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Differential and Mutually Exclusive Expression of CD95 and CD95 Ligand in Epithelia of Normal Pancreas and Chronic Pancreatitis

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SUMMARY: Acinar regression in chronic pancreatitis may be due to immune attack in parenchymal areas neoexpressing HLA-DR molecules. $CD4^+$Th1 cytotoxic T cells induce apoptosis of their targets via oligomerizing CD95 (APO-1/Fas) death receptors on target cells by their CD95 ligand (CD95L). We determined the expression of CD95 and CD95L in epithelia of normal and chronically inflamed pancreatic tissues. We applied RT-PCR and Western blotting for CD95L expression profiles, serial frozen section immunohistochemistry to detect CD95, CD95L, and HLA-DR molecules, CD3, CD4, CD11c, and S-100 protein (S100p). Normal pancreases and chronic pancreatitis contain CD95L message and protein. Immunohistochemistry revealed a mutually exclusive expression of CD95 and CD95L. Physiologically, acini were $CD95^-/CD95L^+$, ducts were $CD95^-/CD95L^-$, and islets were $CD95^-/CD95L^+$. In areas of lymphohistiocytic infiltration, mainly consisting of $CD3^+CD4^+$ T cells and $CD11c^+$, $CD4^{+/-}$, $S100p^+$ interstitial dendritic cells, and in areas of initial fibrosis, acini and ducts were $HLA\text{-}DR^+$, acini $CD95^+/CD95L^-$, and ducts $CD95^+/CD95L^-$. Islet cells were $CD95^-/CD95L^+$ in both conditions. IFNγ levels in protein lysates, as measured by an immunoassay, were significantly higher in chronic pancreatitis than in normal pancreas ($p < 0.0003$). In vitro, IFNγ down-modulated CD95L message and protein in ASPC1 and BxPc3 pancreatic carcinoma cells. In conclusion, pancreatic epithelia differentially express CD95 and CD95L in a mutually exclusive manner. In chronic pancreatitis the $CD95^-/CD95L^+$ status is conserved in islet cells even in the vicinity of lymphohistiocytic infiltrates, whereas it is lost in acini coexpressing HLA-DR. As a potential consequence, and possibly triggered by local release of IFNγ, $CD4^+$Th1 cells may cognately interact with and successfully attack exocrine cells by triggering CD95 on their target without being killed by epithelial, CD95L-mediated, counterattack. (*Lab Invest 2001, 81:317–326*).

Chronic pancreatitis is characterized by a progressive loss and fibrosis of exocrine parenchyma whereas the endocrine part of the organ remains structurally intact for a prolonged period of time (Bockman, 1997; Klöppel and Maillet, 1993; Sarles, 1991). The disease is often accompanied by duct ectasia and might be initiated by ductal obstruction or distortion (Klöppel and Maillet, 1993). Furthermore, foci of lymphohistiocytic infiltrates are commonly found and are also associated with regressing parenchyma (Bockman, 1997). These infiltrates were shown to contain activated cytotoxic T cells (Hunger et al, 1997), suggesting a role of the immune system in the pathogenesis of acinar atrophy and local scarring. Induction of HLA-DR molecules was observed in parenchymal areas affected by chronic pancreatitis (Bedossa et al, 1990; Jalleh et al, 1993) and transcripts for MCP-1 chemokine have been detected in areas of early stage of chronic pancreatitis (Saurer et al, 2000). Local expression of T cell tropic chemokines may recruit T cells, the CD4 subset of which then may cognately interact with HLA-DR neoexpressing epithelial cells. Like $CD8^+$ T cells, $CD4^+$Th1 cells have been shown to exert a cytotoxic function via apoptosis induction by binding the CD95 receptor of target cells (Ju et al, 1994).

CD95 (APO-1/Fas) is a 48-kDa type I membrane glycoprotein and a member of the tumor necrosis factor (TNF) receptor family. On oligomerization of CD95 by antibody or trimerization by its natural ligand, CD95L, it confers an apoptotic signal to apoptosis-sensitive cells (Peter and Krammer, 1998). CD95L is a 40-kDa type II membrane protein that belongs to the TNF family of cytokines (Takahashi et al, 1994). Like other members of this family, CD95L exists in a membrane-bound and soluble form (Dhein et al, 1995). CD95L is expressed on activated T cells (Tanaka et al, 1995), preferentially on $CD4^+$Th1 cells (Ju et al, 1994; Lynch et al, 1995), and in plasma cells (Sträter et al, 1999). Outside the immune system, we detected CD95L transcripts in Paneth cells (Möller et al, 1996). By immunohistochemistry using a polyclonal rabbit antiserum, Lee et al (1999) detected CD95L in a limited number of other epithelial cell types, eg, trophoblast cells, pituitary anterior lobe cells, and gastric parietal cells. However, gastric parietal cells do not express CD95L transcripts (Möller et al, 1996). Although the concept of immunoprivileged sites, defined as such by CD95L expression in resident cells

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potentially counterattacking intruder T cells, is currently very attractive, the CD95L expression profile of many normal human tissues, pancreas included, is not yet convincingly determined. By contrast, more is known about CD95 expression in normal human tissues (Leithäuser et al, 1993). Unlike many other types of epithelial cells, ductal and endocrine epithelia of normal pancreas are constitutively $CD95^-$. Likewise, the majority of acinar cells were found to be $CD95^-$, whereas those few expressing CD95 seemed to be induced by local inflammatory infiltrates, as observed in chronic prostatitis and chronic sialadenitis (Leithäuser et al, 1993).

This study was undertaken to exactly determine the expression of CD95 and CD95L in normal pancreatic epithelia and to analyze the CD95/CD95L status of pancreas epithelia in chronic pancreatitis. Unexpectedly, we found by immunohistochemistry that all pancreas islet and acinar cells constitutively express CD95L. In chronic pancreatitis we observed striking parallels in the induction of CD95 and HLA-DR molecules in exocrine cells close to lymphohistiocytic infiltrates. This local neoexpression of CD95 and HLA-DR was associated with a loss of CD95L in the same parenchymal microareas. By contrast, even in the vicinity of lymphohistiocytic infiltrates, islet cells remained $CD95^-$, HLA-DR^-, and $CD95L^+$.

