

# Parenchymal regression in chronic pancreatitis spares islets reprogrammed for the expression of NF $\kappa$ B 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) $\gamma$ , transforming growth factor (TGF) $\beta$  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 NF $\kappa$ B, which activates antiapoptotic programs. Here, we demonstrate that in insulinoma cells CM, TGF $\beta$ /IFN $\gamma$ /TRAIL in combination induced TRAIL-R4 surface expression. TRAIL/IFN $\gamma$  upregulated NF $\kappa$ B subunits and its target gene survivin while downmodulating I $\kappa$ B $\alpha$  mRNA. RelA transcriptional activity increased upon stimulation with IFN $\gamma$  and IFN $\gamma$ /TRAIL. *In situ*, normal pancreatic epithelia had low mRNA levels of NF $\kappa$ B subunits. These were higher in parenchymal areas of CP with severe fibrosis and highest in islets. NF $\kappa$ B-regulated proteins I $\kappa$ B $\alpha$ , 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 NF $\kappa$ B and its target genes, survivin and cIAP1, to protect themselves from apoptosis.**

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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.<sup>1,2</sup> 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.<sup>3</sup> 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.<sup>4</sup> 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 NF $\kappa$ B constituents, upon binding of TRAIL.<sup>5</sup> We identified pancreatic stellate cells (PSCs) producing the fibrogenic cytokine transforming growth factor (TGF) $\beta$ <sup>6,7</sup> as the major source of TRAIL in CP.<sup>4</sup> 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 NF $\kappa$ B induction and action. Among the multitude of genes regulated by NF $\kappa$ B are antiapoptotic genes such as those for inhibitor of apoptosis proteins (IAPs).<sup>8</sup> IAPs prevent cell death by binding to and inhibiting active caspases.<sup>9</sup> This led us to investigate the role of NF $\kappa$ B 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 NF $\kappa$ B subunits and survivin. Treatment with interferon (IFN) $\gamma$  and IFN $\gamma$  plus TRAIL elevated RelA transcription in CM. Furthermore, in CP we found an induction of NF $\kappa$ B subunits in pancreatic epithelia, which, although at low levels in exocrine pancreas, is massive in pancreatic islet cells. The extent of local NF $\kappa$ B 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  $\mu$ g/ml streptomycin. Cells were maintained at 37°C in 5% CO<sub>2</sub>.

### 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 TGF $\beta$  (Roche) for 24 h followed by 500 U/ml IFN $\gamma$  (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  $\mu$ l propidium iodide (50 mg/ml). After gating for propidium iodide negativity to exclude dead cells, 10<sup>5</sup> 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<sup>6</sup> cells/sample were suspended in 50  $\mu$ l 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')<sub>2</sub> goat anti-mouse immunoglobulins (DAKO) for 30 min on ice. Finally, cells were washed twice and resuspended in 500  $\mu$ l cold FACS medium containing 1  $\mu$ g/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  $8 \mu\text{m}$  thickness and serial sections transferred to PALM<sup>®</sup> 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<sup>®</sup> 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<sup>™</sup> 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  $\text{OD}_{280/260}$  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 ( $2 \times 10^5$ ) were stimulated with TRAIL, IFN $\gamma$  and TRAIL + IFN $\gamma$  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<sup>™</sup> RNA Amplification 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 ( $2.5 \mu\text{g}$ ) 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 NF $\kappa$ B 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  $60^\circ\text{C}$ . 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  $4 \mu\text{l}$  cDNA ( $12.5 \text{ ng}/\mu\text{l}$ ),  $4 \mu\text{l}$  primer mix (forward/reverse) at optimal concentrations,  $12 \mu\text{l}$  sterile distilled water and  $20 \mu\text{l}$  SYBR Green PCR Master Mix (PE Biosystems, Foster City, CA, USA) per reaction. The following cycling conditions were set:  $50^\circ\text{C}$  for 2 min, denaturation at  $95^\circ\text{C}$  for 10 min, followed by 45 cycles at  $95^\circ\text{C}$  for 20 s each,  $60^\circ\text{C}$  for 20 s and  $72^\circ\text{C}$  for 20 s. Gene expression of NF $\kappa$ B 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 ( $\Delta C_T$ ); 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
NF $\kappa$ B1	Forward	5'-ACA ACA TCT CAT TGG CAG GCT-3'	900
	Reverse	5'-GGT CAT AGA GAG GCT CAA AGT-3'	900
NF $\kappa$ B2	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
I $\kappa$ B $\alpha$	Forward	5'-GCT GAT GTC AAT GCT CAG GA-3'	300
	Reverse	5'-CCG 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 CCG-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- $\mu$ m-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), I $\kappa$ B $\alpha$ /MAD-3 (IgG1 isotype), IKK $\gamma$ /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<sup>TM</sup>, 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

