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ARTICLE

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Expression and secretion of alpha1-proteinase inhibitor are regulated by proinflammatory cytokines in human pancreatic islet cells

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Abstract *Aims/hypothesis:* Alpha1-proteinase inhibitor (α1-PI) has been considered a key player in inflammatory processes. In humans, the main production site of α1-PI is the liver, but other tissues, including pancreatic islets, also synthesise this molecule. The aims of this study were to assess the islet cell types that produce α1-PI, to determine whether α1-PI is actually secreted by islet cells, and to assess how its production and/or secretion are regulated. *Methods:* Expression of α1-PI in human islet cells was assessed by immunofluorescence, electron microscopy and western blotting. Release of α1-PI was analysed by reverse haemolytic plaque assay and ELISA. The effects of cytokines on α1-PI synthesis and secretion were tested. *Results:* Immunofluorescence showed that alpha and delta cells do express α1-PI, whereas beta cells do not. By electron microscopy, we demonstrated a colocalisation of α1-PI with glucagon and somatostatin within secretory granules. Immunolabelling also revealed localisation of α1-PI within the Golgi apparatus, related vesicles and lysosomal structures. The expression of α1-PI in islet cells was also demonstrated by western blotting and ELISA of protein extracts. ELISA and reverse haemolytic plaque assay showed that α1-PI is secreted into the culture medium. Treatment of islet cells with IL-1β and oncostatin M for 4 days increased the production and release of α1-PI. *Conclusions/interpretation:* Our results demonstrate that α1-PI is expressed by the alpha and delta cells of human islets, and that proinflammatory cytokines enhance the production and release of this inhibitor.

Keywords Human islets · IL-1β · Oncostatin M · α1-Proteinase inhibitor · Reverse haemolytic plaque assay

Abbreviations OSM: oncostatin M · α1-PI: alpha1-proteinase inhibitor · RHPA: reverse haemolytic plaque assay

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Introduction

Alpha1-proteinase inhibitor (α1-PI) is a 52-kDa acute phase protein that inhibits serine proteases, especially elastase, which is released by human neutrophils during inflammatory processes [1]. Deficiency of α_1-PI is associated with chronic liver disease, and is often associated with pulmonary emphysema. In humans, the liver is the major site of expression of α1-PI. Hepatocytes synthesise and secrete systemic α1-PI, and plasma concentrations of α1-PI acutely increase three to four-fold in response to 'secondary cytokines, such as members of the IL-6 family, including IL-6 itself, leukaemia inhibitory factor and oncostatin M (OSM) [2, 3]. It is thought that α1-PI is involved in the tissue repair reactions that follow inflammation.

The way secreted α1-PI acts as an anti-inflammatory agent is not well understood. The inactivation of proteolytic enzymes, such as serine proteinases of inflammatory cells is undoubtedly of key importance, but various observations indicate that other mechanisms are also involved. Thus, α1-PI has a neutrophil chemoattractant activity [4, 5], induces changes in neutrophil shape, and increases cell-to-substrate adhesion [5] and spreading of smooth muscle cells in a fibrin gel [6]. Moreover, this molecule also acts as

a secretagogue, since it induces degranulation of neutrophils [5] and, if complexed with elastase, increases its own synthesis by macrophages [7]. α1-PI may also have a protective effect against factors inducing apoptosis by a mechanism unidentified to date. Thus, α1-PI has been shown to specifically inhibit the apoptosis of hepatocytes induced by tumor necrosis factor [8], as well as that induced by prolonged ischaemia followed by reperfusion of organs in vivo [9]. It has been also suggested that α1-PI may act as an antioxidant, given its high content in methionine [10].

Although the liver is the major site of expression of α1-PI, many studies have shown that this molecule is also synthesised in other tissues and organs, in response to an inflammatory insult. For instance, lung epithelial cells respond dramatically to OSM, a cytokine of the IL-6 family, by synthesising and secreting α1-PI [11–14]. In addition, local inflammatory conditions have been shown to upregulate the production of α1-PI by monocytes [15], corneal cells [16], enterocytes and Paneth cells [17], and articular chondrocytes [18]. Taken together, these data indicate that local production of α1-PI may contribute to the defence of tissues against different types of injuries.

Preservation of a sufficient cell mass and function by endogenous mechanisms capable of protecting against cell injury is particularly important for the pancreatic islets of Langerhans. Indeed, the integrity of these micro-organs is altered in many pathological conditions. In type 1 diabetes, activation of autoimmune events leads to a local peri-insular inflammation that is particularly aggressive toward beta cells. In type 2 diabetes, amyloid deposits alter the function and viability of beta cells [19, 20], and it is known that α1-PI can act in vitro as an inhibitor of amyloid fibril formation, while promoting disaggregation of β-amyloid fibrils [21]. In pancreatitis, endocrine cells have to defend against the aggression of proteases released as a result of the destruction of the exocrine tissue. Finally, in islet of Langerhans transplantation for the treatment of type 1 diabetes, islets infused into the liver are subject to inflammatory insults at the site of implantation in addition to more specific insults such as immune rejection [22]. In these situations, α1-PI could play an important protective role in the progression of damage to beta cells. Even though previous reports have documented the presence of α1-PI within pancreatic islets [23–26], the type of islet cells producing the inhibitor is still a matter of controversy. In addition, whether islet cells secrete α1-PI, and whether and how this secretion is regulated has not been shown.

Materials and methods

Islet isolation

Pancreases were harvested from multiorgan cadaveric donors. Islets were isolated using a previously described modification of the automated method [27, 28]. Islets were then rinsed with CMRL 1066 medium (Sigma, St Louis, MO), and aliquots of 20,000 islets were incubated in 30 ml CMRL 1066 medium containing 5.6 mmol/l glucose and supplemented with 10% fetal calf serum, 25 mmol/l *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES), 2 mmol/l glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin (hereafter, referred to as complete CMRL). After 12 h at 37°C, culture medium was changed and islets were further cultured at 24°C for 1 to 3 days.

Islet cell preparation

Islets were washed three times with phosphate-buffered saline without Mg^{2+} and Ca^{2+} (PBS). Aliquots of 10^4 islets were resuspended in 1 ml 0.025% trypsin solution containing 1 mmol/l EDTA (Gibco, Paisley, Scotland), and incubated at 37°C for 9–10 min, with occasional pipetting through a 1-ml plastic tip. Trypsinisation was stopped by addition of 10 ml cold complete CMRL, and cells were washed twice with the same medium. Cells were then counted and aliquots of either 3×10^5 or 1×10^6 cells were incubated for 24 h in 10-cm-diameter, non-tissue culture-treated petri dishes containing 10 ml complete CMRL.