Results

CD95L Expression in Normal Pancreas and Chronic Pancreatitis

Normal pancreases and representative tissue specimens of seven cases of chronic pancreatitis were successfully subjected to mRNA and protein extraction. Referred to the amount of message of the housekeeping gene SP1, RT-PCR revealed CD95L transcripts in both normal pancreas and pancreatitis (Fig. 1). In our series, the five normal pancreases expressed CD95L transcripts at a higher level than five of seven inflamed pancreases (Fig. 1, A, B, D, and E). The pancreatic cell line AsPc1, known to express CD95L transcripts (Bernstorff et al, 1999; Ungefroren et al, 1998), was used as the positive control. The colon carcinoma cell line HT29 chosen as the negative control (O'Connell et al, 1996) did not reveal detectable amounts of CD95L message. In parallel, both normal and chronically inflamed pancreatic tissues contained CD95L protein as detected by Western blot analysis (Fig. 1, C and F). Corresponding to the mRNA data, AsPC1 but not HT29 cells expressed CD95L protein. There was, however, no stringent correlation between the amount of transcripts and protein in the tissue extracts. Thus, normal and chronically inflamed pancreatic tissues express CD95L and, at least at the mRNA level, CD95L might be down-regulated in chronic pancreatitis.

Immunodetection of CD95L In Situ

Using CD95L monoclonal antibody G247-4 to frozen tissue sections, CD95L protein was detected in pancreatic epithelia. In normal pancreas, CD95L was present in every acinar cell in a supranuclear cytoplasmic location. Duct epithelia were $CD95L^-$ (Fig. 2). Islet epithelia were $CD95L^+$ in a dust-like microgranular cytoplasmic fashion (Figs. 2 and 3). No membrane-confined immunostaining was detected. Endothelial cells were $CD95L^-$. In chronic pancreatitis, acinar cells were mostly $CD95L^+$. However, scattered groups of acini were found to be $CD95L^-$. These $CD95L^-$ acini were involved in cellular interstitial infiltration or adjacent to mild interstitial fibrosis (Fig. 3). Islet cells, by contrast, were found to be $CD95L^+$, even in the vicinity of inflammatory cellular infiltrates and/or fibrosis (Fig. 2). Within the inflammatory infiltrates, immunodetection of CD95L was restricted to plasma cells and scattered cells of lymphohistiocytic infiltrates (Fig. 3). As revealed by serial-section immunohistochemistry, these inflammatory infiltrates were mainly composed of $CD3^+CD4^+$ T cells and $CD11c^+$ S-100 $protein^+$ ($S100p^+$) $CD4^{+/-}$ interstitial dendritic cells (+/− denotes admixture of positive and negative cells) (Fig. 3). Collectively, these immunohistologic patterns suggested a down-modulation of CD95L in acinar epithelia associated with local inflammatory infiltration and/or fibrosis.

CD95, CD95L, and HLA-DR Immunoprofiles of Pancreatic Epithelial Components

The majority of normal glandular epithelia, pancreatic epithelia included, are mainly $CD95^-$, but may, on induction, express CD95 in the context of chronic inflammation (Leithäuser et al, 1993). Parenchymal areas involved in chronic pancreatitis have also been shown to neoexpress HLA-DR (Ectors et al, 1997). We thus chose CD95 and HLA-DR as molecular targets to further elucidate the potentially inflammation-associated change in CD95L expression. The resulting immunoprofiles are summarized in Table 1 and paradigmatically depicted in Figures 2 and 3. In microareas with HLA-DR^+ lymphohistiocytic infiltrates and in microareas with early fibrosis, the epithelial components of the pancreas showed different patterns of expressional changes. Acini induced for HLA-DR were also $CD95^+$ and had lost CD95L expression (Fig. 3). In moderately fibrotic microareas, some acini had undergone a morphologic change, recently referred to as tubular acini (Bockman, 1997). Cells of these ectatic, tubular acini showed the same expression pattern as the epithelial cells in globular acini (Fig. 2). The duct epithelium being mixed HLA-DR-positive and -negative in the normal state and in normal areas of inflamed pancreases, turned to entirely HLA-DR^+, especially in ectatic ducts, and also neoexpressed CD95 while remaining $CD95L^-$ (Fig. 2). Islet cells in normal pancreas and normal areas of inflamed pancreases were HLA-DR^-, $CD95^-$, and $CD95L^+$. In striking contrast to acinar and ductal epithelia, islet epithelia did not alter their expressional status; ie, islets adjacent to HLA-DR^+ cellular infiltrates and to acini induced for HLA-DR and CD95 still were HLA-DR^- and $CD95^-$ and remained $CD95L^+$. The same

