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Immunohistochemical localization of human kallikreins 6 and 10 in pancreatic islets

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Summary

Tissue kallikreins are thought to be present in the pancreatic islets of Langerhans and to aid in the conversion of proinsulin to insulin. In recent immunohistochemical studies, we observed strong staining of the newly identified human kallikreins 6 and 10 (hK6 and hK10) in the islets of Langerhans. Here, we examine hK6 and hK10 immunoexpression in different types of islet cells of the endocrine pancreas, in order to obtain clues for hK6 and hK10 function in these cells. Ten cases of normal pancreatic tissue, two cases of nesidioblastosis, five insulin-producing tumours and one case of multiple endocrine neoplasia 1 syndrome, containing an insulin-, a somatostatin- and several glucagon-producing tumours, as well as tiny foci of endocrine dysplasia with different predominance of the secreted hormones (mainly glucagon and pancreatic polypeptide) were included in the study. A streptavidin–biotin–peroxidase and an alkaline phosphatase protocol, as well as a sequential immunoenzymatic double staining method were performed, using specific antibodies against hK6, hK10, insulin, glucagon, somatostatin, pancreatic polypeptide, and serotonin. hK6 and hK10 immunoexpression was observed in the islets of Langerhans, including the pancreatic polypeptide-rich islets, in the normal pancreas. Scattered hK6 and hK10 positive cells were localized in relationship with pancreatic acinar cells. In the exocrine pancreas, a cytoplasmic and/or brush border hK6 and hK10 immunoexpression was observed in the median and small sized pancreatic ducts, while the acinar cells were negative. Foci of nesidioblastosis and endocrine dysplasia expressed both kallikreins. hK6 and hK10 were also strongly and diffusely expressed throughout all insulin-, glucagon- and somatostatin-producing tumours. The double staining method revealed co-localization of each hormone and hK6/hK10 respectively, in the same cellular population, in the normal as well as in the diseased pancreas. Our results support the view that hK6 and hK10 may be involved in insulin and other pancreatic hormone processing and/or secretion, as well as in physiological functions related to the endocrine pancreas.

Introduction

Kallikreins are a group of serine proteases that are found mainly in glandular cells, neutrophils and biological fluids. They are divided into two main groups: tissue and plasma kallikreins. By means of enzymatic action, kallikreins release vasoactive peptides (kinins) from endogenous substrates called kininogens. Although the primary function of some tissue kallikreins may be to release kinins, additional functions may include processing of protein precursor molecules, enzymes, receptors and hormones (Bhoola *et al*. 1992). A single gene codes for plasma kallikrein, whereas tissue kallikreins are members of a multigene family that displays a diverse pattern of tissue-specific gene expression. The human kallikrein gene family was, until recently, known to include only three members: pancreatic/renal kallikrein (KLK1, encoding for human kallikrein 1 (hK1) protein), human glandular kallikrein 2 (KLK2, encoding for hK2 protein) and human kallikrein 3 (KLK3, encoding for hK3 protein or prostate-specific antigen [PSA]). The human tissue kallikrein multigene family is now known to include 15 genes, all located on chromosome 19q13.4 (Yousef & Diamandis 2000, 2001, Diamandis *et al*. 2000a).

The KLK6 gene, encoding for human kallikrein 6 (hK6), was cloned independently by three different groups and was given three different names, zyme (Little *et al*. 1997), protease M (Anisowicz *et al*. 1996) and neurosin (Yamashiro *et al*. 1997). The KLK10 gene, initially named NES1 (normal epithelial cell-specific 1), encoding for human kallikrein 10 (hK10), was cloned by subtractive hybridization (Liu *et al*. 1996). Steroid hormones upregulate both genes in a dose-dependent manner (Yousef *et al*. 1999, Luo *et al*. 2000).

The KLK6 and KLK10 genes and the encoded hK6 and hK10 proteins share significant homologies with PSA and other kallikreins and the enzymes are predicted to be secreted serine proteases (Anisowicz *et al*. 1996, Liu *et al*. 1996, Little *et al*. 1997, Yamashiro *et al*. 1997, Yousef *et al*. 1999). We recently developed sensitive immunofluorometric procedures and rabbit polyclonal and mouse monoclonal hK6 and hK10 antibodies. The assays were used to quantify the proteins in various biological fluids and tissue extracts (Diamandis *et al*. 2000c, Luo *et al*. 2001b).