Culture conditions and cytokine treatment

To study the effect of cytokines on α1-PI expression by western blotting, aliquots of 4,500 islets were seeded in non-tissue culture-treated petri dishes (60 mm diameter) and incubated for 4 days in the presence of 4 ml complete CMRL medium supplemented, or not, with 1 mmol/l dexamethasone, 20 ng/ml IL-1β (Biosource International, Nivelles, Belgium), 20 ng/ml OSM (Biosource International) and 0.5 mmol/l isobutyl–methylxanthine (IBMX; Sigma). To study the effect of cytokines on α1-PI synthesis by enzyme-linked immunosorbent assay (ELISA) and immunofluorescence, aliquots of 15×10^4 islet cells were seeded onto coverslips (15 mm diameter), and placed into a 24-well plate. Then, cells were incubated for 4 days in the presence of 0.5 ml complete CMRL, supplemented, or not, with 1 mmol/l dexamethasone, 20 ng/ml IL-1β, 20 ng/ml OSM and 0.5 mmol/l IBMX. To analyse the secretion of α1-PI, islet cells were incubated as described above except that a serum-free complete CMRL medium was used.

Antibodies

Goat antihuman α1-PI polyclonal antibody (A-1845) was purchased from Sigma, and rabbit antihuman α1-PI polyclonal antibodies were purchased from Dako (A-0012; Glostrup, Denmark), Sigma (A-0409) and Zymed (18-0002; San Francisco, CA, USA). Rabbit antiglucagon (A565), somatostatin (A0566) and pancreatic polypeptide (A0619) polyclonal antibodies were purchased from Dako.

A guinea-pig anti-insulin polyclonal antibody was developed in our laboratory, using porcine insulin as immunogen, as previously described [29].

Immunofluorescence

To analyse the expression of α1-PI and islet hormones by immunofluorescence, aliquots of 10^4 islets cells were left to attach for 60 min at 37°C into Cunningham chambers. These chambers were rinsed with PBS, fixed in a 4% paraformaldehyde-PBS solution for 20 min, rinsed again with PBS and stored at 4°C. Samples of human pancreases were snap frozen and embedded in optimal cutting temperature (OCT) compound (Sakura Finetek Europe Zoeterwoude, The Netherlands). Five-micrometre sections were prepared and fixed in 4% paraformaldehyde-PBS solution for 20 min. Then, sections were rinsed and used immediately for immunofluorescence labelling.

The whole immunofluorescence procedure was carried out at room temperature and all solutions were prepared using PBS. After fixation, cells and sections were rinsed three times for 5 min in PBS, treated for 15 min with 0.1% Triton X-100 and exposed to a 0.1% bovine serum albumin (BSA) solution for 20 min. Cells were then incubated for 2 h with one, two or three of the primary antibodies, as indicated in the Results section. The dilutions were 1:1,200 for the anti-insulin antibody, and 1:400 for the both anti-glucagon and the antisomatostatin antibodies. For the anti-α1-PI, dilutions were 1:1,200, 1:400 and 1:200, for the Sigma goat antibody, and the rabbit antibodies from Sigma and Dako, respectively.

Immunogold electron microscopy

Pellets of isolated islets were washed twice in 0.1 mol/l phosphate buffer, followed by 5-min fixation at room temperature in 4% paraformaldehyde and 0.1% glutaraldehyde, 60-min fixation in 4% paraformaldehyde (all fixatives diluted in 0.1 mol/l phosphate buffer, pH 7.4). The islets were washed three times in 0.1 mol/l phosphate buffer, embedded in 12% gelatin and cooled on ice. Small blocks of gelatin-embedded islets were infused with 2.3 mol/l sucrose, snap frozen in liquid nitrogen, and sectioned with an EMFCS cryoultramicrotome (Leica Microsystems, Wetzlar, Germany). Ultrathin sections were mounted on Parlodion-coated copper grids. The sections were treated as per a modification [30] of a previously described technique [31]. Briefly, sections were sequentially incubated as follows: twice for 2 min in 0.1% glycine (in PBS), once for 2 min in 1% BSA (in PBS), 1 h at room temperature in the presence of the first primary antibody, rinsed four times for 2 min in 0.1% BSA, and exposed 20 min at room temperature to either protein A-coated gold particles (15 nm diameter, for islet hormones) or to goat antimouse or antirabbit antibodies, whichever appropriate, coupled to 15-nm and to 10-nm diameter gold particles, respectively. After two 2-min rinses in 0.1% BSA, and two 2-min rinses in PBS, the sections were exposed for 5 min to 1% glutaraldehyde (in 0.1 mol/l sodium phosphate buffer, pH 7.4), rinsed twice for 5 min in PBS, exposed for 1 h at room temperature to a second primary antibody (when required), rinsed four times for 2 min in 0.1% BSA, exposed for 20 min at room temperature to either protein A-coated gold particles or an appropriate gold-conjugated goat antibodies, rinsed twice 2 min in 0.1% BSA, rinsed twice for 2 min in PBS, exposed for 5 min to 1% glutaraldehyde (in 0.1 mol/l sodium phosphate buffer, pH 7.4), rinsed twice for 5 min in PBS, rinsed six times for 1 min in distilled water, and eventually stained for 5 min in 2% uranyl acetate oxalate (pH 7), followed by 2% methylcellulose containing 0.5% uranyl acetate. Cryosections were screened and photographed in a CM10 electron microscope (Philips, Eindhoven, The Netherlands).

Single immunolabelling for α1-PI was carried using the rabbit polyclonal serum (Dako) diluted 1:40, and revealed with a goat anti rabbit IgG serum coupled to 10-nm gold particles (British Biocell International, Cardiff, Wales) and diluted 1:5. Double immunolabelling for islet hormones and α1-PI was performed using the same anti α1-PI serum and one of the following antibodies: a mouse monoclonal antibody against glucagon (Sigma), diluted 1:100, and revealed with a goat antimouse IgG serum coupled to 15-nm gold particles (British Biocell International) diluted 1:5; a goat antisomatostatin antibody (a kind gift of M.P. Dubois), diluted 1:40, and revealed with 15-nm protein A-coated gold particles diluted 1:150; a mouse monoclonal against insulin (a kind gift of M.J. Storch), diluted 1:100, and revealed with 15-nm protein A-coated gold particles diluted 1:150.