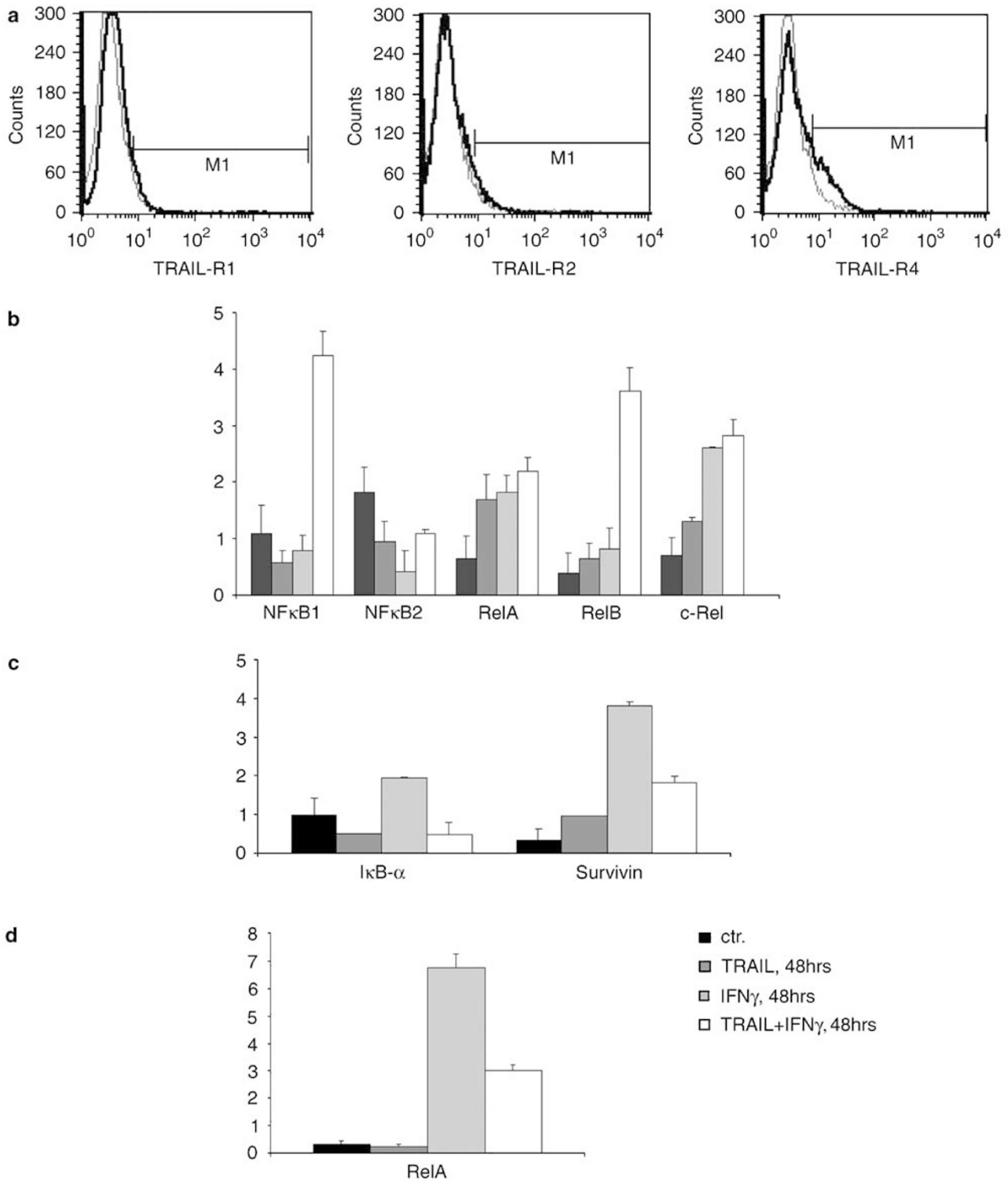
DNA-binding activity of RelA of the NF $\kappa$ B transcription factor family in CM treated with 500 U/ml

