Distribution of Indian Hedgehog and its receptors patched and smoothened in human chronic pancreatitis

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Abstract

Indian hedgehog (IHH) and its receptors patched (PTC) and smoothened (SMO) belong to the hedgehog family of signaling molecules, which are essential for a variety of patterning events during mammalian tissue development. IHH plays a role in pancreas organogenesis and differentiation, as well as in the regulation of insulin production. In the present study, the expression of IHH and its receptors was analyzed in normal human pancreatic and chronic pancreatitis (CP) tissues using Northern blotting, immunohistochemistry and Western blotting, and was correlated with clinicopathological parameters. In addition, the effects of inhibition and stimulation of the hedgehog signaling pathway on cell growth were determined in TAKA-1 normal pancreatic ductal cells. IHH mRNA was expressed in the normal human pancreas and CP tissues, with slightly higher expression levels in CP. Using immunohistochemistry, IHH and its receptors were

localized mainly in the islet cells of the normal pancreas. In CP, IHH and its receptors were present in the cells forming tubular complexes and in the islets with a different signal pattern compared with the islets in the normal pancreas. Correlation between diabetic and non-diabetic CP patients revealed no significant difference in IHH, SMO, or PTC immunoreactivity. Inhibition of hedgehog signaling in TAKA-1 pancreatic ductal cells using cyclopamine significantly reduced their growth through cell cycle arrest, while stimulation of the IHH pathway enhanced the growth of these cells. In conclusion, IHH and its receptors are expressed in the normal human pancreas and in CP, yet with a different distribution and cellular localization. IHH signaling may be involved in the pathogenesis of CP, i.e. in the formation and proliferation of tubular complexes and in islet cell dysfunction.

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Introduction

Indian hedgehog (IHH) belongs to the hedgehog family of signaling molecules, which are essential for a variety of patterning events during development (Echelard *et al.* 1993, Roelink *et al.* 1994). IHH undergoes autocatalytic internal cleavage, yielding N-terminal and C-terminal domains (Porter *et al.* 1996). The C-terminal peptide is released from the cell surface whereas the N-terminal peptide remains associated with the cell surface. The C-terminal displays autoproteolytic and cholesterol transferase activity, resulting in the cleavage of the full-length protein and covalent attachment of a cholesterol moiety to the newly generated N-terminal domain. The N-terminal peptide, but not the C-terminal peptide, is active in both local and systemic signaling. It binds to the patched (PTC) receptor to mediate downstream IHH signaling (Stone *et al.* 1996, Zhang *et al.* 2001). However, the exact mechanisms by which binding of IHH to PTC influences signaling, and the function of smoothened (SMO) in this process, are still not completely known (Kalderon 2000). PTC might directly inhibit SMO activity, and IHH

binding might induce a conformational change within the PTC/SMO complex which liberates SMO activity from the repressive influence of PTC, resulting in downstream signaling (Stone *et al.* 1996). Alternatively, IHH binding to PTC might result in a dissociation of the PTC/SMO complex, resulting in direct SMO-mediated signaling (Taipale *et al.* 2002). It has also been suggested that PTC could repress SMO activity without directly interacting with SMO, through a diffusible intermediate molecule (Taipale *et al.* 2002). IHH binding to PTC could alter the activity of this molecule, resulting in the loss of the repressive effect on SMO activity and thereby initiating signaling. Cyclopamine, a steroidal alkaloid, may block the hedgehog signaling pathway by mimicking the action of such small molecules (Cooper *et al.* 1998, Incardona *et al.* 1998, Kim & Melton 1998, Taipale *et al.* 2002).

Hedgehog genes have been shown to be expressed in the gut endoderm in vertebrates (Krauss *et al.* 1993, Bitgood & McMahon 1995, Ekker *et al.* 1995, Roberts *et al.* 1995, Stelow & Shi 1995), playing a crucial role in epithelial stem cell proliferation and cell differentiation of the gastrointestinal tract (Ramalho-Santos *et al.* 2000).

In the pancreas, for example, increased hedgehog signaling might disrupt organogenesis (Hebrok *et al.* 2000). Thus, targeted inactivation of IHH in mice allows ectopic branching of ventral pancreatic tissue, resulting in an annulus that encircles the duodenum, a phenotype observed in humans suffering from a rare disorder known as annular pancreas. IHH mutants have an increase in pancreas mass and pancreatic endocrine cell number. In contrast, mutations in PTC reduce pancreas-specific gene expression and impair glucose homeostasis. Thus, islet cells, pancreatic mass and pancreatic morphogenesis are regulated by hedgehog signaling molecules expressed within and adjacent to the embryonic pancreas. In addition, loss of hedgehog signaling results in ectopic pancreas formation in chicken embryos (Roberts *et al.* 1995, Cooper *et al.* 1998), as confirmed by local exposure of the foregut of embryonic chicks to cyclopamine, an inhibitor of IHH signaling (Cooper *et al.* 1998, Kim & Melton 1998). Also, defective hedgehog (Hh) signaling in the endocrine pancreas may contribute to the pathogenesis of type 2 diabetes mellitus (Thomas *et al.* 2000). Furthermore, Hh signaling regulates insulin production in the clonal β -cell line INS-1 (Thomas *et al.* 2000). Thus, after cyclopamine treatment, insulin secretion is inhibited dose dependently in these cells and insulin content is reduced (Thomas *et al.* 2000).