Controls included exposure of the sections to only the protein A-coated gold particles or to the gold-conjugated goat antibodies against either rabbit or mouse IgGs, whatever was appropriate. None of these incubations resulted in a sizeable, specific staining of the sections.

Western blot analysis

For preparation of total protein extracts, islets were incubated under the different conditions detailed in the results, rinsed three times in PBS and lysed by sonication in 80 mmol/l Tris–HCl buffer (pH 7.4), containing 5% SDS, 5 mmol/l EDTA and a cocktail of protease inhibitors (Complete Mini Protease Inhibitor Cocktail Tablets, Roche, Rotkreuz, Switzerland), which was used as per the manufacturer's recommendations. Protein content was determined by the Bio-Rad DC protein assay reagent kit (Bio-Rad, CA). Ten-micrograms aliquots of total proteins were diluted in gel loading buffer (0.025 mol/l Tris pH 6.8, 0.5% SDS, 1% 2-mercaptoethanol, 0.025% bromophenol blue, 17.5% glycerol). Aliquots were loaded on a 7.5% polyacrylamide-SDS gel. After separation, proteins were transferred to a nitrocellulose membrane (Protran; Schleicher & Schuell, Keene, NH), which was incubated overnight in Tris-buffered saline (TBS; Tris 0.01 M, pH 7.4, 0.15 mol/l NaCl) containing 5% non-fat dried milk and 0.1% Tween,

and subsequently incubated for 1 h with a polyclonal rabbit antihuman α1-PI antibody (Dako), diluted 1:2,000 in the same buffer. After extensive washes in TBS containing 0.1% Tween (TBS-Tween), the membrane was probed for 1 h with a horseradish-peroxidase-conjugated secondary antibody against rabbit IgG (Biorad), diluted 1:6,000 in TBS-Tween, rinsed again in TBS-Tween, revealed with the SuperSignal West chemiluminescent substrate kit (Amersham-Pharmacia, Buckinghamshire, UK) according to manufacturer's instructions, and exposed for 1 min to Hyperfilm (Amersham-Pharmacia).

Expression and release of α1-PI

The islet content in α1-PI and the amounts of inhibitor secreted in the medium were measured by ELISA (Immundiagnostik, Bensheim, Deutschland).

A reverse haemolytic plaque assay (RHPA) was used as described [32] to attribute the secretion of α1-PI to specific islet cell types. Briefly, isolated islet cells were suspended in a Krebs–Ringer-bicarbonate buffer containing 12.5 mmol/l HEPES and 0.1% BSA (control KRB) and mixed with 5% (v/v) packed sheep red blood cells (SRBC, Behring Institute, Marburg, Germany) that had been previously coated with protein A. Then, the cells were rinsed and incubated for 2 h at 37°C in the presence of a goat antihuman α1-PI polyclonal antibody (Sigma), diluted 1:30 in control KRB supplemented or not with 16.7 mmol/l glucose, 10 μmol/l forskolin and 100 nmol/l phorbol 12-myristate 13 acetate (PMA). The cells were rinsed with control KRB and incubated for 1 h at 37°C in the presence of guinea pig complement (Behring Institute), diluted 1:40 in control KRB. Chambers were filled with 0.04% (wt/vol) solution of trypan blue in control KRB, rinsed with control KRB, and filled with Bouin's fixative or 4% paraformaldehyde. After immunofluorescence for insulin or α1-PI, the proportion of secreting cells (as determined by the presence of a surrounded haemolytic plaque that was identified as a circular area centered on an islet cell and containing the dark lysed erythrocytes) was determined under a microscope equipped with a 40× objective and a combined phase-contrast and fluorescence illumination. Analysis was restricted to single cells that excluded trypan blue at the end of the plaque assay. Haemolytic plaques were not detected when SRBC not coated with protein A were used as controls, nor when either the anti-α1-PI antibody or complement were omitted.

Statistical analysis

Statistical analysis was performed by analysis of variance (ANOVA) and LSD Post Hoc tests using SPSS (Statistical Package for Social Sciences, Chicago, IL). Student's *t*-test was performed for comparison between two groups using the Statistica software package (StatSoft, Tulsa, OK). Values of $p<0.05$ were considered significant.

Results

α1-PI is selectively expressed in alpha and delta cells

Expression of α1-PI was studied by immunofluorescence on dispersed islet cells prepared from seven human pancreases, and cultured under conditions favouring survival of endocrine cells. In all preparations, 13–27% of the cells stained positively for α1-PI (means±SEM: 22.1± 2.0%), (Fig. 1a). To identify the cell types expressing α1-PI, double staining was performed for α1-PI and either insulin, glucagon, somatostatin or pancreatic polypeptide (Fig. 1b). Virtually no insulin-producing beta cell and only few pancreatic polypeptide-producing cells (PP-cells) expressed α1-PI. In contrast, most glucagon- (alpha cells) and somatostatin-producing cells (delta cells) were labelled for α1-PI. This pattern was confirmed using triple immunolabelling. Thus, we found no beta cell co-expressing α1-PI, but observed that most glucagon- and somatostatin-immunoreactive cells also expressed α1-PI (Fig. 2).

On frozen sections of four normal pancreases, immunofluorescence showed α1-PI predominantly within cells situated at the periphery of islets, most of which stained for either somatostatin or glucagon, but did not detect the inhibitor in the insulin-immunoreactive cells which occupy the core of the islets (Fig. 3).