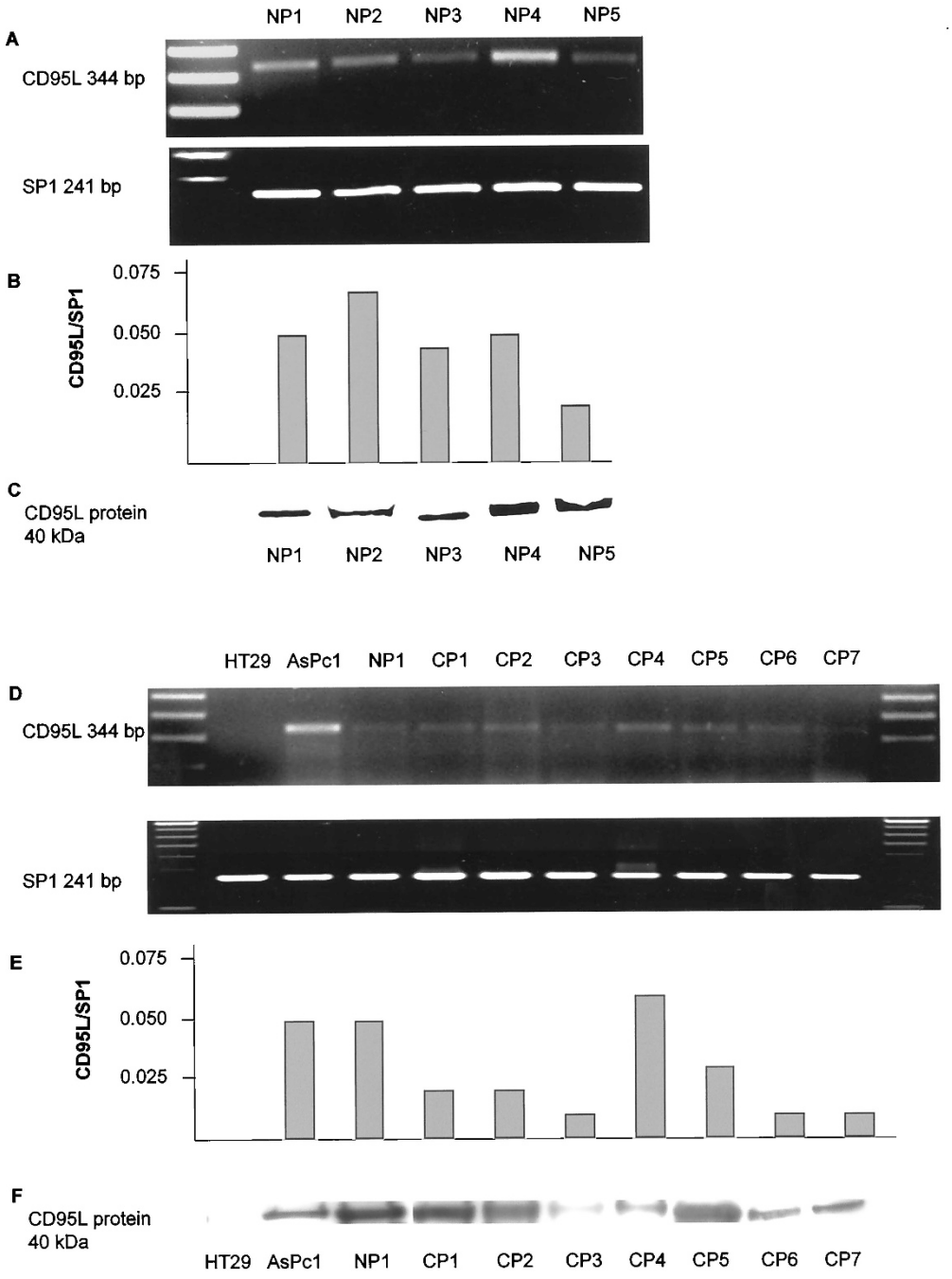

Figure 1.

CD95L transcript and protein expression in five normal pancreases (NP1 to NP5) (A to C) and in seven pancreases with chronic pancreatitis (CP1 to CP7) (D to F). A and D, CD95L and SP1 mRNA detected in an ethidium bromide–stained agarose gel after RT-PCR. Pancreatic carcinoma cell line AsPc1 and colon carcinoma cell line HT29 served as positive and negative control, respectively. NP1, blotted twice, is the standard allowing the direct comparison of intensity of bands. B and E, Ratio profiles of integrated optical densities. C and F, Immunoblots of Western blotted CD95L protein detected by CD95L monoclonal antibody G247-4, visualized by chemiluminescence. NP1 protein, blotted twice, is the standard allowing the direct comparison of intensity of bands.

was true for islets in the vicinity (Fig. 2) of, or surrounded by, fibrosis. In areas with advanced fibrosis, no further immunophenotypic changes were observed. Taken together, these findings suggest that islet cells are nonresponsive to the inflammatory stimuli acting in chronic pancreatitis, which, in acinus cells, leads to loss of CD95L and acquisition of CD95, HLA-DR and causes duct epithelium to up-regulate HLA-DR and induce CD95.

IFNγ is Locally Increased in Chronic Pancreatitis

One key inductor of CD95 is the proinflammatory cytokine IFNγ (Möller et al, 1994). In constitutively negative glandular epithelia, IFNγ also leads to expression of major histocompatibility complex (MHC) class II products. We therefore determined the content of IFNγ in protein lysates of normal and chronically inflamed pancreatic tissue by enzyme immunoassay