Chronic pancreatitis (CP) is an inflammatory disorder characterized by exocrine and later endocrine insufficiency as well as morphological, proliferative and degenerative changes (DiMagno 1993, Adler & Schmid 1997, Kleeff *et al.* 2000). Compared with acinar cell destruction, the islets of Langerhans are well preserved except in advanced stages of CP, where they show reduction in number and morphological changes (DiMagno 1993, Adler & Schmid 1997, Kleeff *et al.* 2000). In the current study we evaluated the expression of IHH and its receptors PTC and SMO in normal human pancreatic tissues and CP tissue samples, and analyzed the relationship between IHH expression and histopathological parameters. We also examined the effect of inhibition and stimulation of IHH signaling on the growth of a normal pancreatic ductal cell line.

Materials and Methods

Tissue sampling

Tissue specimens were obtained from 31 patients in whom resection for CP was performed. There were 25 men and 6 women with an age range from 22 to 66 (mean 44) years. Normal human pancreatic tissue samples were obtained through an organ donor program from 10 previously healthy individuals. All samples were histologically normal. In all cases, freshly removed tissue samples were fixed in paraformaldehyde solution for 12–24 h and paraffin embedded for histological analysis. In addition, a portion of the tissue sample was snap-frozen in liquid

nitrogen immediately upon surgical removal and maintained at -80°C until use. The Human Subjects Committees of the University of Bern, Switzerland, and the University of Heidelberg, Germany, approved all studies.

Clinical endocrine status

Thirty-one patients with CP were categorized into three groups according to the preoperative glucose tolerance test (Koliopoulos *et al.* 2001): group A consisted of 14 patients with no history of disturbance of glucose metabolism and a normal preoperative glucose tolerance test; group B comprised 9 patients with no history of disturbance of glucose metabolism, but latent diabetes mellitus in the preoperative glucose tolerance test; and group C was made up of 8 patients with manifest diabetes mellitus (6 cases of insulin-dependent diabetes mellitus and 2 cases on oral hypoglycemic therapy).

Immunohistochemistry

Paraffin-embedded tissue sections (2–3 μm thick) were immunostained using the HistoMark Red alkaline phosphatase-labeled reagents (KPL, Gaithersburg, MD, USA). Tissue sections were deparaffinized in xylene and rehydrated in progressively decreasing concentrations of ethanol. Thereafter, the slides were placed in washing buffer (10 mM Tris-HCl, 0.85% NaCl, 0.1% bovine serum albumin, pH 7.4) and subjected to immunostaining. After antigen retrieval performed by boiling the tissue sections in 10 mM citrate buffer for 10 min in the microwave oven, sections were incubated with normal rabbit serum (for IHH and SMO) and normal goat serum (for PTC) (DAKO Corporation, Carpinteria, CA, USA) for 45 min to block non-specific binding sites. Next, the sections were incubated with the goat polyclonal anti-IHH antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) diluted 1:50, goat polyclonal anti-SMO antibody (Santa Cruz Biotechnology Inc.) diluted 1:25, and rabbit polyclonal anti-PTC antibody (Santa Cruz Biotechnology Inc.) diluted 1:25 for 18 h at 4°C . For control, sections were incubated with the respective isotype-matched control IgGs instead of the primary antibodies. The slides were then rinsed with washing buffer and incubated with biotinylated rabbit anti-goat (for IHH and SMO) and goat anti-rabbit immunoglobulins (for PTC) for 45 min at room temperature. Thereafter, tissue sections were incubated with 60 μl streptavidin-phosphatase (KPL) for 35 min at room temperature. Tissue sections were then washed in washing buffer and each section was subjected to 100 μl PhThaloRED-Activator-Buffered substrate mixture (KPL) and counterstained with Mayer's hematoxylin.

Double staining

Paraffin-embedded tissue sections were deparaffinized, rehydrated as mentioned above, and incubated with peroxidase block (DAKO Corporation) for 5 min. Sections were washed in washing buffer as mentioned before, then incubated with an insulin mouse monoclonal IgG (Santa Cruz Biotechnology Inc.), 1:2000 dilution, for 30 min at room temperature. Tissue sections were washed in washing buffer, then incubated with anti-rabbit-labeled polymer HRP (DAKO Corporation) for 30 min, then washed and incubated with a buffered substrate for liquid DAB/liquid DAB chromogen mixture (DAKO Corporation) for 20 s. Next, the sections were incubated in doublestain universal block (DAKO Corporation) for 3 min. Anti-IHH antibody diluted 1:50 was applied, and specimens were incubated at 4 °C overnight. On the following day, application of the secondary biotinylated rabbit anti-goat antibody and other steps were performed as described above.