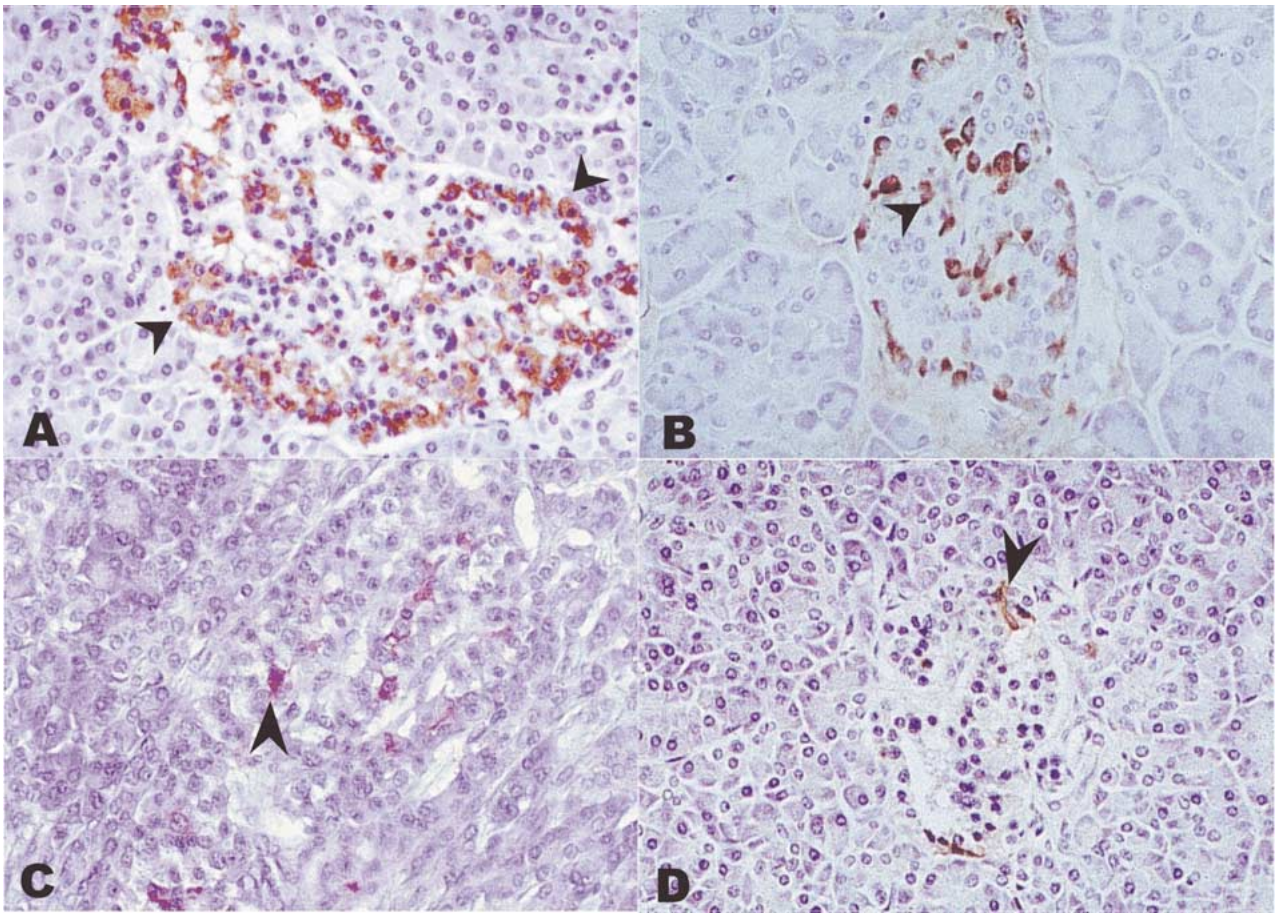

Figure 1.

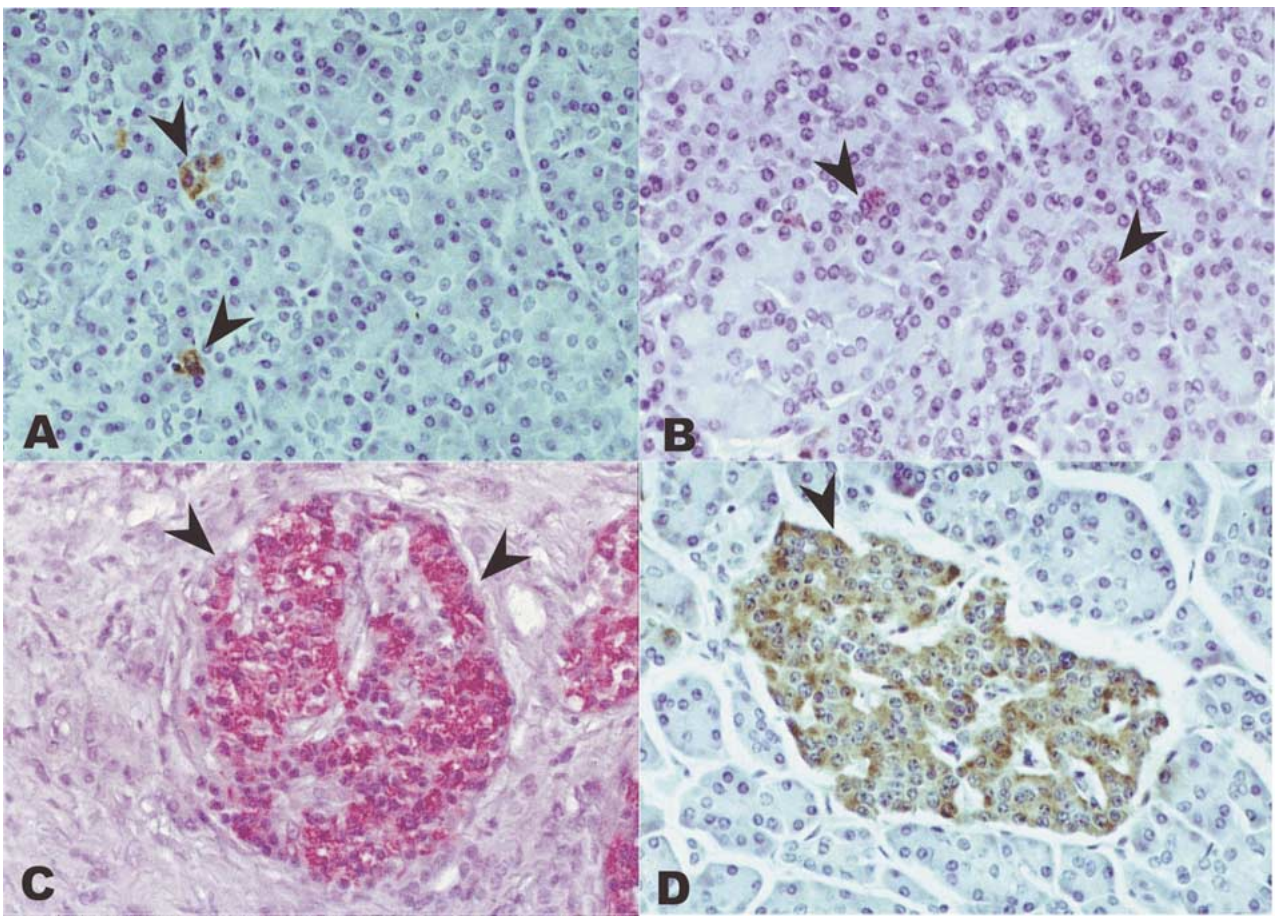

Figure 2.

Using rabbit polyclonal and mouse monoclonal hK6 and hK10 antibodies, we have recently mapped in detail hK6 and hK10 expression in normal human tissues (Petraki *et al*. 2001, 2002). In short, hK6 and hK10 were widely expressed by many tissues. Glandular epithelia constituted the main immunoexpression sites. Choroid plexus epithelium, peripheral nerves and some neuroendocrine cells in several sites expressed the proteins strongly and diffusely (Petraki *et al*. 2001). Recent data suggest that hK6 and hK10 may represent new biomarkers for diagnosis and monitoring of ovarian cancer and may have tumour suppressor activity (Goyal *et al*. 1998, Diamandis *et al*. 2000b, Luo *et al*. 2001a,c).

