their role as Notch ligands. *Delta-like1* and *Jagged1* were up-regulated in cervical cancers and were also shown to be active ligands in glioma cell lines [11,13]. *Jagged1* was identified as the gene responsible for Alagille syndrome [25] and activates Notch signalling through any of the Notch receptors. There is high Jagged1 expression in pancreatic cancer [26,9] and in mucinous cystic neoplasms of the pancreas [27].

We found that the mRNA of the Notch target HERP1 increased by 2.4-fold in ectatic ducts, while *HES1* mRNA was down-modulated (Figure 2c). This reflects the observation that expressions of HERP and HES are not always up-regulated simultaneously by Notch, as certain tissues express only one of them [28]. HERP1 was identified as a novel primary target of Notch and is regulated in a cell type-specific manner [29]. The epithelium of ectatic ducts withstands increased intraductal pressure over a prolonged period. Therefore, it is most likely that the Notch alterations we found in microdissected ectatic ducts of CP represent functional re-programming of the epithelium, effected through the physical stress of duct obstruction and PDH.

To check whether or not externally applied pressure induced Notch alterations in duct epithelial cells, we subjected human pancreatic duct epithelial (HPDE) cells to an external pressure for 24 h. Interestingly, Notch1 in pressure-stimulated HPDE increased by 1.6-fold (Figure 3a), along with a rising tendency in Jagged1 (Figure 3b), while Notch2 levels tended to decrease (Figure 3a). HERP1 mRNA was detected at very low levels: therefore, we conclude that HPDEs are HERP1-poor cells (Figure 3c). No changes were noted in the HES1 levels (Figure 3c). In sum, externally applied hydrostatic pressure over a day induced a set of Notch alterations. Some Notch pathway component alterations in duct ectasia of CP were closely mirrored by HPDEs when subjected to elevated pressure, pointing to PDH as an initiator of these events. Thus, in the ectatic ducts of CP, pressure-induced Notch pathway activation may lead to transformation, eventually giving rise to metaplastic and early neoplastic lesions.

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