IFN $\gamma$  (Roche) and 100 ng/ml of human recombinant Killer<sup>TM</sup> TRAIL (Alexis, San Diego, CA, USA) separately and together for 48 h was determined using an ELISA-based assay (TransAM Kit<sup>TM</sup>) obtained from Active Motif (Rixensart, Belgium). CM ( $2 \times 10^5$ /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  $\mu$ g of nuclear extract was added to microwells coated with a cold oligonucleotide containing the consensus-binding site for NF $\kappa$ B. 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<sup>-</sup>, TRAIL-R2<sup>-</sup> and TRAIL-R4<sup>+</sup>,<sup>4</sup> 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.<sup>4</sup> The TRAIL-R profile of CM upon stimulation with TGF $\beta$  (24 h), IFN $\gamma$  (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, IFN $\gamma$  and TRAIL + IFN $\gamma$  compared with untreated cells. (c) Semiquantitative real-time PCR for NFκB targets IκB $\alpha$  and survivin in CM cells stimulated with cytokines for 48 h. IκB $\alpha$  mRNA levels increased with IFN $\gamma$ , while a decrease was noted with TRAIL and TRAIL + IFN $\gamma$ . Survivin mRNA increased considerably with TRAIL, IFN $\gamma$  and TRAIL + IFN $\gamma$ . (d) RelA transcriptional activity in CM activated by TRAIL, IFN $\gamma$  and IFN $\gamma$  + 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,<sup>4</sup> in being TRAIL-R1<sup>-</sup>, TRAIL-R2<sup>-</sup>, TRAIL-R3<sup>-</sup> (not shown) and TRAIL-R4<sup>+</sup> in a major subset of cells (Figure 1a).

### TRAIL Modulates NF $\kappa$ B Subunit Expression in CM

Next, CM cells were stimulated with TRAIL, IFN $\gamma$  and TRAIL + IFN $\gamma$  for 48 h to determine the mRNA expression of NF $\kappa$ B subunits and NF $\kappa$ B targets I $\kappa$ B $\alpha$  and survivin.

The combination of TRAIL and IFN $\gamma$  brought about a four-fold rise in NF $\kappa$ B1 mRNA levels. On the other hand, mRNAs of transcriptionally active NF $\kappa$ B subunits RelA, RelB and c-Rel were between two- to 9.5-fold increased compared to untreated cells on TRAIL + IFN $\gamma$  stimulation (Figure 1b). Additionally, mRNA of NF $\kappa$ B target I $\kappa$ B $\alpha$  was downmodulated, while survivin message increased considerably (Figure 1c).

Thus, TRAIL-R4 acts as a signal transducer in modulating NF $\kappa$ B 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 NF $\kappa$ B family of transcription factors in CM cells as determined by an ELISA-based assay increased significantly following 48 h stimulation with IFN $\gamma$  (20-fold rise) and TRAIL + IFN $\gamma$  (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 NF $\kappa$ B subunits. These were increased in CP in parenchymal areas with fibrosis and most intensely in islets. The expression of NF $\kappa$ B-regulated proteins I $\kappa$ B $\alpha$ , 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,  $12.4 \pm 1.8\%$ , moderate,  $41.2 \pm 0.6\%$ , and severe fibrosis,  $73.9 \pm 6\%$ ; stroma constituted  $6.3 \pm 1.9\%$  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 NF $\kappa$ B 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 NF $\kappa$ B 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