In light of our previous finding of strong hK6 and hK10 immunoexpression in the islets of Langerhans, we here examine hK6 and hK10 expression in many normal and diseased pancreatic tissues, with emphasis on identifying the endocrine cell types associated with hK6 and hK10 expression. This knowledge may provide clues for the biological function of these kallikreins in the endocrine pancreas.

Materials and methods

Formalin fixed archival and current material was examined in order to establish the precise hK6 and hK10 immunoexpression in the human pancreas and, mainly, in the islets of Langerhans. The immunohistochemical expression of the classical peptides that are produced in the endocrine pancreas, including insulin, glucagon, somatostatin and pancreatic polypeptide (PP), as well as serotonin, was also studied in order to compare their expression in the different cell types with the expression of the two kallikreins. The following were included in this study, (a) 10 cases of normal pancreatic tissue taken from pancreas excised for adenocarcinomas of the exocrine part, in sites far from the tumours. Parenchyma from the PP-rich region in the head of the pancreas was also available. The islets in this region are notably irregular in outline, have somewhat elongated cells arranged in trabecular patterns, and are rich in PP cells (Oertel *et al*. 1992, Martella *et al*. 1997). (b) Two cases of nesidioblastosis, which is a pancreatic disorder affecting newborns and rarely adults, characterized by conspicuously hypertrophic beta-cells, lying in usually irregularly sized islets and by inappropriate insulin secretion (Jaffe *et al*. 1980, Oertel *et al*. 1992). (c) Five cases of insulin-producing tumours and (d) one case of a patient with multiple endocrine neoplasia 1 syndrome (MEN1), containing an insulin-, a somatostatin- and several glucagon-producing tumours, as well as tiny foci of endocrine dysplasia with different predominance of the secreted hormones (mainly glucagon and PP). MEN1 corresponds to an endocrine growth of usually less than 0.5 mm in size, which deviates from the normal architecture of the islets in having a trabecular structure, abnormal prevalence of one or another of the four islet cell types and mild cellular atypia (Jaffe *et al*. 1980, Martella *et al*. 1997).

The immunohistochemical staining was performed on 4-μm thick paraffin sections of tissues fixed in buffered formalin, according to a streptavidin–biotin protocol using the DAKO LSAB + Kits (peroxidase and alkaline phosphatase). The following antibodies were used.

(A) An hK6-specific rabbit polyclonal antibody (1 : 500) and an hK6 mouse monoclonal antibody (1 : 150) that were raised in-house against full-length mature hK6, produced recombinantly in a mammalian stable cell line system (Little *et al*. 1997, Diamandis *et al*. 2000c). The recombinant hK6 protein was purified by HPLC as previously described (Diamandis *et al*. 2000c). The specificity of the antibodies was evaluated during development of the immunofluorometric assay and by Western blotting (Diamandis *et al*. 2000c). We found no detectable cross-reactivity from other closely related antigens, including hK2, hK3, hK10 and hK11 (Diamandis *et al*. 2000c).

(B) An hK10-specific rabbit polyclonal antibody (1 : 400) and an hK10 (5D3) mouse monoclonal antibody (1 : 100) that were raised by immunizing with a full-length hK10 protein, produced in a yeast expression system (Luo *et al*. 2001b). The recombinant hK10 protein was purified by HPLC as previously described (Luo *et al*. 2001b). The specificity of the antibodies was evaluated during development of the immunofluorometric assay (Luo *et al*. 2001b). We found no detectable cross-reactivity from other closely related antigens, including hK2, hK3, hK6 and hK11 (Luo *et al*. 2001b).

(C) A mouse insulin monoclonal antibody (1 : 1000, Sigma), a rabbit glucagon polyclonal antibody (1 : 50, Novocastra), a rabbit somatostatin polyclonal antibody (1 : 1000, Dako), a rabbit PP polyclonal antibody (1 : 500, Dako) and a mouse serotonin monoclonal antibody (1 : 10, DAKO).

Staining procedures included deparaffinization in warm xylene for 5 min with two changes of xylene at room temperature, followed by rehydration by transfer through graded alcohols. Endogenous peroxidase activity was blocked with 0.5% H_2O_2 in methanol for 10 min. The sections were pretreated with 10 mmol/l citrate buffer (pH 6.1) in a microwave oven for 5 min and incubated overnight at 4 °C with the hK6 and hK10 primary rabbit polyclonal and mouse monoclonal antibodies in 3% BSA. With the insulin, glucagon, somatostatin, PP and

Figures 1 and 2. (1) Immunohistochemical localization of hormones in human islets of Langerhans. A: Insulin; B: Glucagon; C: Somatostatin; D: Pancreatic polypeptide. All magnifications ×200. Panels A, B, D: staining with an immunoperoxidase protocol (DAB). Panel C: staining with an alkaline phosphatase protocol (Fuchsin). (2) A: Insulin-positive cells between acinar cells in normal pancreas; B: Serotonin-positive cells between acinar cells in normal pancreas; C: Immunohistochemical localization of hK6 in pancreatic islets with a monoclonal antibody; D: Immunohistochemical localization of hK10 in pancreatic islets with a monoclonal antibody. All magnifications ×200. Panels A, B, D: staining with an immunoperoxidase protocol (DAB); Panel C: staining with an alkaline phosphatase protocol (Fuchsin).