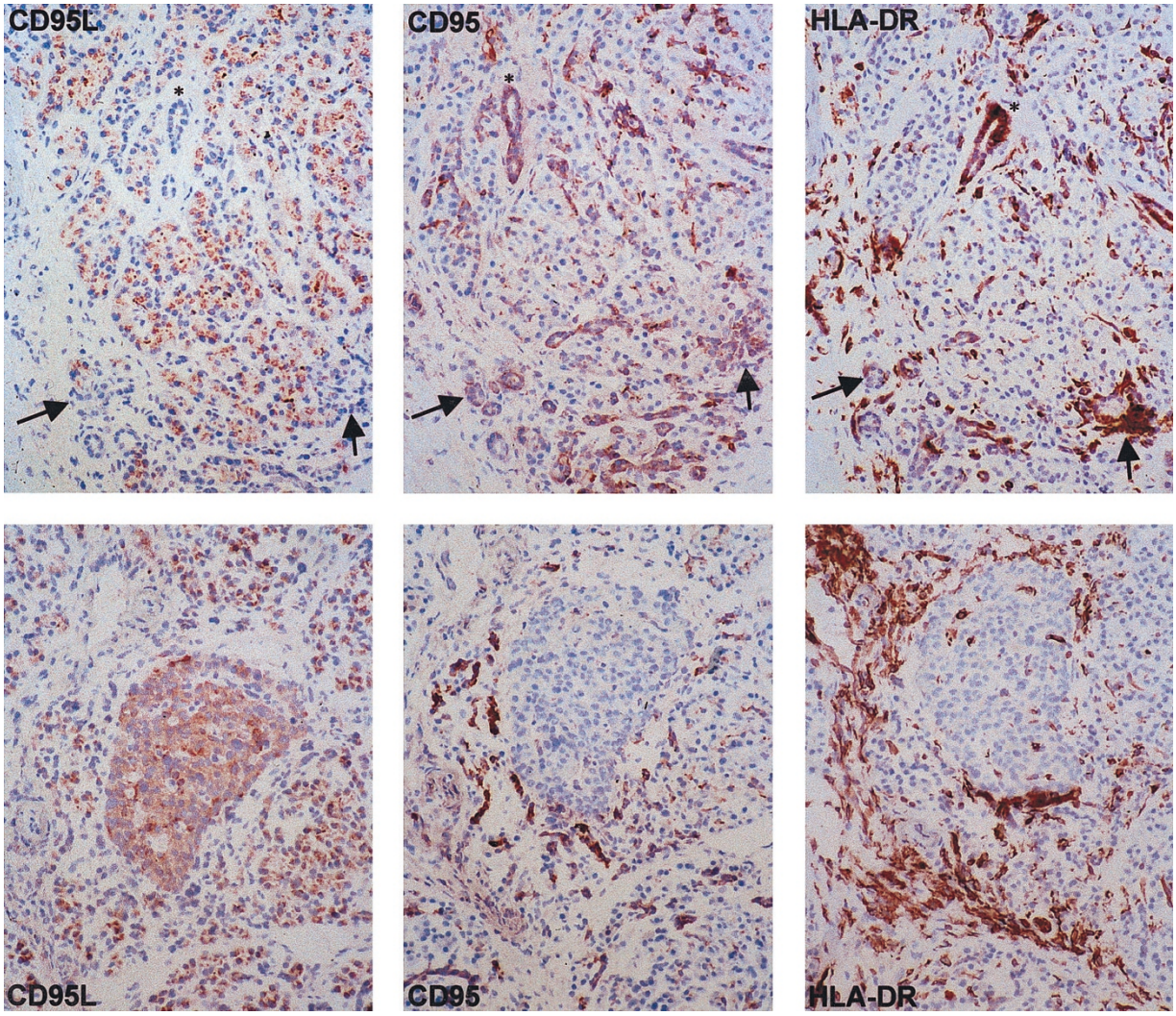

Figure 2.

Serial frozen section immunohistochemistry on a case of chronic pancreatitis (CP6) showing expression and tissue distribution of CD95L, CD95, and HLA-DR (original magnification, ×104). *Arrows* in the upper panels indicate corresponding areas. The *right arrow* indicates an exocrine parenchymal microarea infiltrated by HLA-DR–expressing inflammatory cells. The acini at this site are $CD95L^{-}$ and $CD95^{+}$. The *left arrow* marks a tubular acinus that is $CD95L^{-}$, $CD95L^{+/-}$, and $HLA\text{-}DR^{+/-}$. The small duct marked by an *asterisk* is $CD95L^{-}$, $CD95^{+}$, and heavily $HLA\text{-}DR^{+}$. The lower panels depict an islet surrounded by an inflammatory infiltrate and fibrosis (light interspersed areas). Note that directly adjacent to the islet, there is induction of CD95 in ductular structures and a loss of CD95L in surrounding acinar cells. Endocrine cells of the islet retain their $CD95L^{+}$, $CD95^{-}$, $HLA\text{-}DR^{-}$ immunoprofile.

(EIA). As shown in Figure 4A, chronic pancreatitis specimens contained higher amounts of IFNγ than normal pancreas. This difference was statistically significant ($p < 0.0003$).

Down-Modulation of CD95L in AsPc1 and BxPc3 by IFNγ

These data and the immunophenotypic changes observed in chronic pancreatitis in situ lead to the question whether IFNγ can down-modulate CD95L expression in exocrine pancreatic epithelium. As a surrogate, we used the AsPc1 and BxPc3 pancreatic carcinoma cell lines and stimulated the cells with IFNγ for 48 hours. As expected (Moldenhauer et al, 1999) this treatment led to HLA-DR surface expression (data not shown). In accordance with published data (Bernstorff et al, 1999), CD95 was constitutively expressed at the mRNA and protein level. After IFNγ treatment there was no significant change in either CD95 mRNA or protein expression. However, CD95 ligation-induced apoptotic rate was increased in both cell lines (data not shown). On IFNγ treatment there was a considerable reduction of CD95L mRNA relative to SP1 mRNA (Fig. 4, B and C) that accompanied a decrease in CD95L protein expression (Fig. 4D).

Discussion

We have shown that normal pancreases and pancreases removed for chronic pancreatitis express CD95L mRNA and also CD95L protein. As revealed by immunohistochemistry, the cells expressing CD95L protein are pancreatic acinar cells and islet cells, whereas duct epithelium is $CD95L^{-}$. In the course of chronic pancreatitis, acinar cells in areas of lymphocytic infiltration change to $CD95L^{-}$, whereas islet cells, even in