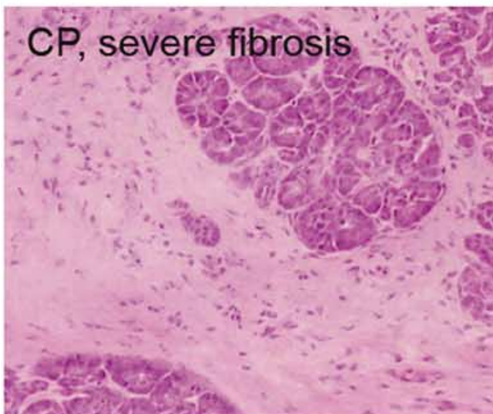
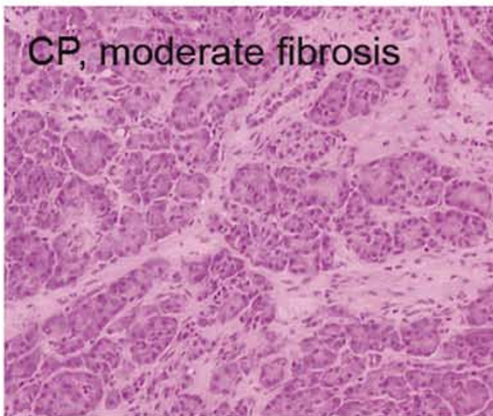
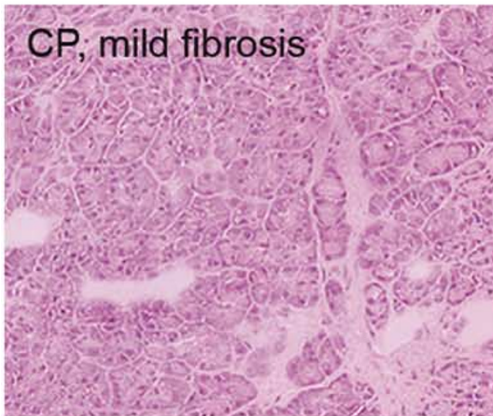
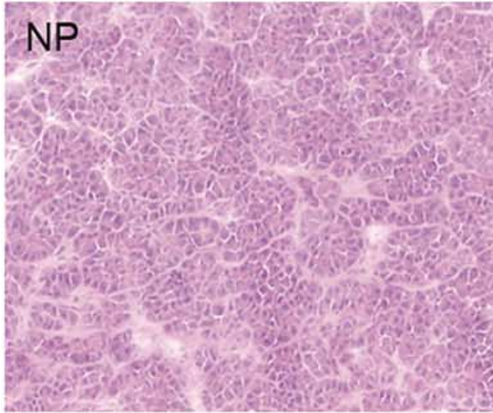
In severe fibrosis, there was a sharp increase in RelA transcripts in islet cells but not in acini and ducts (Figure 3). Normal acini, ductal epithelium and islets showed no expression of RelA protein. In CP, RelA protein was detectable and the intensity of staining increased with progressive severity 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).