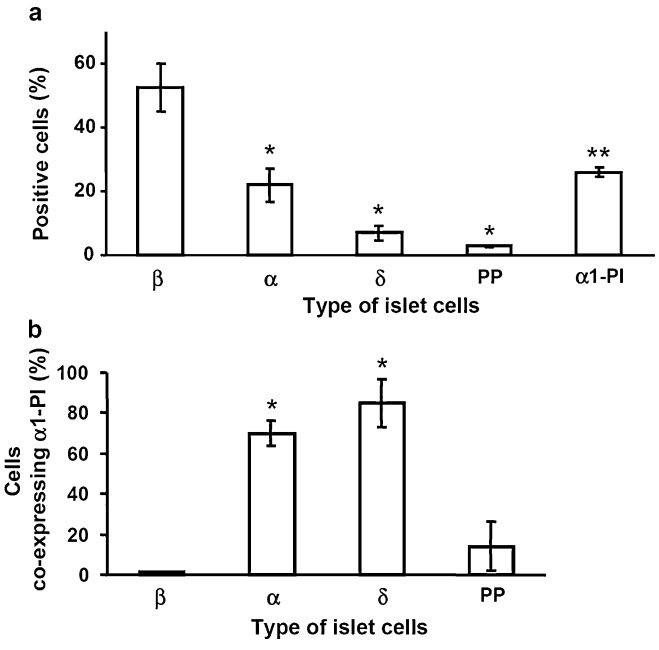

Fig. 1 α1-PI immunoreactive cells are abundant among islet cells and correspond to alpha and delta cells. **a** The percentages of cells labelled for insulin (*β*), glucagon (*α*), somatostatin (*δ*), pancreatic polypeptide (*PP*) and α1-PI are shown. Values are the means±SEM of three independent experiments. A minimum of 200 cells was scored per condition and experiment. *$p<0.0001$ and **$p<0.001$ when compared with the beta-cell group (*β*). **b** The percentage of cells co-expressing α1-PI and either insulin (*β*), glucagon (*α*), somatostatin (*δ*) or pancreatic polypeptide (*PP*) are shown. Values are means±SEM of three independent experiments. A total of 305 insulin-, 231 glucagon-, 82 somatostatin- and 123 pancreatic-polypeptide-expressing cells was scored. *$p<0.0001$ when compared with the beta-cell group (*β*)

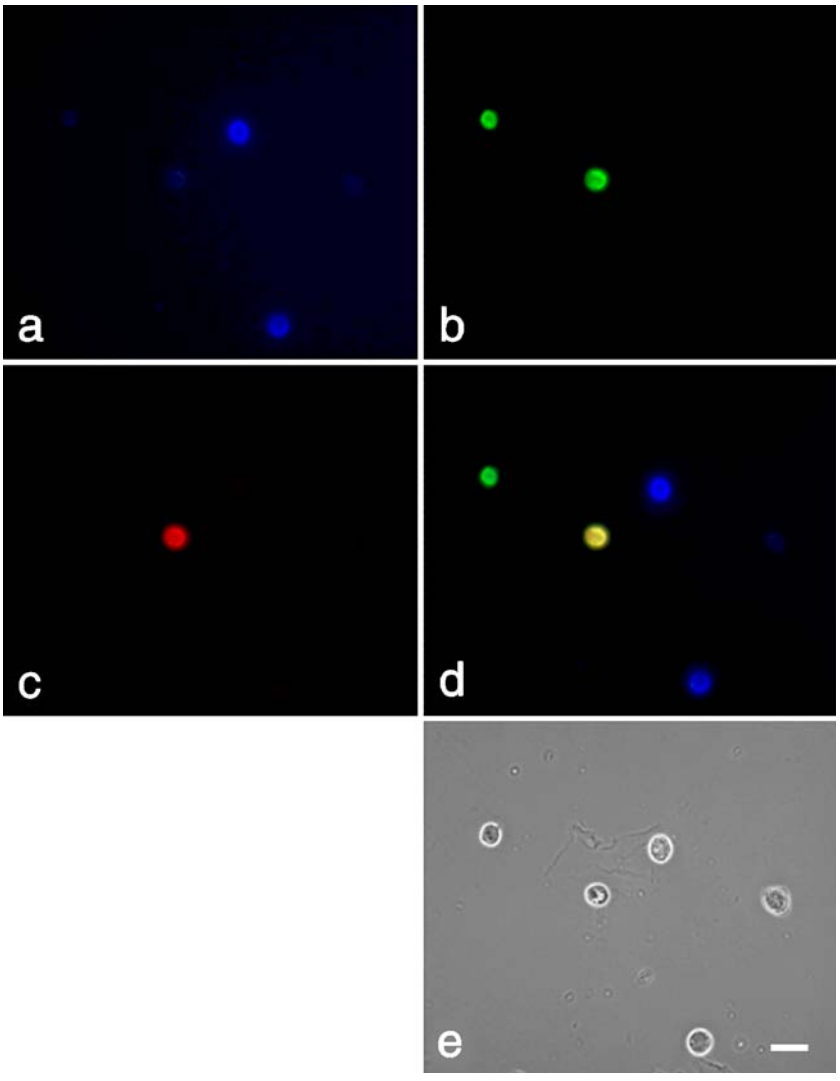

Fig. 2 α1-PI was detected in somatostatin- but not insulin-containing cells after triple immunofluorescence labelling. Insulin-expressing cells are stained in *blue* (**a**), α1-PI-expressing cells in *green* (**b**) and somatostatin-expressing cells in *red* (**c**). *Merged images* (**d**) show that beta cells do not contain detectable levels of α1-PI, whereas it is abundant in one somatostatin-expressing cell (in *yellow*). A phase-contrast view of the same cells (**e**). *Scale bar*: 20 μm

α1-PI is localised in multiple secretory compartments of glucagon- and somatostatin-containing cells

Immunolabelling of ultrathin cryosections of human islets resulted in a specific staining for α1-PI within glucagon- and somatostatin-expressing cells, but not within insulin-expressing cells (Fig. 4a). In alpha and delta cells, α1-PI was found within multiple secretory compartments, including the stacks and vesicles of the Golgi apparatus (Fig. 5a), the hormone-containing secretory granules (Fig. 4a–d) and a variety of lysosomal structures (Fig. 5a). In contrast, no labelling was observed within nuclei, mitochondria and cell membranes (Fig. 4a, Fig. 5a).

α1-PI synthesis by islet cells is regulated by cytokine stimulation

Isolated islet cells were cultured for 4 days in the absence or presence of 0.5 mmol/l IBMX, 20 ng/ml IL-1β and 20 ng/ml OSM, with, or without, 1 μmol/l dexamethasone, before α1-PI content of islet cells was measured by ELISA after protein extraction. In the absence of dexame thasone, IL-1β and OSM affected α1-PI synthesis poorly (Fig. 6a). In contrast, in the presence of dexamethasone, treatment of islet cells by both cytokines resulted in a two- to three-fold increase in α1-PI content (Fig. 6a). Irrespective of the presence of dexamethasone, incubation with IBMX with the aim to increase cAMP had no effect on α1-PI synthesis (Fig. 6a).

The effect of cytokines on α1-PI expression was further assessed by quantifying the immunofluorescence labelling of α1-PI, related to that of the cell hormone content. In the presence of dexamethasone, IL-1β and OSM, but not IBMX, significantly increased the labelling of α1-PI compared with that of glucagon (Fig. 6b). This change was due to an actual increase in immunolabelling for α1-PI, inasmuch as the ratio of glucagon to insulin staining remained unchanged (Fig. 6b).