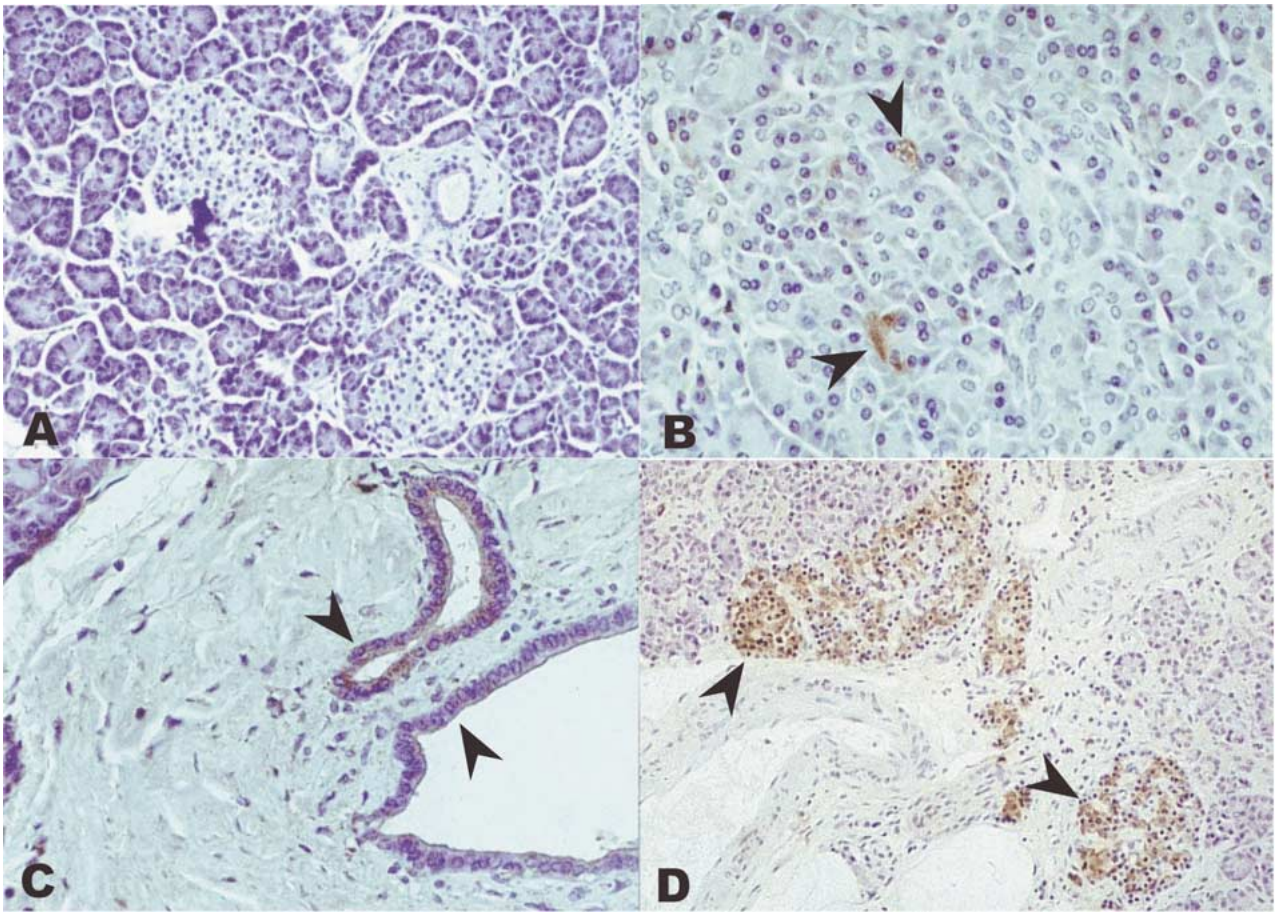

Figure 3.

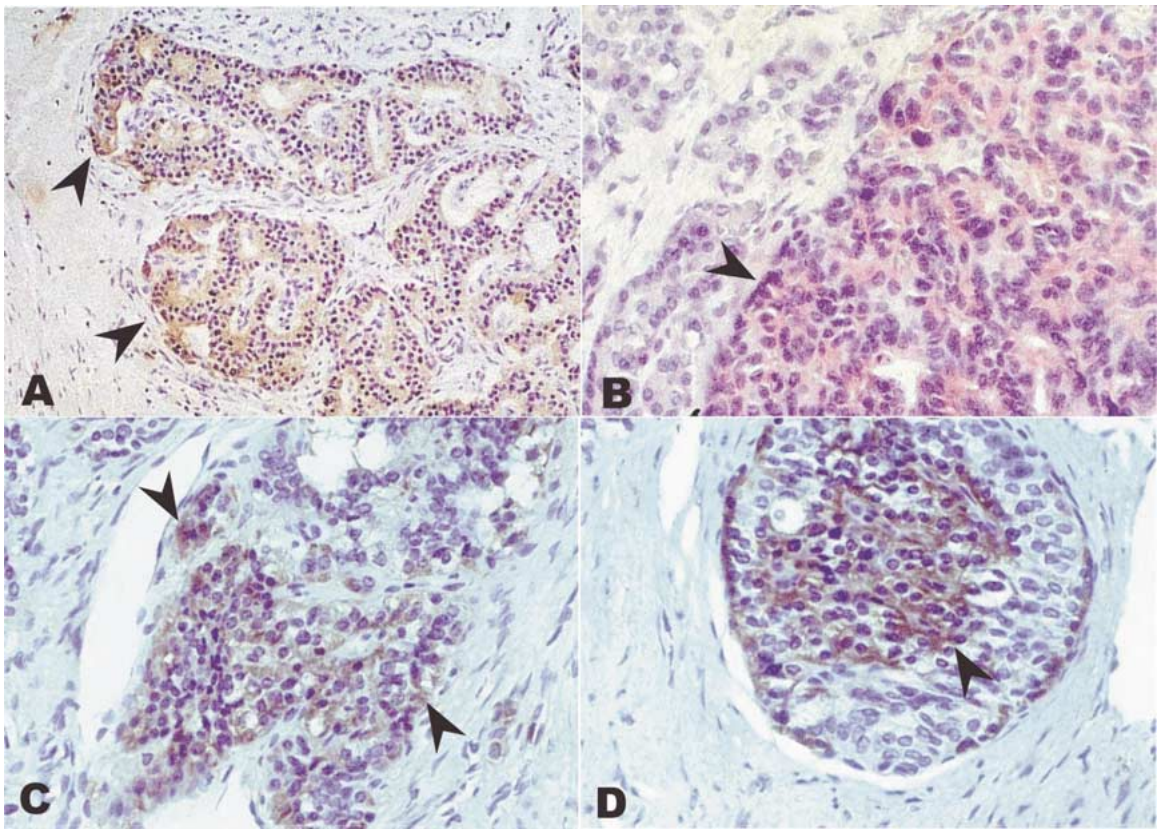

Figure 4.

serotonin antibodies, the sections were incubated for 45 min at room temperature, after a pretreatment in a pressure cooker for 2 min. After two washes of all the sections in 50 mM Tris buffer (pH 7.6), the biotinylated Link (DAKO Corporation USA) was applied for 15 min. A streptavidin–peroxidase or streptavidin–alkaline phosphatase conjugate (DAKO) was then added for 15 min. The enzymatic reaction was developed in a freshly prepared solution of 3,3′-diaminobenzidine tetrahydrochloride using DAKO Liquid DAB Substrate–Chromogen Solution (brown colour) for 10 min for a first group of sections and DAKO Fuchsin Chromogen (red colour) for 20 min for a second group of sections. The first group of sections were then counterstained with Haemalum, dehydrated, cleared in xylene and mounted while the second one was counterstained with aqueous medium without any further dehydration.