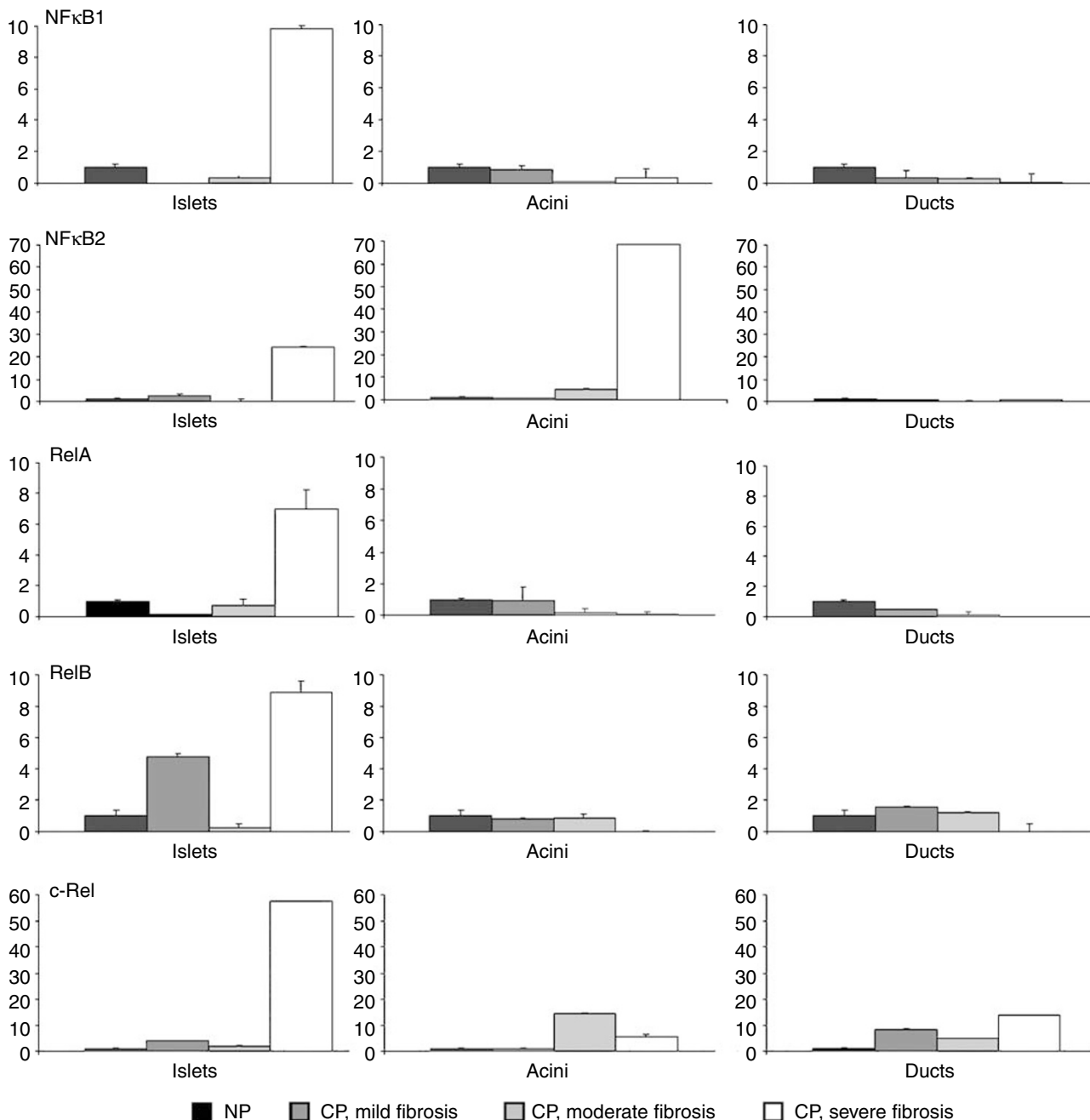
### NF $\kappa$ B1 (p105)

There was a sharp, about 10-fold, increase in NF $\kappa$ B1 (p105) transcript numbers in islet cells in areas of severe fibrosis (Figure 3). As was the case for NF $\kappa$ B1 (p100), no protein data are available for NF $\kappa$ B2 (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,  $12.4 \pm 1.8\%$ , moderate,  $41.2 \pm 0.6\%$ , and severe fibrosis,  $73.9 \pm 6\%$ , and NP,  $6.3 \pm 1.9\%$  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).