In further experiments, cultured intact human islets were exposed for 4 days to cytokines, in the presence or absence of dexamethasone, and the expression of α1-PI was analysed by western blotting. Increased levels of immunoreactive α1-PI were observed in islets treated with IL-1β and

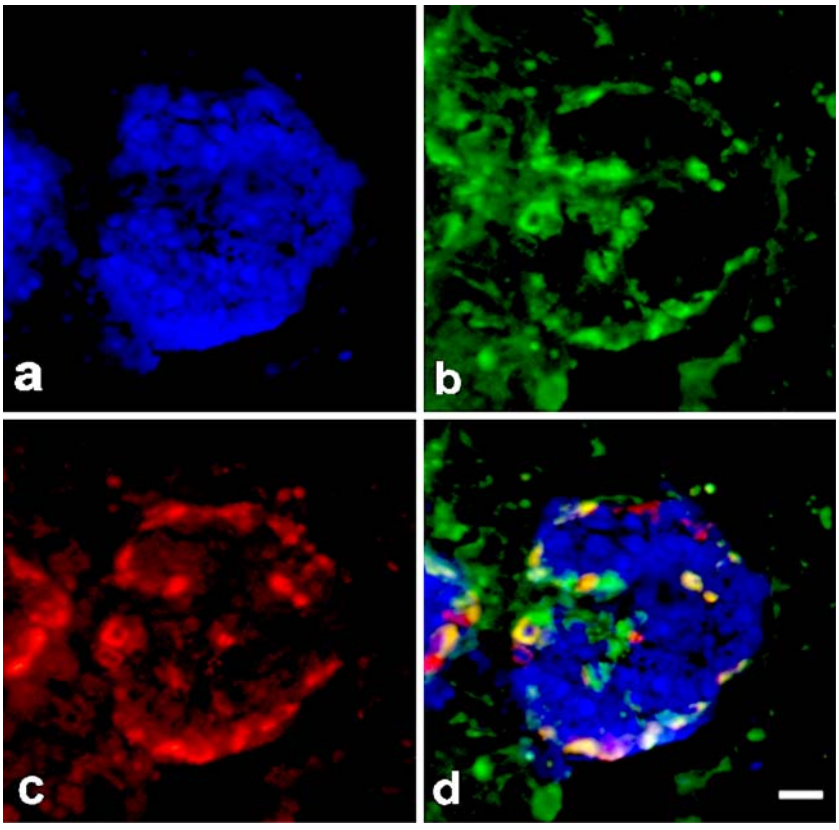

Fig. 3 α1-PI colocalises with glucagon, but not insulin, within intact human pancreas. Insulin-containing cells appear in *blue* (**a**), α1-PI-expressing cells in *green* (**b**) and glucagon-expressing cells in *red* (**c**). *Merged images* (**d**) demonstrate that beta cells (*blue*) did not contain detectable levels of α1-PI, whereas the inhibitor was present in several glucagon-containing cells (in *yellow*). Cells labelled only for α1-PI (*green* in **d**) could be either delta cells or intra-insular endothelial cells [24]. *Scale bar*: 40 μm

OSM, as compared with control islets (Fig. 6c). Addition of dexamethasone resulted in a much stronger stimulation of α1-PI expression (Fig. 6c).

α1-PI is secreted by islet cells

To assess whether α1-PI is secreted by islet cells, we developed a RHPA that allows for a direct visualisation of α1-PI release by individual islet cells. After a 2 h-incubation in the presence of antibodies against α1-PI, and a 1-h incubation in the presence of complement, several islet cells were surrounded by a haemolytic plaque, as judged by the presence of dark, non-refringent red blood cell ghosts (Fig. 7a). Immunofluorescence for insulin showed that only non-beta cells were surrounded by a haemolytic plaque. Immunofluorescence for α1-PI confirmed that cells surrounded by a haemolytic plaque contained α1-PI (Fig. 7a). Quantitative analysis showed that 16.7±0.7% (mean±SEM of three independent experiments) of the cells that did not stain for insulin were surrounded by a haemolytic plaque. This percentage did not change when cells were stimulated with glucose (16.7±0.9, n=3) or with 0.5 mmol/l IBMX+10 μmol/l forskolin+100 nmol/l PMA (16.0±1.0, n=3).

To assess whether the release of α1-PI was regulated by dexamethasone and cytokines, islet cells were exposed for 4 days to these agents and both the cumulated (4 day culture, Fig. 7b) and acute release (1 h in control medium, Fig. 7d) of α1-PI was measured by ELISA, and compared with the release of insulin (Fig. 7c). Results show that both chronic and acute releases of α1-PI were significantly increased by IL-1β and OSM, but only when dexamethasone was present in the medium (Fig. 7b,d). In contrast, IL-1β and OSM did not affect insulin secretion, which was stimulated by IBMX and decreased by dexamethasone (Fig. 7c).

Discussion

Previous reports have documented that α1-PI was preferentially expressed by peripheral islet cells, but failed to positively identify these cells [25, 26, 33]. Using different approaches, our study documents now that, in pancreases from brain-dead heart-beating donors, α1-PI is expressed in differentiated alpha, delta and PP-cells, accounting for about 22% of the isolated islet cells.

The reason why α1-PI is located preferentially in the alpha and delta cells of human pancreatic islets, within only a few PP-cells and not in beta cells, remains to be understood. One possibility is that it is related to lineage relationships between the different islet cell types. Indeed, it has been shown that there is a close ontogenetic relationship between pancreatic polypeptide- and insulin-producing cells, whereas adult glucagon- and insulin-expressing cells originate from different precursors [34, 35].