In most cases, a sequential immunoenzymatic double staining method was also performed, in order to compare the localization of pancreatic hormones and the kallikreins in the different lesions. The tissue sections were incubated using the first antibody (kallikrein or pancreatic hormone), as described above (DAKO LSAB + Kit Peroxidase), then rinsed in distilled water and sequentially incubated with the second antibody (pancreatic hormone or kallikrein, respectively) [DAKO LSAB + Kit Alkaline Phosphatase]. A combination of antibodies of different origin (mouse or rabbit) was also applied. The sections were counterstained with aqueous medium without any further dehydration. The co-localization of a kallikrein (hK6 or hK10) and a hormone (insulin, glucagon, somatostatin or PP) in a cell or a cell population was marked by a dark brownish colour that can be characterized as a mixed colour between brown (DAB) and red (Fuchsin).

In selected tissues, the primary hK6 and hK10 antibody was replaced by a non-immune rabbit serum (1 : 500) in 3% BSA in order to abolish the immunostaining. For the same reason, an immunoabsorbtion test was also performed, in which the primary hK6 and hK10 antibodies were incubated for 1 h at room temperature with excess of recombinant hK6 and hK10 antigens.

Results

The immunohistochemical expression of all the hormones as well as of hK6 and hK10 was cytoplasmic. All the hK6 and the hK10-specific antibodies, the polyclonal and the monoclonal ones, revealed the same expression pattern and distribution of the antigens in the normal endocrine and exocrine pancreas, as well as in the pathological conditions. The detailed distribution of all the peptides, in the tissues examined, is described below. Replacement of the primary antibodies by non-immune rabbit serum and the immunoabsorbtion tests resulted in disappearance of the immunostaining in all examined tissues.

Normal pancreatic tissue

The immunohistochemical expression and characteristic distribution of insulin, glucagon, somatostatin and PP in the different cells of the pancreatic islets are shown in Figure 1A–D. PP cells predominated in the special, irregular in outline and somewhat elongated islets in part of the pancreatic head. A small number of cells in close relationship with acinar cells and in small and medium sized pancreatic ducts were positive mainly for PP, but also for the other hormones (Figure 2A). A variable number of serotonin-expressing cells (enterochromaffin cells) were located in the islets and in the exocrine pancreas (Figure 2B). Strong hK6 and hK10 immunoexpression was observed in the vast majority of the islet cells (Figures 2C,D and 3A). Scattered hK6 and hK10 positive cells were localized in relationship with pancreatic acinar cells (Figure 3B). In the exocrine pancreas, a cytoplasmic and/or brush border hK6 and hK10 immunoexpression was observed in the medium and small sized pancreatic ducts, while the acinar cells were negative (Figure 3C).

Nesidioblastosis

All hormones were expressed in the islets. Their distribution was similar to that in the normal pancreas with the exception that the number of insulin-positive cells was higher and the enlarged islets contained some cells with large nuclei that were insulin positive. The immunoexpression of hK6 and hK10 was strong and diffuse (Figure 3D).

Insulin-producing tumours

Insulin was diffusely and strongly expressed in all insulin-producing tumours (Figure 4A). Only a few scattered cells with a focal localization were positive for PP and somatostatin. hK6 and hK10 were also strongly and diffusely expressed throughout the tumours (Figure 4B–D).

Glucagon-producing tumours

These tumours expressed glucagon diffusely and only in a few scattered cells, PP. Both kallikreins were immunoexpressed throughout the tumours (Figure 5A).

Figures 3 and 4. (3) Immunohistochemical staining of human pancreas. A: Immunoabsorption of monoclonal hK10 antibody (no staining); B: hK6-positive cells between acinar cells in normal pancreas (hK6 polyclonal antibody); C: hK10 positivity in epithelium of medium-sized pancreatic ducts (hK10 monoclonal antibody); D: hK6 positivity in nesidioblastosis (hK6 polyclonal antibody). Immunoperoxidase protocol (DAB). Magnification ×100 (A), ×200 (B, C and D). (4) Immunohistochemical staining of an insulin-producing tumour. A: Staining for insulin in an insulin-producing tumour; B: staining for hK6 with a monoclonal antibody; C: staining for hK10 with a monoclonal antibody; D: staining for hK10 with a polyclonal antibody. Panels A, C, D, staining with an immunoperoxidase protocol (DAB). Panel B: staining with an alkaline phosphatase protocol (Fuchsin). Magnification ×100 (A), ×200 (B, C and D).