**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.

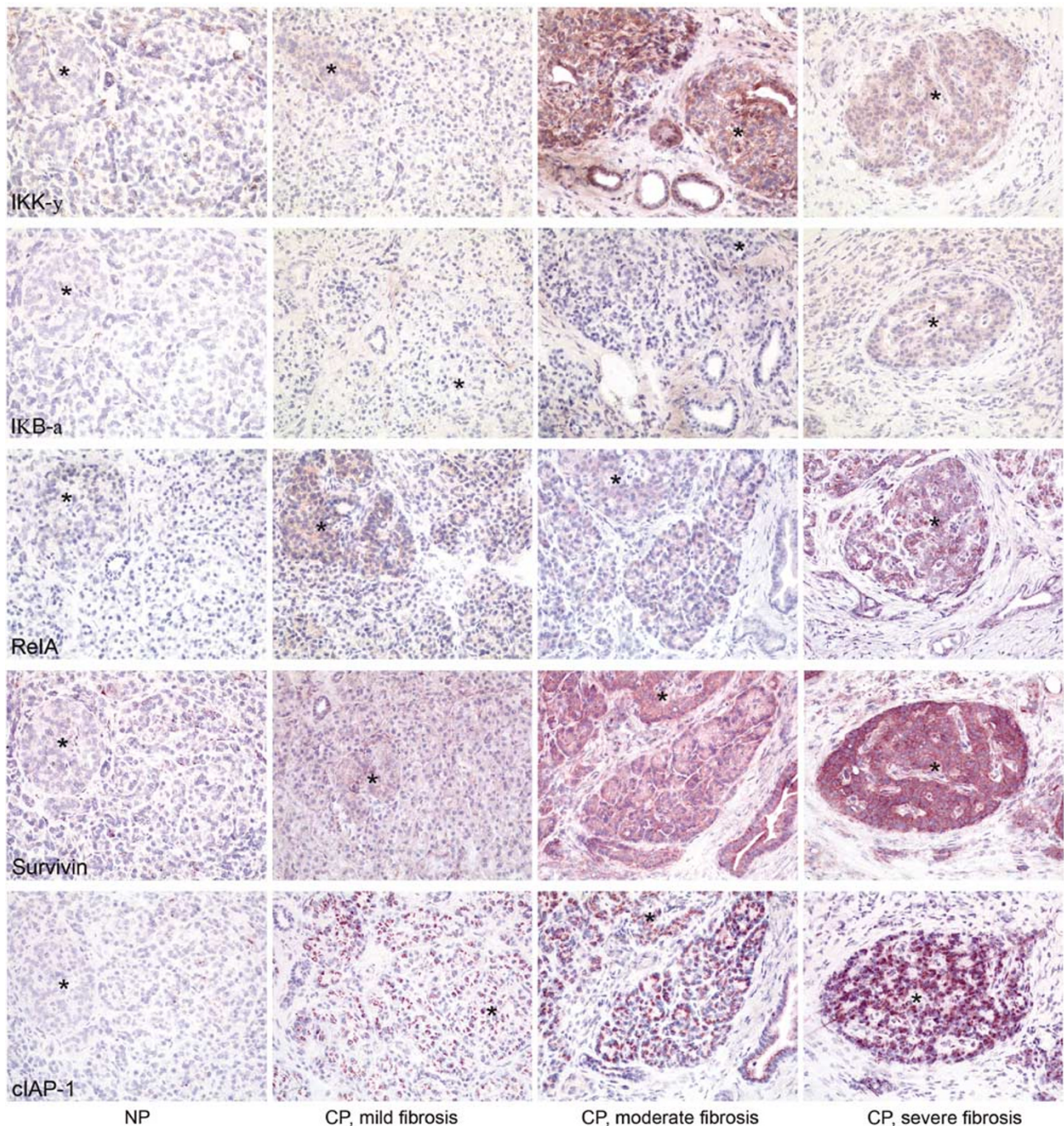
### cIAP1

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.<sup>5</sup> 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 IKK $\gamma$ , I $\kappa$ B $\alpha$ , RelA, survivin and cIAP1. IKK $\gamma$ : NP tissues show no or only a very weak expression of IKK $\gamma$ . 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. I $\kappa$ B $\alpha$ : NP, CP with mild and moderate fibrosis are largely devoid of I $\kappa$ B $\alpha$ . 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 NF $\kappa$ B 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 NF $\kappa$ B1, NF $\kappa$ B2, RelA, RelB and c-Rel in islet cells. Together with our findings that transcriptionally active NF $\kappa$ B 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.<sup>10</sup>