Our experiments show that α1-PI is expressed in multiple secretory compartments, including the Golgi apparatus, lysosomes and secretory granules. While the

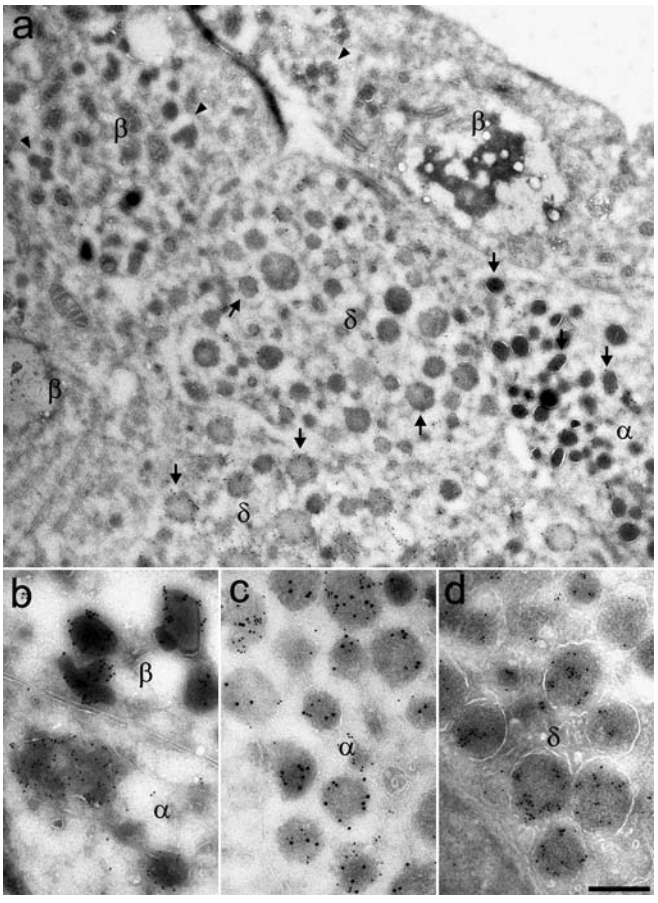

Fig. 4 α1-PI was found in glucagon- and somatostatin-containing granules, but not in insulin-containing granules. **a** Low-power view of a cryosection immunolabelled for α1-PI showing the inhibitor within the secretory granules (*arrows*) of glucagon- (α) and somatostatin-containing cells (δ), but not within the granules (*arrowheads*) of insulin-containing cells (β). **b**–**d** Double immunolabelling for α1-PI and one islet hormone demonstrated co-expression of the inhibitor (small gold particles) and either glucagon or somatostatin (large gold particles) within individual secretory granules of (**c**) alpha and (**d**) delta cells. In contrast, no α1-PI was detected within the insulin-containing granules of beta cells (**b**), when an antibody was used that resulted in intense labelling of the granules of an adjacent alpha cell (**b**). *Scale bar*: 1 μm (**a**), 500 nm (**b**–**d**)

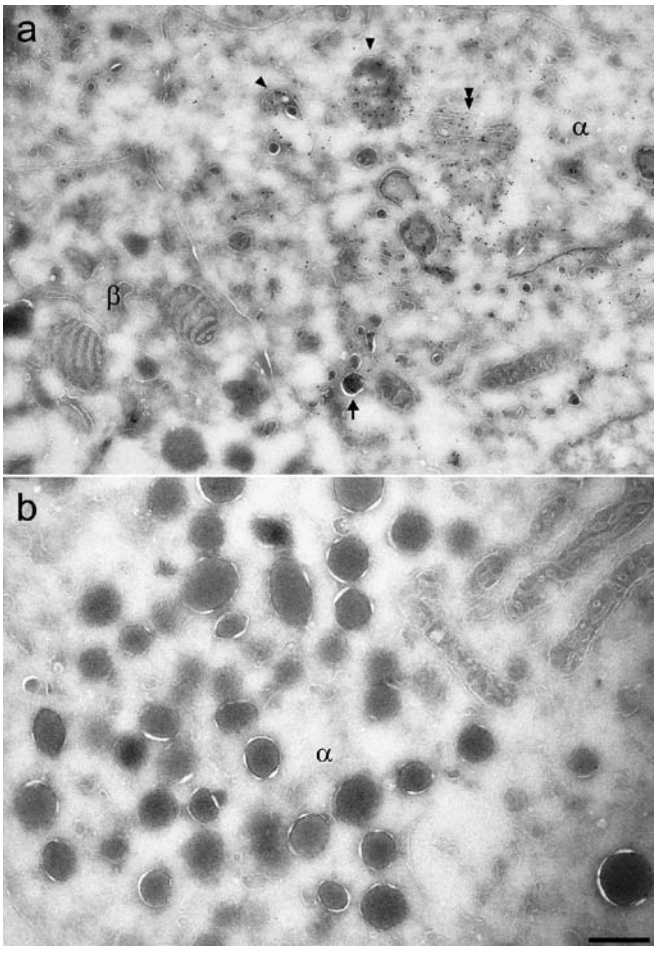

Fig. 5 α1-PI was detected in multiple secretory compartments of glucagon-producing cells. **a** Immunolabelling revealed α1-PI within the Golgi apparatus (*double arrowhead*), the secretory granules (*arrows*) and the lysosomes (*single arrowheads*) of a glucagon-producing alpha cell. Note the absence of labelling in an adjacent beta cell (β). **b** Omission of the antibody against α1-PI resulted in no gold labelling of secretory granules of an alpha cell (α). *Scale bar*, 1 μm (**a**), 500 nm (**b**)

presence of α1-PI in the Golgi apparatus and in lysosomes is not surprising, the finding of this inhibitor in secretory granules containing glucagon or somatostatin is unexpected, in view of previous reports considering this inhibitor as a constitutive secretory marker [36]. In secretory cells, trafficking from the Golgi apparatus onwards involves vesicles that are either directly conveyed to the cell surface (constitutive secretion), targeted to the lysosomal compartments or stored in granules belonging to the regulated secretory pathway. A genetic variant of α1-PI with a single Arg→Met^{358} substitution was shown to be transported into granules and to inhibit conversion of hormones during their transit through the regulated secretory pathway [37–39]. Also, the native form of α1-PI cells was found at low levels in secretory granules after transfection of MIN6 [39], suggesting that α1-PI could be allocated to the regulated pathway. However, this finding does not demonstrate that the α1-PI released and detected in the medium comes from a regulated secretory pathway. Rather, our results showing that IBMX and PMA did not stimulate α1-PI secretion support the hypothesis that this inhibitor is predominantly released by a constitutive pathway. Our finding that α1-PI is localised in hormone-containing granules, argues also for a role of this inhibitor in the regulation of glucagon and somatostatin conversion within differentiated islet cells. This process starts in the Golgi apparatus and immature granules, and involves proteolytic cleavage by a family of proteases called convertases [40].

We further show here that α1-PI expression and release by human islets was increased by cytokines. The plasma levels of α1-PI increase during acute and chronic inflammatory processes [1–3]. Certainly, the main source of this inhibitor in humans is the liver, and the secretion of α1-PI by hepatocytes was shown to increase in response to proinflammatory cytokines, such as IL-1β and IL-6 [2].