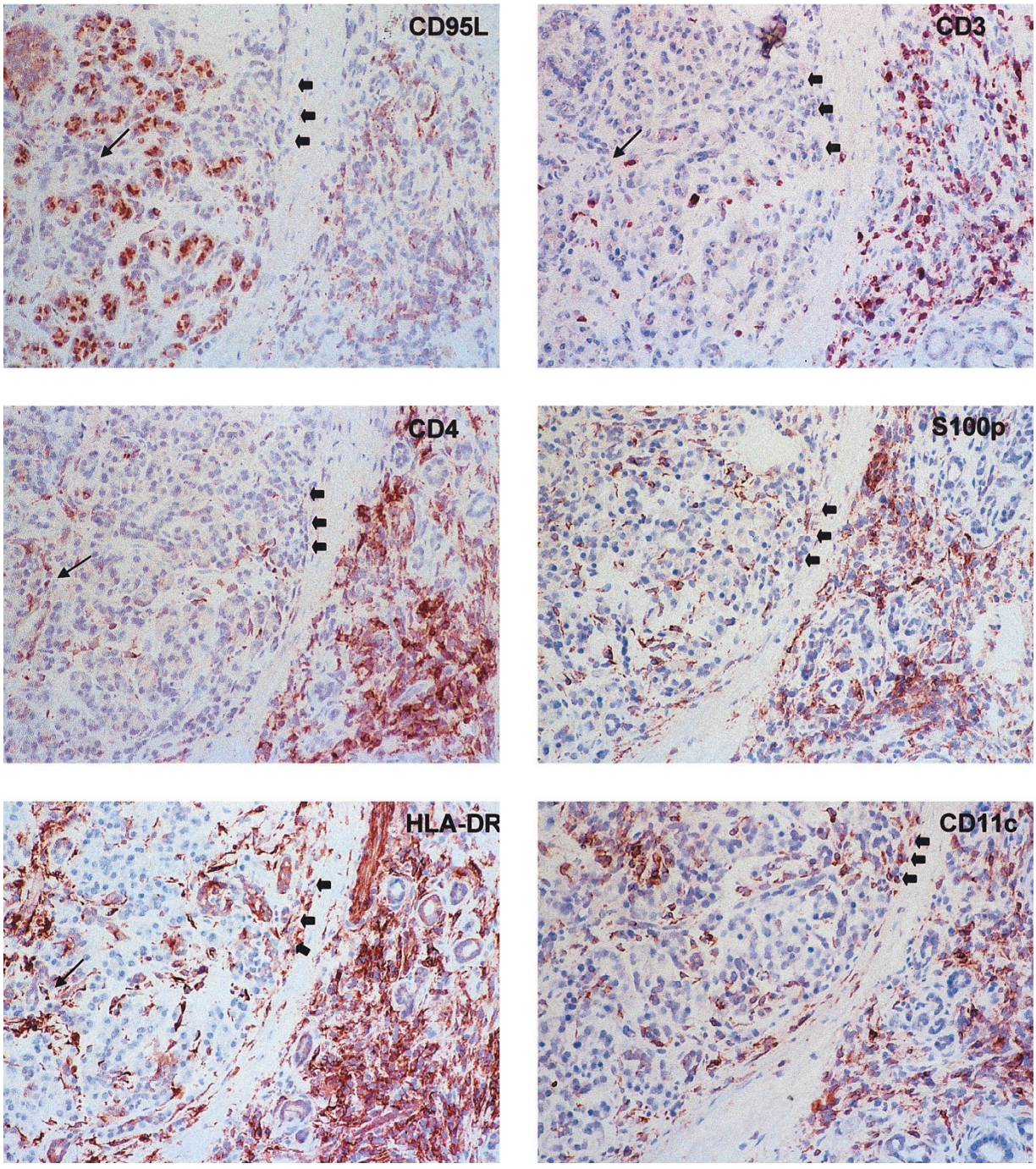

Figure 3.

Serial frozen section immunohistochemistry on a case of chronic pancreatitis (CP6) showing six different antigens. The micrographs show an informative situation of a sclerotic band in the middle, a parenchymal area affected by a minimal to moderate interstitial fibrosis, and a minimal inflammatory infiltrate on the left, and a heavily inflamed parenchymal area with displacement of ductules and acini by the lymphohistiocytic infiltrate on the right. Note a pancreatic islet at the upper left corner. *Long, thin arrows* and *triple short arrows* mark corresponding microareas in different immunostainings (original magnification, ×121). CD95L is heavily expressed in exocrine acini of unaffected parenchyma and in islet cells. *Arrows* mark regions where acini are $CD95L^-$. The *triple arrows* mark an exocrine area induced for HLA-DR and infiltrated by scattered $CD11c^+/S100p^+$ dendritic cells. Note that there are no $CD3^+/CD4^+$ T lymphocytes at this side. The parenchymal microarea indicated by the *single arrow* is $CD95L^-$, is slightly induced for HLA-DR, and contains some scattered $CD3^+/CD4^+$ T lymphocytes. Accidentally, the corresponding areas are not included in the serial sections stained for CD11c and S100p. At the right side, cells of tubular (ie, ectatic and atrophic) acini are $CD95L^-$, $HLA\text{-}DR^+$ and are surrounded by cells of the lymphohistiocytic infiltrate that is heavily $HLA\text{-}DR^+$ and contains scattered $CD95L^+$ cells.

the vicinity of inflammatory infiltration, conserve their high levels of CD95L protein.

These data are at variance with previous reports stating that normal pancreatic tissue is devoid of CD95L transcripts (as tested on a single tissue specimen using RT-PCR; Xerri et al, 1997) and CD95L reactivity in immunohistochemistry (Bernstorff et al, 1999; Lee et al, 1999). These negative results may be due to antigen accessibility that is impaired by formalin fixation and/or antibody reactivity (Sträter et al,

Table 1. Immunoprofile of Pancreatic Epithelia Summarized

	CD95L n/cp	CD95 n/cp	HLA-DR n/cp
Acinar cells	+ → −	− → +	− → +
Cells of tubular acini[a]	−	+	+
Duct epithelium	− → −	− → +	+/− → +
Island epithelium	+ → +	− → −	− → −

n, normal pancreatic tissue; cp, chronic pancreatitis; −/+, admixture of positive and negative cells.

[a] Sometimes referred to as "ectatic, atrophic acini."

1999). At least in our hands, anti-CD95L monoclonal antibody G247-4 is the only reliable clone for immunohistology that is commercially available at present; others like NOK-1, although potently neutralizing in functional assays (Kayagaki et al, 1995), do not stain sufficiently well, even in frozen tissue preparations (Sträter et al, in press). Even G247-4 loses most of its reactivity in paraffin sections due to formalin sensitivity of its epitope (Sträter et al, 1999). Ungefroren et al (1998) correctly state that pancreatic duct epithelium lacks CD95L, but did not give information on CD95L in exocrine and endocrine pancreatic cells.