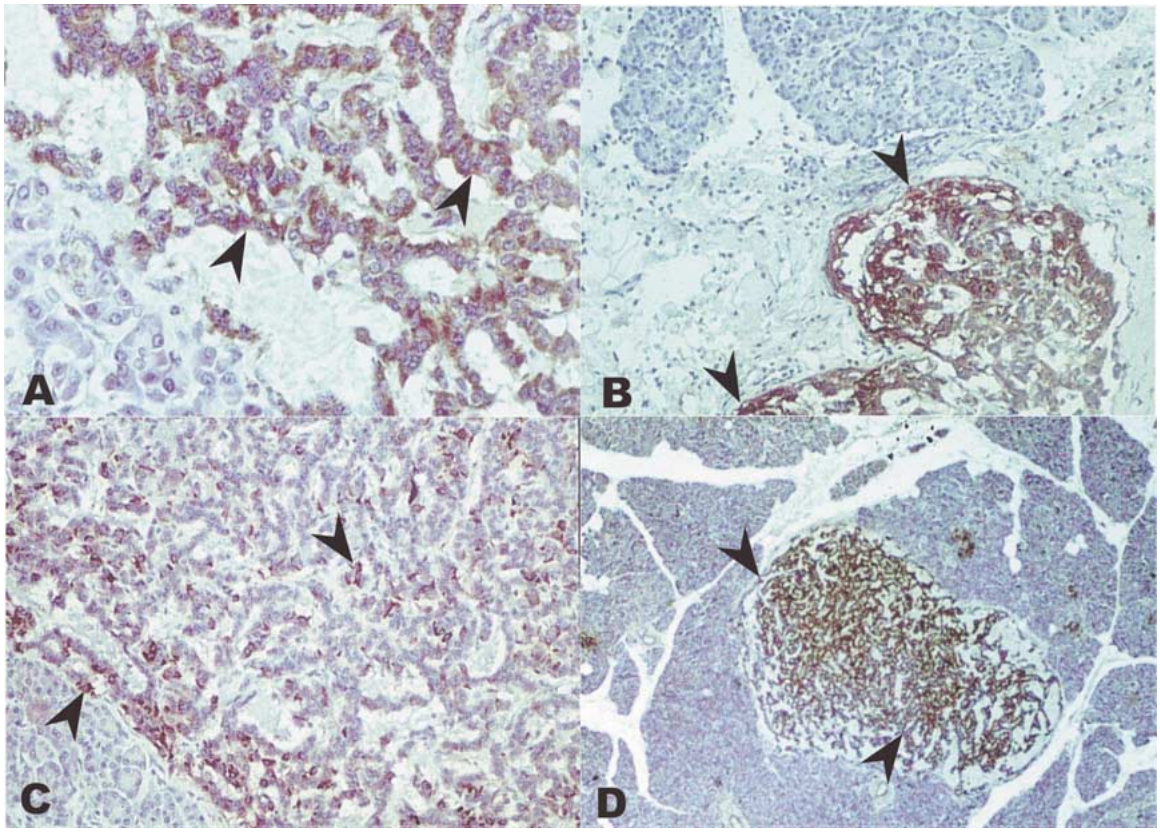

Figure 5.

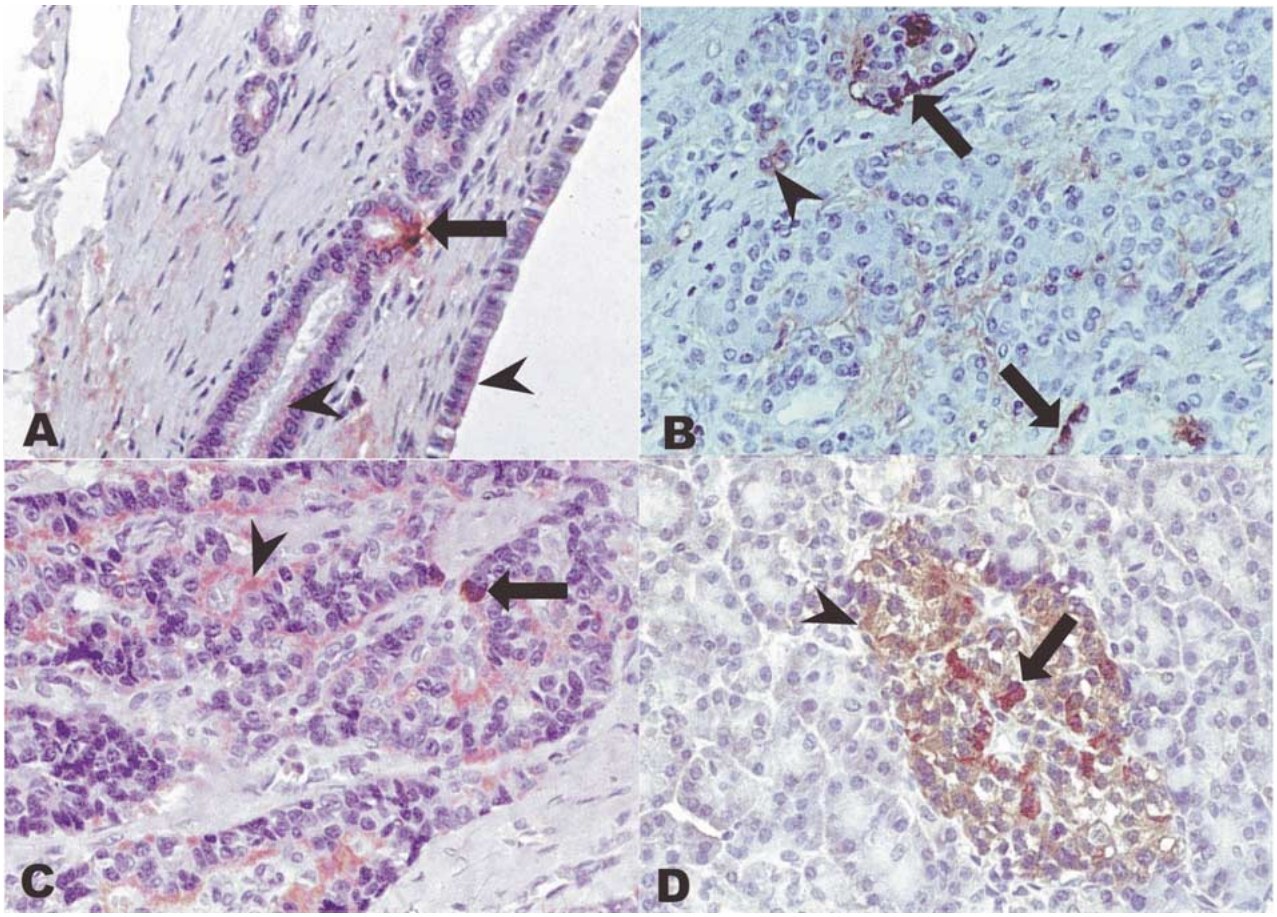

Figure 6.

Somatostatin-producing tumours

Somatostatin was strongly expressed by these tumours. Both kallikreins were also expressed (Figure 5B).