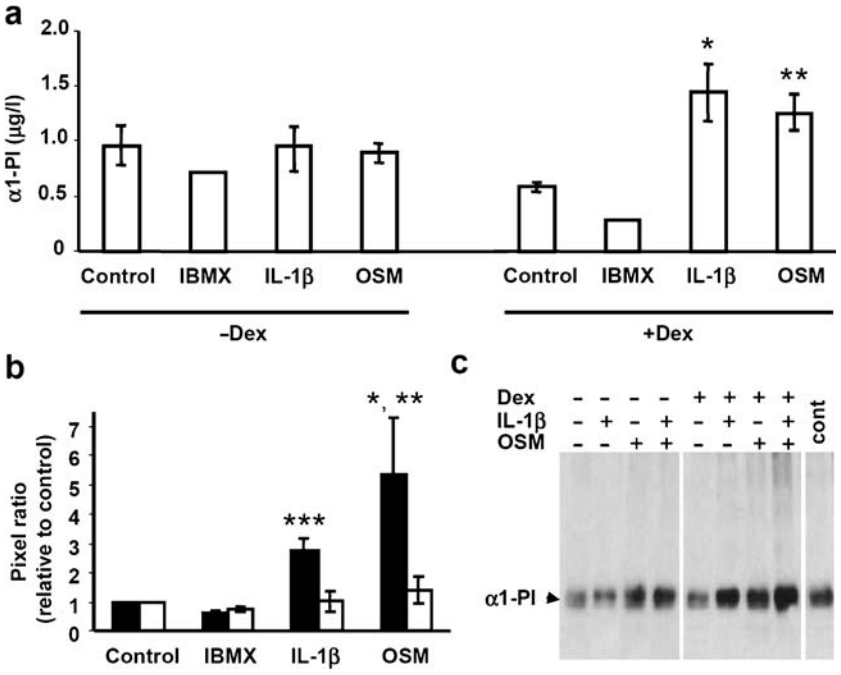

Fig. 6 α1-PI expression within islets and islet cells is increased by cytokines. Islet cells were incubated for 4 days, in the absence (−*Dex*) or presence (+*Dex*) of 1 µmol/l dexamethasone. Standard medium (*control*) was supplemented with 0.5 mmol/l IBMX, 20 ng/ml IL-1β or 20 ng/ml OSM. **a** Proteins were extracted and α1-PI measured by ELISA. In the absence of dexamethasone, neither IBMX nor cytokines caused significant changes in α1-PI expression. In contrast, in the presence of dexamethasone, IL-1β and OSM induced a 2 to 3-fold increase in α1-PI expression. Results for control, IL-1β and OSM conditions are expressed as means±SEM of three experiments; only one experiment was performed with IBMX. *p<0.02 and **p<0.05 when compared with the control in the presence of dexamethasone. **b** After fixation and triple immunofluorescence for α1-PI, glucagon and insulin, multiple microscopic fields from three independent experiments were quantified. Pixel measurements for α1-PI and insulin staining were compared with those for glucagon, and expressed as α1-PI/glucagon (*solid columns*) and insulin/glucagon (*open columns*) ratios. After exposure to either IL-1β or OSM for 4 days in the presence of dexamethasone, labelling of α1-PI compared with that of glucagon increased, while labelling of insulin was unchanged. *p<0.005 when compared with control, **p<0.05 and ***p<0.01 when compared with the corresponding insulin/glucagon group. **c** Human islets were incubated for 4 days in the absence (−) or presence (+) of 1 µmol/l dexamethasone, in standard medium (−) or in this medium containing (+) either 20 ng/ml IL-1β or 20 ng/ml OSM. Total proteins were extracted from islets, separated by SDS-PAGE and reacted with an antibody against α1-PI. Under all conditions tested, α1-PI was detected as a single band, with an apparent molecular weight similar to that of purified human α1-PI (*control*). Both IL-1β and OSM increased the expression of α1-PI, with a synergistic effect in the presence of dexamethasone. This panel is representative of three different experiments

Presumably, this increase results in the inhibition of neutrophil elastase, which breaks down connective tissue components causing tissue damage during inflammatory diseases. It has been suggested that extrahepatic α1-PI may also contribute locally, within specific organs, to the response initiated by an inflammatory or infectious process [14, 41]. Our study extends this possibility to human pancreatic islets, raising the question of whether α1-PI is implicated in the protection of peripherally located islet cells, which are preferentially exposed to the autoimmune attacks leading to islet destruction. As in other epithelial cell types [12, 14], dexamethasone was shown to enhance cytokine-induced production and release of α1-PI by islet cells. It is widely accepted that corticosteroid hormones are involved in the regulation of immune responses and have a potent anti-inflammatory action, via an up- or down-regulation of several genes. Our study shows that α1-PI is one of such genes in the islets. The effects of glucocorticoids on cell death and survival depend on the cell type. For instance, these steroids induce apoptosis of cells involved in inflammatory reactions, such as monocytes, macrophages and T lymphocytes, but they protect some cells of epithelial origin against apoptotic signals [42]. Many studies have examined the effects of glucocorticoids on the function of beta cells. Under many conditions, glucocorticoids have been shown to have a deleterious effect on insulin secretion [43], whereas in others they have been shown to protect islet cells partially against IL-1β-induced inhibition of insulin secretion [44, 45]. Another protective effect of glucocorticoids on pancreatic islets results from the induction in the expression of *Reg* gene, which has been shown to activate regeneration of beta cells, when dexamethasone is combined with inflammatory mediators [46].

Therefore, it is possible that the α1-PI expressed by alpha and delta cells has an anti-inflammatory function in human islets. Proinflammatory cytokines are critical in the development of type 1 diabetes. These molecules are found within the islets at the onset of diabetes [47], and have been shown to inhibit the function of islets, by inducing DNA damage and beta cell death [48–50].