Coexpression of CD95 and CD95L is thought to be a major prerogative for autocrine suicide in T lymphocytes (Nagata, 1997). Our immunohistologic examination of serial frozen sections strongly suggests that in pancreatic epithelia, expression of CD95 and CD95L is mutually exclusive at the level of cell types and, in chronic pancreatitis, also at the single-cell level in areas where inflammatory changes in expression of CD95 and CD95L are observed. This corresponds to the situation we encountered in other cell types such as colonic epithelium ($CD95^+/CD95L^-$), Paneth cells ($CD95^-/CD95L^+$), other crypt and villus enterocytes of small intestine ($CD95^+/CD95L^-$), and plasma cells ($CD95^-/CD95L^+$) (Leithäuser et al, 1993; Möller et al, 1993, 1994; Sträter et al, 1997, 1999). Thus, at the phenomenologic level, these cell types seem to be protected against CD95-mediated autocrine death.

Evidently, the induction and surface expression of CD95 is a prerequisite for becoming susceptible to CD95L- mediated signals. We show that in chronic pancreatitis, the $CD95^-$ organ neoexpresses CD95 in acinar cells and duct epithelium in a focal manner. These foci of induction correspond to those infiltrated by lymphocytes, mainly $CD4^+$ T cells, and early interstitial fibrosis and also to those rich in $HLA\text{-}DR^+$ $CD11c^+$, $CD4^{+/-}$ $S100p^+$ interstitial dendritic cells (Grabbe et al, 2000). At the microtopographic level, CD95 induction in exocrine cells is paralleled by HLA-DR induction in these epithelia. Inflammation-associated HLA-DR induction in pancreatic epithelia has been previously described (Bedossa et al, 1990; Pavlovic et al, 1997). This setting strongly suggests an induction of these three molecules by inflammatory cytokines released by the local mononuclear infiltrate. Interestingly, in chronic pancreatitis, these infiltrate-associated changes in expression of CD95, CD95L, and HLA-DR did not occur in islet cells. If CD95-mediated apoptosis plays a role in parenchymal damage during pancreatitis, which still has to be proven, our observations would readily explain why islets are frequently unaffected even at advanced stages of this disease.

One major inductor of CD95 in constitutively $CD95^-$ cells is the Th1 cytokine INFγ (Leithäuser et al, 1993; Möller et al, 1994). IFNγ also induces HLA-DR in various constitutively MHC class II^- epithelia (Koretz et al, 1987, 1989). This is also the case in exocrine pancreatic epithelia but not in pancreatic islets (Pavlovic et al, 1997). In our study IFNγ tissue levels were significantly higher in chronically inflamed pancreases, suggesting that IFNγ is critically involved in this induction. IFNγ also up-regulates sensitivity toward CD95-mediated cell death (Reyher et al, 1998; Xu et al, 1998). These phenomena were also observed in AsPc1 pancreatic carcinoma cells. In addition, we detected a down-modulation of CD95L at the mRNA and protein level. This again fits very well in the pattern of immunophenotypic changes in chronic pancreatitis described herein. Data on regulatory pathways of CD95L expression are sparse. Sata and Walsh (1999) showed that cyclosporin down-regulates CD95L expression in vascular endothelial cells. Xu et al (1998) state that IFNγ and TNFα, but not PDGF, EGF, and FGF, up-regulated CD95L in HT29 colon carcinoma cells. However, the bands depicted in this publication do not convincingly show the inducing effect of IFNγ and TNFα. Furthermore, the authors failed to find CD95L message by RT-PCR, which agrees with our finding of CD95L negativity of HT29 cells. In the same report, Xu et al show a down-modulation of CD95L by IFNγ but not by TNFα in U3A, a human fibroblast line lacking STAT-1. STAT-1 transfection into U3A cells was followed by an IFNγ-mediated up-regulation of CD95L. The question arises whether this transfection restored physiologic levels or led to overexpression of STAT-1. In LS174T human colon carcinoma cells, constitutive CD95L expression was up-regulated by high-dose TNFα, whereas IFNγ or IL-2 failed to modulate CD95L (Wimmenauer et al, 1999). In critical conclusion, it is too early to generalize the fact that IFNγ down-modulates CD95L transcripts and protein in AsPc1 and BxPc3 cells. Nevertheless, IFNγ seems to be a good candidate inductor of local changes in the CD95/CD95L status of exocrine pancreatic cells.

We confirm that pancreatic islet cells of normal pancreas are $CD95^-CD95L^+$ (Loweth et al, 1998). With regard to the CD95 death receptor system, this is the phenotype of an immunoprivileged site. There is an ongoing discussion on the role that the CD95/CD95L (Fas/FasL) system plays in immune diabetes (NOD) (Signore et al, 1997; Suarez-Pinzon et al, 1999). Although the wealth of experimental data obtained in mouse systems (Chervonsky et al, 1997; Lau and Stoeckert, 1997) tentatively favors the view that the breakdown of the $CD95^-/CD95L^+$ status of pancreatic islets may be the critical event in NOD mice, open questions and contradictory results remain (Allison and Strasser, 1998; Itoh et al, 1997; Kang et al, 1997;