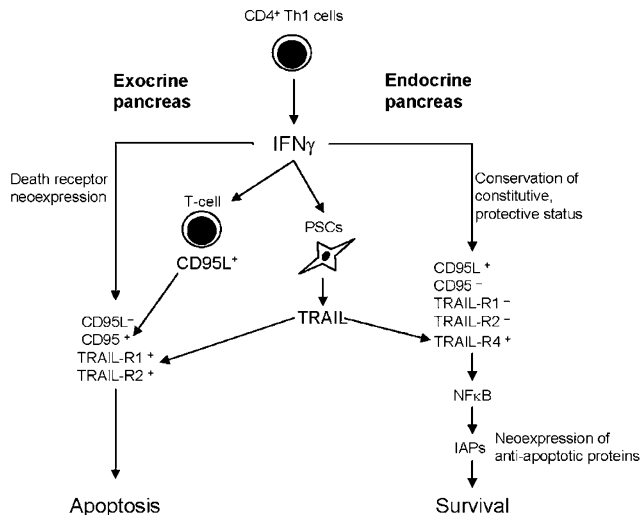
At present, there is a wealth of data on the defensive and protective role NF $\kappa$ B plays in the acute phase response in inflammation.<sup>11,12</sup> It was suggested that RelA and c-Rel are functional antagonists with respect to apoptosis, RelA over-expression inhibiting apoptosis, while c-Rel acts in a proapoptotic way by enhancing TRAIL-R1, -R2 or suppressing cIAP1, cIAP2 and survivin.<sup>13</sup> It was, however, also suggested that NF $\kappa$ B2 (p100) might be a direct activator of programmed cell death.<sup>14,15</sup> NF $\kappa$ B was shown to be involved in acute pancreatitis.<sup>16</sup> Activation of NF $\kappa$ B 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 NF $\kappa$ B activation had an adverse effect.<sup>16</sup> Conversely, intraductally administered *in vivo* gene transfer of RelA led to NF $\kappa$ B activation, acinar damage and acute pancreatitis in rats.<sup>17</sup> Thus, it remains unclear whether NF $\kappa$ B activation in pancreas is protective or deleterious.<sup>18</sup> Remarkably, in the acini of CP with severe fibrosis, we found a 69-fold NF $\kappa$ B2 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 NF $\kappa$ B-regulated transcriptional activity, the functional consequences of which, at the single-cell level, are largely unclear. One family of genes regulated by NF $\kappa$ B are IAPs, acting as suppressors of apoptosis by inhibiting caspase-3, -7 and -9.<sup>9,19</sup> It was suggested that cancer cells, for example, of pancreatic carcinoma,<sup>20,21</sup> 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.<sup>22</sup> The low levels or lack of survivin expression in NP is in line with data

on the absence of mRNA in normal adult tissues<sup>22</sup> and the absence of protein in NP reported by Satoh *et al*.<sup>21</sup> However, survivin expression has been reported in human fetal islets.<sup>23</sup> 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 NF $\kappa$ B subunits and, consequently, IAPs are lifeguards for endocrine cells in the adverse microenvironment of CP, while the pattern of NF $\kappa$ B 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.<sup>24</sup> 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*<sup>25</sup> 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 insulinitis 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 insulinitis, while the exocrine parenchyma is unaffected. In both processes, apoptotic cell death is the key event.<sup>26</sup> 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,<sup>27</sup> 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<sup>3,4</sup> 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 IFN $\gamma$  locally released by CD4<sup>+</sup>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 IFN $\gamma$ -triggered PSCs. The effects of IFN $\gamma$  and TRAIL on endocrine cells are completely opposite. In the setting of CP, islet cells retain their protective CD95L<sup>+</sup>, death receptor<sup>-</sup> state but respond to IFN $\gamma$ /TRAIL via TRAIL-R4 signalling with NF $\kappa$ B 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.

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## References

- 1 Bockman DE. Morphology of the exocrine pancreas related to pancreatitis. *Microsc Res Technol* 1997;37:509–519.
- 2 Kloppel G, Maillet B. Pathology of acute and chronic pancreatitis. *Pancreas* 1993;8:659–670.