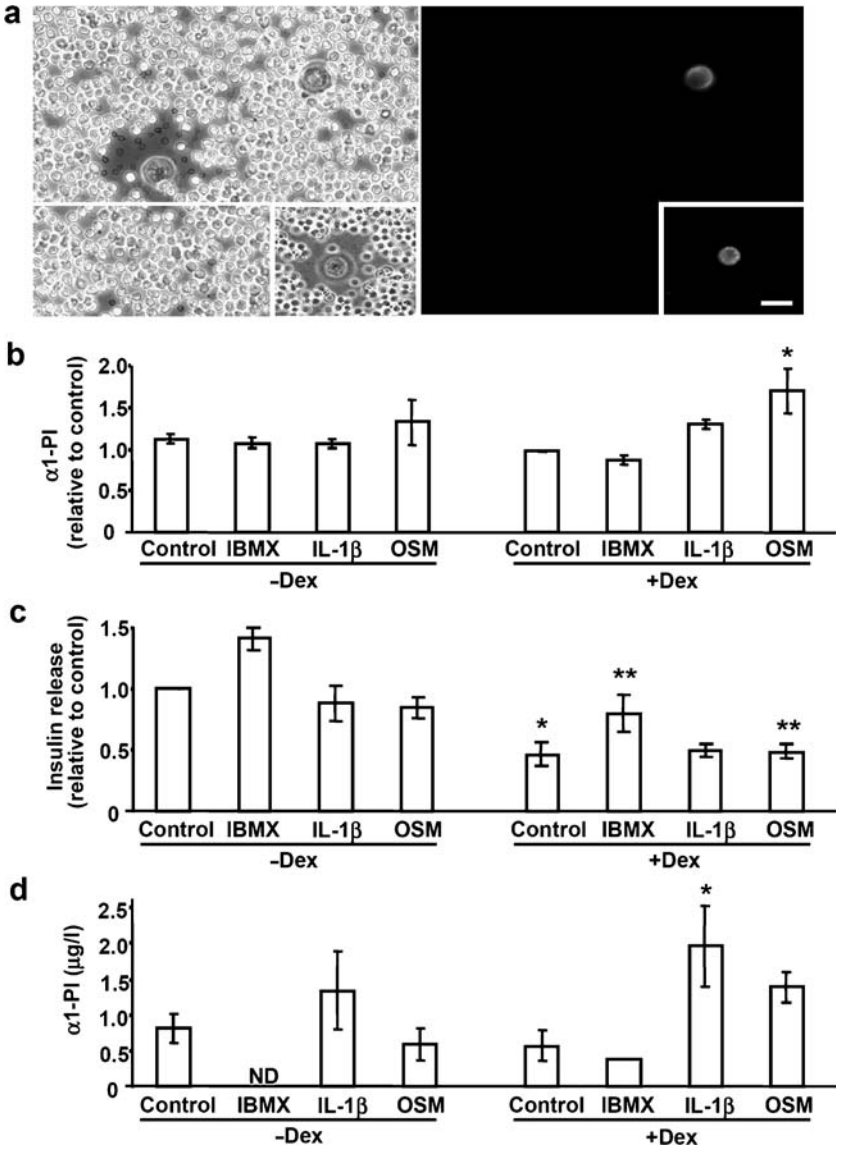

Fig. 7 α1-PI is released by islet cells. **a** After a 2-h incubation in control medium, a minority of islet cells secreted α1-PI, and are seen surrounded by circular haemolytic plaques, containing dark ghosts of complement-lysed erythrocytes (*left*). Immunostaining for insulin (*right*) revealed that beta cells were not surrounded by haemolytic plaques, which were always centred on non-beta cells. Inserts show one cell surrounded by a haemolytic plaque, which stained positively for α1-PI. *Scale bar*: 30 μm. **b** Islet cells were cultured in serum-free medium, in the absence (−*Dex*) or presence (+*Dex*) of 1 μmol/l dexamethasone. Standard medium (*control*) was supplemented with 0.5 mmol/l IBMX, 20 ng/ml IL-1β or 20 ng/ml OSM. Aliquots of supernatant were taken after 4 days in culture and α1-PI content measured by ELISA. Results of control, IL-1β, OSM (four experiments) and IBMX (two experiments) are means±SEM. **p*<0.001 when compared to control in the presence of dexamethasone. **c** Islet cells were cultured as described (**b**). Aliquots of medium were taken after 4 days of culture and insulin release measured by ELISA. Data show that only IBMX affected insulin release, both in the absence (−*Dex*) and presence of dexamethasone (+*Dex*). Results are expressed as means±SEM of six experiments for control, IL-1β and OSM, and four experiments for IBMX. **p*<0.005 and ***p*<0.05 when compared with the corresponding condition without dexamethasone. **d** Islet cells were cultured as described (**b**). After 4 days, the cells were rinsed and incubated in control medium for 1 h. Aliquots of culture supernatant were taken and α1-PI content measured by ELISA. In the absence of dexamethasone (−*Dex*), results are means±SEM of two experiments. *ND*, non-determined. In the presence of dexamethasone (+*Dex*), results are means±SEM of three experiments for all conditions, except for IBMX where *n*=1. **p*<0.05 compared with the control in the presence of dexamethasone

Further in-vitro experiments are needed to verify that α1-PI is really involved in protecting islet cells from the injury caused by cytokines, such as IL-1β, TNF-α and IFNγ [51]. In this regard, it is interesting to note that IL-6, a cytokine related to OSM, was shown to confer protection to pancreatic islet beta cells from cytokine-induced cell death [52]. It will also be important to assess the expression of α1-PI in islets of diabetic patients. One implication of our study is that the expression of α1-PI should be affected in islets showing a well-characterised insulitis and beta-cell destruction. In some diabetic patients, the plasma concentration and activity of α1-PI was found to be decreased

compared with controls [53–55], but the pathogenesis of this finding was not investigated.

In addition to a possible role in regulating cell injury induced by inflammatory cytokines, we propose that α1-PI, produced by the pancreatic alpha and delta cells, may also act as an antiprotease against enzymes released by the surrounding exocrine tissue. In this perspective, alpha and delta cells could protect the more centrally located islet cells, mostly beta cells. This function could be particularly important during the development of acute pancreatitis when leakage of aggressive enzymes from surrounding inflamed acinar cells is expected.

Finally, our results show that islet cells are able to release α1-PI. We measured a higher concentration of α1-PI in the culture supernatant after cytokine treatment. This increase is accounted for by a higher synthesis of α1-PI after cytokine treatment, than by a major increment in the release of the inhibitor. When islet cells treated with cytokines were further incubated in control medium, they continued to release more α1-PI than untreated cells. These observations suggest that α1-PI in islet cells is secreted in a constitutive manner.

In summary, our results clearly show that human islet alpha and delta cells express and secrete α1-PI, and that these events are modulated by cytokines. Taken together our data suggest that local production of α1-PI in islets may be triggered by inflammatory processes that occur at the onset of diabetes, the development of acute pancreatitis and conditions perturbing the native islet environment, such as transplantation. Further studies should determine whether and how α1-PI is involved in these pathological conditions.

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