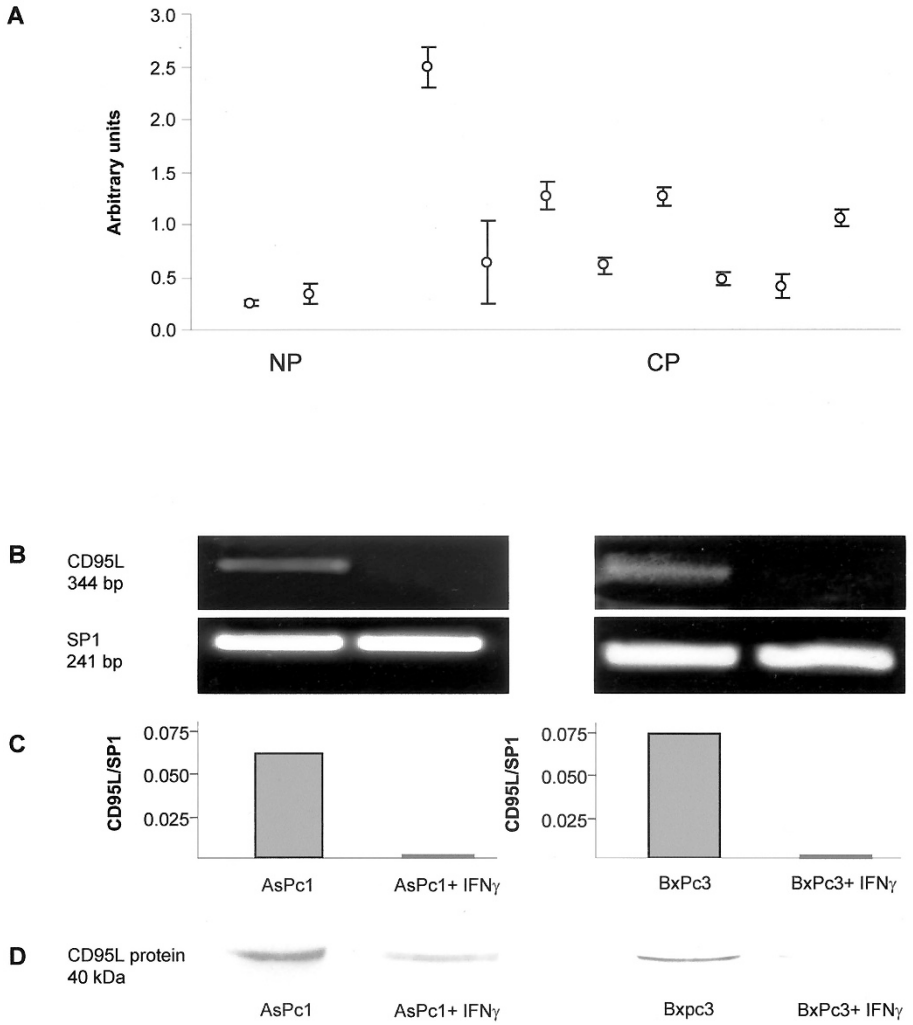

Figure 4.

A, Content of IFNγ in protein lysates of specimens of normal pancreas (*NP*) and chronic pancreatitis (*CP*), as determined by an enzyme immunoassay. B to D, CD95L transcript and protein expression in AsPc1 and BxPc3 pancreatic carcinoma cells in the absence vs presence of 100 U/ml IFNγ for 48 hours. B, CD95L and SP1 mRNA detected in an ethidium bromide-stained agarose gel after RT-PCR. C, Ratio profiles of integrated optical densities. D, Immunoblot of Western blotted CD95L protein detected by CD95L monoclonal antibody G247-4 visualized by chemiluminescence

Kim et al, 1999; Thomas et al, 1999). The situation is even less clear in humans. Moriwaki et al (1999) report immunohistochemical findings in biopsy specimens of recent-onset type I diabetic patients. Intact islets were $CD95^-$, whereas insulitis was associated with CD95 expression in islet cells, preferentially in β cells. Using an antiserum to CD95L, these authors did not detect CD95L in normal and infiltrated islets. We have shown that islets conserve their $CD95^-/CD95L^+$ phenotype even in the vicinity of inflammatory infiltrates of chronic pancreatitis. It is tempting to speculate that, for this reason, islets are often so well conserved in pancreases with advanced atrophy and scar formation in the course of chronic pancreatitis. Therefore, the elusive mechanism(s) initiating NOD by breakdown of the $CD95^-/CD95L^+$ status of islets might be different from those operative in chronic pancreatitis. The reported switch of the $CD95^-/CD95L^+/HLA\text{-}DR^-$ protector status of pancreatic acinar cells to $CD95^+/CD95L^-/HLA\text{-}DR^+$, probably triggered by IFNγ, may confer susceptibility to $CD4^+$Th1-mediated cytotoxicity (Ju et al, 1994; Lynch et al, 1995) and hence be a crucial step in parenchymal destruction during chronic pancreatitis.

Materials and Methods

Pancreatic Tissues

Eighteen patients, whose informed consent was obtained before surgery, were chosen for this study. Thirteen of these patients underwent partial pancreatectomy for chronic pancreatitis, eight of them had alcoholic pancreatitis, three had chronic non-alcoholic pancreatitis connected with pancreas divisum, one developed a cyst after initial surgery, and one had cryptogenic chronic pancreatitis. Five patients underwent partial pancreatectomy for pancreas divisum. The specimens of these five patients were histopathologically diagnosed as normal pancreas and were 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, ie, RNA and protein isolation by methods described below. Other parts of the quick-frozen tissues were stored at −80° C to be later used for frozen section immunohistochemistry.

Cell Culture

AsPc1 and BxPc3, two human pancreatic carcinoma cell lines, and HT29, a human colon carcinoma cell line, were purchased from American Type Culture Collection (Rockville, Maryland), kept in a humidified incubator at 37° C in 5% CO_2 atmosphere and cultured in Iscove's modified Dulbecco's medium (IMDM) (Biochrom, Berlin, Germany)/Roswell Park Memorial Institute medium (RPMI) (Life Technologies, Paisley, Scotland) supplemented with 10% FCS (PAA, Linz, Austria), 5 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin (BioWhittaker, Veviers, Belgium). To examine CD95L expression under IFNγ-stimulated conditions, cells were seeded in Ø 25 mm^2 culture plates (Nalge Nunc, Naperville, Illinois), allowed to adhere for 24 hours, then treated with IFNγ (Boehringer Mannheim, Mannheim, Germany) for another 48 hours. Cells were washed once with PBS w/o (Life Technologies) and harvested with trypsin (500 mg/l)/EDTA (200 mg/l) 1:250 (BioWhittaker).