Foci of endocrine dysplasia

In most of them there was a prevalence of glucagon-expressing cells. Some were composed by an admixture of glucagon- and PP-expressing cells (Figure 5C). Insulin-producing cells were either absent or present in small numbers. Both hK6 and hK10 were strongly expressed by all these foci (Figure 5D).

Double staining method

The performance of the double staining method revealed co-localization of each hormone and hK6/hK10, respectively, in the same cellular population in the normal as well as in the diseased pancreas. The following combinations were studied: kallikrein (hK6 or hK10)–insulin, kallikrein–glucagon, kallikrein–PP and kallikrein–somatostatin. The co-localization was documented by the presence of a dark brownish colour. A brown or red colour, depending on the chromogen, was observed in cells that expressed only the kallikrein and not the studied hormone (Figure 6A–D).

Discussion

The hK6 and hK10 genes (KLK6 and KLK10) are two of the recently cloned members of the tissue kallikrein gene family (Anisowicz *et al*. 1996, Liu *et al*. 1996, Little *et al*. 1997, Yamashiro *et al*. 1997, Yousef *et al*. 1999, Diamandis *et al*. 2000a, Yousef & Diamandis 2001). Messenger RNA encoding for hK6 and hK10 proteins has been detected in many tissues, including breast, brain, spinal cord, cerebellum, kidney, uterus, salivary gland, thymus, spleen and testis (Liu *et al*. 1996, Goyal *et al*. 1998, Yousef *et al*. 1999, Diamandis *et al*. 2000a, Luo *et al*. 2001c, Yousef & Diamandis 2001). The recent development of monoclonal and polyclonal antibodies against hK6 and hK10 and of immunofluorometric assays for quantifying the proteins has helped in defining the distribution of hK6 and hK10 in serum, biological fluids (milk of lactating women, cerebrospinal fluid, nipple aspirate fluid, breast cyst fluid) and tissue extracts (Diamandis *et al*. 2000c, Luo *et al*. 2001b). Both genes may control normal cell growth, function as tumour suppressors and KLK10 is down-regulated during progression of some cancers (Goyal *et al*. 1998, Diamandis *et al*. 2000a, Yousef & Diamandis 2000, 2001, Luo *et al*. 2001c).

We recently described in detail the immunohistochemical expression of hK6 (Petraki *et al*. 2001) and hK10 (Petraki *et al*. 2002) in normal human tissues. The localization of both proteins in the pancreas was very characteristic: strong immunoexpression by the islets of Langerhans (endocrine pancreas) and a mainly brush border immunoexpression by the epithelium of the pancreatic ducts. The acini of the exocrine pancreas were negative. It is worth mentioning that the term 'Kallikrein' was introduced in the 1930s by Werle and colleagues (Kraut *et al*. 1930, Werle 1934) who found high levels of their original isolates in the pancreas (in Greek, the 'Kallikreas'). In the present study, we examined in detail hK6 and hK10 immunoexpression in the different cells of the endocrine pancreas, in order to implicate them in the production and secretion of the islet hormones and perhaps with the related endocrinopathies.

The diverse sites of tissue kallikrein expression has led to the suggestion that the functional role of these enzymes may be specific to cell types (Bhoola *et al*. 1992). Apart from the kininogenase activity of one member (KLK1), tissue kallikreins have been implicated in the processing of growth factors and peptide hormones. It has been reported that nerve growth factor gamma subunit, epidermal growth factor-binding protein and kallikrein (hK1) are very closely related enzymes and belong to a larger family of similar enzymes, all of which are involved in the processing of precursors to polypeptide hormones and growth factors (Bothwell *et al*. 1979, Mason *et al*. 1983). This idea has been supported by the finding of immunoreactive pancreatic kallikrein (hK1) in the beta-cells of the pancreatic islets (ole-MoiYoi *et al*. 1979, Pinkus *et al*. 1983) and in the lactotroph cells of the rat (Vio *et al*. 1990) and human anterior pituitary (Jones *et al*. 1990). Insulin is synthesized in the beta-cells as part of a larger preprohormone, preproinsulin, which includes a 23 amino acid leader sequence, attached to proinsulin. This leader sequence is lost upon entrance of the molecule into the endoplasmic reticulum. Proinsulin consists of three

Figures 5 and 6. (5) Immunohistochemical staining of human pancreas. A: staining for an hK6 (polyclonal antibody) in a glucagon-producing tumour; B: staining for hK10 (monoclonal antibody) in a somatostatin-producing tumour; C: staining for pancreatic polypeptide in endocrine dysplasia; D: staining for hK6 (monoclonal antibody) in endocrine dysplasia. Immunoperoxidase protocol (DAB). Magnification ×200 (A), ×100 (B, C), ×20 (D). (6) A: Immunoexpression of hK6 by the epithelium of the medium sized pancreatic ducts (red colour) and co-expression of insulin and hK6 in a cell in the wall of a duct (dark brownish colour – indicated by an arrow). Double staining: Insulin (DAB); hK6 monoclonal antibody (alkaline phosphatase; Fuchsin). Magnification ×200. B: Immunoexpression of hK6 by the islet cells and scattered cells between acinar cells (brown colour – indicated by an arrowhead) and co-expression of glucagon and hK6 in some cells in the islet and in scattered cells between the acinar cells (dark brownish colour – indicated by two arrows). Double staining: hK6 monoclonal antibody (DAB); glucagon (alkaline phosphatase; Fuchsin). Magnification ×200. C: Immunoexpression of hK6 by the cells of an insulin-producing tumour (red colour) and co-expression of PP and hK6 in a cell (dark brownish colour – indicated by an arrow). Double staining: PP (DAB); hK6 polyclonal antibody (alkaline phosphatase; Fuchsin). Magnification ×200. D: Immunoexpression of hK10 by the islet cells (brown colour) and co-expression of somatostatin and hK10 in some cells (dark brownish colour – indicated by an arrow). Double staining: hK10 monoclonal antibody (DAB); somatostatin (alkaline phosphatase; Fuchsin). Magnification ×200.