- 3 Hasel C, Rau B, Perner S, *et al*. Differential and mutually exclusive expression of CD95 and CD95 ligand in epithelia of normal pancreas and chronic pancreatitis. *Lab Invest* 2001;81:317–326.
- 4 Hasel C, Durr S, Rau B, *et al*. In chronic pancreatitis, widespread emergence of TRAIL receptors in epithelia coincides with neoexpression of TRAIL by pancreatic stellate cells of early fibrotic areas. *Lab Invest* 2003;83:825–836.
- 5 Degli-Esposti MA, Dougall WC, Smolak PJ, *et al*. The novel receptor TRAIL-R4 induces NF-kappaB and protects against TRAIL-mediated apoptosis, yet retains an incomplete death domain. *Immunity* 1997;7:813–820.
- 6 Bachem MG, Schneider E, Gross H, *et al*. Identification, culture, and characterization of pancreatic stellate cells in rats and humans. *Gastroenterology* 1998;115:421–432.
- 7 Kruse ML, Hildebrand PB, Timke C, *et al*. TGFbeta1 autocrine growth control in isolated pancreatic fibroblastoid cells/stellate cells *in vitro*. *Regul Pept* 2000;90:47–52.
- 8 Aggarwal BB, Bhardwaj U, Takada Y. Regulation of TRAIL-induced apoptosis by ectopic expression of antiapoptotic factors. *Vitam Horm* 2004;67:453–483.
- 9 Deveraux QL, Reed JC. IAP family proteins—suppressors of apoptosis. *Genes Dev* 1999;13:239–252.
- 10 Ashkenazi A, Pai RC, Fong S, *et al*. Safety and antitumor activity of recombinant soluble Apo2 ligand. *J Clin Invest* 1999;104:155–162.
- 11 Barkett M, Gilmore TD. Control of apoptosis by Rel/NF-kappaB transcription factors. *Oncogene* 1999;18:6910–6924.
- 12 Schmid RM, Adler G. NF-kappaB/rel/IkappaB: implications in gastrointestinal diseases. *Gastroenterology* 2000;118:1208–1228.
- 13 Chen X, Kandasamy K, Srivastava RK. Differential roles of RelA (p65) and c-Rel subunits of nuclear factor kappa B in tumor necrosis factor-related apoptosis-inducing ligand signaling. *Cancer Res* 2003;63:1059–1066.
- 14 Wang Y, Cui H, Schroering A, *et al*. NF-kappa B2 p100 is a pro-apoptotic protein with anti-oncogenic function. *Nat Cell Biol* 2002;4:888–893.
- 15 Hacker H, Karin M. Is NF-kappaB2/p100 a direct activator of programmed cell death? *Cancer Cell* 2002;2:431–433.
- 16 Steinle AU, Weidenbach H, Wagner M, *et al*. NF-kappaB/Rel activation in cerulein pancreatitis. *Gastroenterology* 1999;116:420–430.
- 17 Chen X, Ji B, Han B, *et al*. NF-kappaB activation in pancreas induces pancreatic and systemic inflammatory response. *Gastroenterology* 2002;122:448–457.
- 18 Weber CK, Adler G. From acinar cell damage to systemic inflammatory response: current concepts in pancreatitis. *Pancreatol* 2001;1:356–362.
- 19 Tamm I, Wang Y, Sausville E, *et al*. IAP-family protein survivin inhibits caspase activity and apoptosis induced by Fas (CD95), Bax, caspases, and anticancer drugs. *Cancer Res* 1998;58:5315–5320.
- 20 Fulda S, Debatin KM. Sensitization for tumor necrosis factor-related apoptosis-inducing ligand-induced apoptosis by the chemopreventive agent resveratrol. *Cancer Res* 2004;64:337–346.
- 21 Satoh K, Kaneko K, Hirota M, *et al*. Expression of survivin is correlated with cancer cell apoptosis and is involved in the development of human pancreatic duct cell tumors. *Cancer* 2001;92:271–278.

- 22 Verhagen AM, Coulson EJ, Vaux DL. Inhibitor of apoptosis proteins and their relatives: IAPs and other BIRPs. *Genome Biol* 2001;2 (reviews 3009. 1–10).
- 23 Liggins C, Orlicky DJ, Bloomquist LA, *et al*. Developmentally regulated expression of survivin in human pancreatic islets. *Pediatr Dev Pathol* 2003;6:392–397.
- 24 Mi QS, Ly D, Lamhamedi-Cherradi SE, *et al*. Blockade of tumor necrosis factor-related apoptosis-inducing ligand exacerbates type 1 diabetes in NOD mice. *Diabetes* 2003;52:1967–1975.
- 25 Lamhamedi-Cherradi SE, Zheng S, Tisch RM, *et al*. Critical roles of tumor necrosis factor-related apoptosis-inducing ligand in type 1 diabetes. *Diabetes* 2003;52:2274–2278.
- 26 Ou D, Metzger DL, Wang X, *et al*. TNF-related apoptosis-inducing ligand death pathway-mediated human beta-cell destruction. *Diabetologia* 2002;45: 1678–1688.
- 27 Nagata S. Apoptosis by death factor. *Cell* 1997;88: 355–365.

Supplementary Information accompanies the paper on Laboratory Investigation website (<http://www.nature.com/labinvest>)