mRNA Preparation and RT-PCR

RNA from tissue samples was prepared as described by Chomczynski and Sacchi (1987), then digested with RNase-free DNase I (Boehringer Mannheim). RNA from cell lines was extracted with TriZol Reagent (Life Technologies) according to the manufacturer's instructions, reversely transcribed using Superscript Reverse Transcriptase (Life Technologies), random hexamer primer, and addition of a RNase-inhibitor following the manufacturer's protocol. Semiquantitative triplex PCR was performed on cDNA using the *Taq* PCR Core Kit (Qiagen, Hilden, Germany) and the following sense and antisense primers: human transcription factor SP1: 5'-ACT ACC AGT GGA TCA TCA GGG-3 and 5'-CTG ACA ATG GTG CTG CTT GGA-3'; Fas Ligand: 5'-GGA TTG GGC CTG GGG ATG TTT CA-3' and 5'-TTG TGG CTC AGG GGC AGG TTG TTG-3. The length of amplicons are 241 bp and 344 bp. Thermocycling included the following steps: denaturation at 95° C for 5 minutes, 25 cycles: 95° C for 1 minute, 56° C for 1 minute, 72° C for 1 minute, and prolongation for 10 minutes at 72° C. Primers were used in a final concentration of 0.5 μM each, dNTPs at 10 μM, 2.5 U of *Taq* DNA polymerase in a total of 50 μl, 10× buffer and 5× Q-Solution according to the instructions. Omitting RT reaction resulted in no detectable PCR products. Electrophoretically separated PCR-products were ethidium-bromide stained and the fluorescence image of the amplicons were analyzed by ImageMaster VDS (Pharmacia Biotec, San Francisco, California). The ratio of the background corrected integrated optical densities of the DNA bands related to SP1 expression were calculated. RNA extraction and further processing was successful in 12 of 18 pancreases, but the RNA from 6 pancreases was degraded, probably due to endogenous RNase.

Immunohistochemisty

Serial, 2-μm thick cryosections were immediately fixed in ice-cold acetone for 10 minutes, air-dried and incubated for 1 hour with the following mouse antihuman monoclonal antibodies in appropriate dilutions: CD95L clone G 247-4, IgG1 isotype (PharMingen, San Diego, California), CD95 (anti-APO-1), IgG1 isotype, (DAKO, Copenhagen, Denmark), CD3(Leu4), IgG1 isotype (Becton Dickinson, Mountain View, California), CD4 (OKT4), IgG2a isotype, and CD11c(BU15), IgG1 isotype (Immunotech, Marseille, France). Anti-HLA-DR clone 1B5, IgG1 isotype, was generously contributed by Gerhard Moldenhauer (Deutsches Krebsforschungszentrum, Heidelberg, Germany). A purified rabbit antiserum to bovine S100p reactive with human S100p (DAKO) was also used. Bound primary antibody was detected via goat antimouse or antirabbit immunoglobulins conjugated to peroxidase-labeled dextran polymer in Tris-HCL buffer containing carrier protein (EnVision; DAKO). 3-amino-9-ethyl-carbazole (Sigma, St. Louis, Missouri) was used for substrate. Counterstain was performed with hemalum. Controls were performed by omitting the first-step antibody and yielded negative results, with the exception of stained granulocytes inconsistently present. Granulocyte staining was due to endogenous peroxidase that was not blocked for the sake of unimpaired antigenicity of the tissue.

Immunoblotting of CD95L Protein

Tissue samples were picked up in ice-cold lysis buffer (50 mmol/l Tris-HCl [pH 7.4], 150 mmol/l $NaCl_2$, 10 mmol/l EDTA [pH 8.0], 1% nonidet P-40, 10 μM PMSF, 1% sodium vanadate, 1 tablet of complete [Boehringer Mannheim]), a protease-inhibitor cocktail, then homogenized, sonified, and incubated for 1 hour at 4° C. Cells harvested as described above were lysed in boiling lysis buffer for 3 minutes. After centrifugation supernatant was removed and protein concentration determined using Bradford reagent (Bio-Rad, Munich, Germany). One hundred micrograms of every total protein lysate was separated on a 10% to 20% Tricine precast gel (Novex, San Diego, California) and transferred to a polyvinylidene difluoride membrane. Membranes were incubated with antihuman CD95L, clone G247-4, for 1 hour after blocking in PBS containing 5% non-fat dried milk at room temperature. Membranes were washed three times with PBS/0,02% Tween 20, then incubated with sheep antimouse biotinylated immunoglobulins (1:5,000) and streptavidin (1:5,000; Amersham, Arlington Heights, Illinois). Protein lysates of AsPc1 and HT29 cells served as positive and negative control, respectively. After another three washes, the blots were developed by enhanced chemiluminescence using the ECL system (Amersham).

Human IFNγ Sandwich EIA

To determine tissue concentrations of IFNγ, we prepared protein lysates extracted from snap-frozen tissue specimens of two normal pancreases and eight pancreases with chronic pancreatitis. We applied a commercial EIA kit containing a recombinant human IFNγ preparation as the standard (CYTELISA; CYTImmune Sciences, College Park, Maryland). For antigen capture, 100 μl of each protein lysate, together with a dilution series of the standard, were dispensed in triplicate in 96-well microtiter plates precoated with murine monoclonal antibodies against human IFNγ. Simultaneously, 25 μl of IFNγ specific rabbit antihuman polyclonal antibody per well were added for detection. Plates were sealed and incubated for 3 hours at room temperature. After repeated washing, 50 μl of goat antirabbit conjugated alkaline phosphatase were added, plates were resealed and incubated for 45 minutes at room temperature and then washed again. A two-step color-generating system was used according to the manufacturer's instructions. Briefly, the system is based first on dephosphorylation of NADH to NAD by alkaline phosphatase, and then by NADH serving as a cofactor activating a cycling redox reaction maintained by alcohol dehydrogenase and diaphorase. This reaction leads to the formation of the red product formazan, which absorbs light at 492 nm. Two hundred microliters of the color reagent were dispensed in each well. The light absorption was measured and calibrated against the standard with a MRX microplate reader (Dynatech Laboratories, Chantilly, Virginia). All wells were measured in triplicate. Results were analyzed using the Revelation software (Dynatech) and were given in arbitrary units as means with standard deviations. The Wilcoxon test was applied for statistical comparison of values of normal pancreas and chronic pancreatitis.

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