domains: an amino-terminal B chain, a carboxy-terminal A chain and a connecting peptide in the middle, known as the C peptide. There is experimental evidence that kallikrein enzyme, present in the islets, aids in the conversion of proinsulin to insulin. In most reports, the type of tissue kallikrein that sequentially cleaves proinsulin to form the active molecule, is specified as hK1 (pancreatic kallikrein) (Yoi *et al*. 1979, Bhoola *et al*. 1992). In other studies, it has also been shown that hK1 cleaves low density lipoprotein, the precursor of atrial natriuretic factor, prorenin, vasoactive intestinal peptide, procollagenase, and angiotensinogen (Bhoola *et al*. 1992). Nevertheless, there are contradictory findings regarding hK1 localization in different parts of the exocrine and endocrine pancreas. In one study, the immunohistochemical localization of glandular pancreatic kallikrein, using a monospecific antibody against the antigenically identical urinary kallikrein (urokallikrein), to the beta-cells of the human pancreatic islets was the same as that of insulin in normal human pancreas and in two islet-cell tumours. When beta-cells were lacking in islet-cell tumours or in the pancreas of a patient with juvenile-onset diabetes, kallikrein antigen was not detectable. Anti-urokallikrein absorbed with purified urinary or pancreatic kallikrein no longer identified a pancreatic antigen, whereas absorption with insulin had no effect. The authors concluded that the beta-cell localization of human pancreatic kallikrein, an endopeptidase that, in concert with carboxypeptidase B, converts bovine proinsulin to a polypeptide with the electrophoretic mobility of insulin, suggests that pancreatic kallikrein may be involved in the physiologic activation of proinsulin (ole-MoiYoi *et al*. 1979). Pinkus *et al*., using anti-urinary and anti-pancreatic kallikrein sera, observed moderate acinar and ductal immunostaining in the absence of pretreatment of the tissue with trypsin or pronase. Short incubation with either enzyme permitted the discrete localization of islet beta-cell kallikrein antigen, while increased pronase concentrations decreased kallikrein antigen in both islets and exocrine tissue and led to islet destruction. The authors concluded that both antibody specificity and tissue preparation influence kallikrein localization in human pancreas (Pinkus *et al*. 1983). The studies of Orstavik *et al*. strongly indicate that glandular pancreatic kallikrein is predominantly located in the acinar cells of the exocrine pancreas, as the concentration of kallikrein in pancreatic homogenates was unchanged after beta-cell destruction, whereas it was greatly decreased following acinar cell atrophy. Kallikrein was, by immunohistochemistry, demonstrated only in the acinar cells (Orstavik *et al*. 1981). In another study, the authors found kallikrein-specific fluorescence in the glandular portion of the acinar cells, whereas the islets of Langerhans and ductal cells were unstained (Orstavik *et al*. 1980). Dietl *et al*. also localized kallikrein in the acinar cells of the pancreas, but not in the islets of Langerhans, or in the interlobular ducts (Dietl *et al*. 1978). Chao *et al*. reported that it is unlikely that glandular kallikrein is involved in the *in vivo* conversion of proinsulin to insulin, as appreciable quantities of the enzyme were not detected in pancreatic islets by direct radioimmunoassay and bioassay (Chao *et al*. 1980).

The immunoexpression of hK6 and hK10 in the human endocrine pancreas has not been studied before, mainly due to the lack of suitable antibodies. In the present work, a strong hK6 and hK10 immunoexpression was observed in the vast majority of the islet cells in the normal pancreas. Scattered hK6 and hK10 positive cells were localized in relationship with pancreatic acinar cells. Foci of nesidioblastosis and endocrine dysplasia expressed both kallikreins. The immunoexpression of the studied kallikreins by the insulin-, the glucagon- and the somatostatin-producing tumours supports their co-localization in cells producing insulin, glucagon and somatostatin, respectively. The strong and diffuse expression of the two kallikreins in the PP-rich islets supports their co-localization in cells producing PP as well. The double staining method revealed co-localization of each hormone and hK6/hK10, respectively, in the same cellular population, in the normal as well as in the diseased pancreas. Furthermore, recent data suggest that islet alpha and beta-cell lineages appear to arise independently during ontogeny, probably from a common precursor (Edlund 1999, Herrera 2000, Peters *et al*. 2000). In either case, the immunohistochemical localization of the two kallikreins in all islet cell types would support the presence of a common precursor for all cell types.

Since we show here that both kallikreins are highly expressed in the islets, it is possible that they may play, as hK1, a role in insulin processing. Analogously to insulin, glucagon, somatostatin and PP are cleaved from prohormones, named proglucagon, prosomatostatin and propancreatic polypeptide, respectively. The exact processing pathway is not clear, especially for somatostatin and PP. It will be worth examining the possible role of hK6 and hK10 in processing multiple hormones of islet cells.

There is some confusion about the prohormone convertases (PCs) that participate in the processing of islet cells prohormones. PCs are a family of mammalian enzymes that participate in the synthesis of peptide hormones. PC1 (also known as PC3) and PC2 are found exclusively in neuroendocrine cells and have a role in the processing of several prohormones by cleaving pairs of basic amino acids. Light and electron microscopic studies have shown that both PC1/PC3 and PC2 are normally co-localized with insulin in the islet beta-cells and participate in proinsulin processing (Itoh *et al*. 1996, Furuta *et al*. 1998, Nie *et al*. 2000). The possible interaction of these convertases with the two kallikreins, which co-localize in these cells, has not been investigated. According to some researchers, kallikreins (serine proteases with a trypsin-, chemotrypsin-like or other specific enzymatic activity) are endopeptidases within the endoplasmic reticulum, which could excise the C peptide, thereby generating the mature form of insulin (ole-MoiYoi *et al*. 1979). On the other hand, PC2 and PC1/PC3 are calcium-dependent serine proteases related to the bacterial enzyme subtilisin. These endopeptidases cleave selectively at Lys–Arg or Arg–Arg sites in precursors, generating products with C-terminal basic residues that are then removed by carboxypeptidase E, an exopeptidase (Steiner *et al*. 1996). The combination of these data would suggest a possible cooperation of the PCs and

the kallikreins in insulin and other islet hormone synthesis (Rouille *et al*. 1994, Rothenberg *et al*. 1995, Dhanvantari & Brubaker 1998).

In conclusion, our results support the view that hK6 and hK10 are present in the four types of islet cells of the pancreas. We speculate that these serine proteases may be involved, along with the convertases PC1 and PC2, in insulin and other pancreatic hormone processing and secretion. More studies will be necessary to further examine these possibilities.

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