Subcloning

A 355 bp IHH cDNA fragment (Genebank accession number XM-050846) was produced by RT-PCR. The PCR product was generated using standard conditions: denaturing at 95 °C, 35 cycles (94 °C for 1 min, 59 °C for 1 min, 72 °C for 1 min), followed by 8 min extension at 72 °C; the product was then subcloned into the pGEM-T Easy vector, and authenticity was confirmed by sequencing. The IHH primer pair was sense 5' TGAGAGCCT TCCAGGTCATC, and antisense 5'-CATGCCAAGCT GTGAAAGAG.

RNA extraction and Northern blot analysis

Total RNA was extracted by the guanidinium thiocyanate phenol chloroform method, as described previously (Korc *et al.* 1992). RNA was size fractionated on 1.2% agarose/1.8 M formaldehyde gels, electrotransferred onto nylon membranes, and cross-linked by UV irradiation. Blots were prehybridized for 8 h and hybridized for 16 h with IHH riboprobes radiolabeled with [α -³²P]CTP (Amersham Pharmacia Biotech.). Blots were washed under high-stringency conditions as previously reported (Korc *et al.* 1992, Friess *et al.* 1993). Blots were then exposed at -80 °C to Kodak BiomaxMS films, and the resulting autoradiographs were scanned to quantify the intensity of the radiographic bands. A fragment of human 7S cDNA that cross-hybridizes with human cytoplasmatic RNA was used to confirm equal RNA loading and transfer (Korc *et al.* 1992, Friess *et al.* 1993).

Western blotting

Cells were homogenized in ice-cold suspension buffer (10 mM Tris-HCl, pH 7.6, 100 mM NaCl) containing

the Complete Proteinase Inhibitors (Roche Diagnostics GmbH, Mannheim, Germany). The homogenized material was collected and centrifuged at 4 °C for 30 min at 14 000 g to remove the insoluble material. The protein concentration of the supernatant was measured by spectrophotometry using the BCA protein assay method (Pierce, Rockford, IL, USA). A total of 40 µg protein/lane was separated by SDS-polyacrylamide gel electrophoresis. After transfer to nitrocellulose membranes, blots were incubated with specific goat antibodies detecting human IHH and SMO, and rabbit polyclonal antibodies to PTC. After washing, blots were incubated with anti-rabbit IgG (Amersham International, Amersham, Bucks, UK) or anti-goat IgG (Santa Cruz Biotechnology) conjugated with horseradish peroxidase. Visualization was performed by the enhanced chemiluminescence method (Amersham International).

Cell growth assay

TAKA-1 cells were routinely grown in RPMI medium, supplemented with 10 or 0.5% FBS and 100 U/ml penicillin. To perform growth assays, cells were plated overnight at a density of 5000 cells/well in 96-well plates. Subsequently, cells were incubated in the absence or presence of the indicated concentrations of cyclopamine (Toronto Research Chemicals Inc., Ontario, Canada), Hh-Ag 1.2 and Hh-Ag 1.3 (Curis Inc., Cambridge, MA, USA) (Frank-Kamenetsky *et al.* 2002) for 48 h. Then, 20 µl MTT (2 mg/ml) dissolved in PBS pH 7.4 were added to each well and incubated for 5 h at 37 °C. Subsequently, cellular MTT was solubilized with 0.04 mM HCl-isopropanol. Optical density was measured at 570 nm with an ELISA plate reader (Behring ELISA processor II). All experiments were performed in triplicate.

Flow cytometry analysis

TAKA-1 cells were seeded in 12-well plates at a density of 100 000 cells/well before addition of 25 µg/ml cyclopamine for 36 h. Subsequently, cells were collected and centrifuged at 200 g. The cell pellet was gently resuspended in 1.5 ml hypotonic fluorochrome solution (50 µg/ml propidium iodide (PI) in 0.1% sodium citrate and 0.1% Triton X-100). Then, the resuspended cells were incubated in polypropylene tubes (Falcon, Franklin Lakes, NJ, USA), and kept in the dark at 4 °C overnight before flow cytometry analysis. Control and cyclopamine-treated cells were also collected in PBS (pH 7.4) containing 2% fetal calf serum and kept in polypropylene tubes on ice. Then, 2 µl PI (1 mg/ml) were added to each tube. All measurements were performed using the FACScan (Becton Dickinson and Company, NJ, USA).

Figure 1 Northern blot analysis of Indian hedgehog (IHH) mRNA in pancreatic tissue samples obtained from normal pancreatic and chronic pancreatitis (CP) tissues. Total RNA (20 µg) was subjected to Northern blot analysis and probed with the ^{32}P -labeled IHH riboprobe, as described in the Materials and Methods section. Blots were subsequently rehybridized with a 7S cDNA probe to verify equivalent RNA loading and transfer. The ribosomal size markers (5.1 kb for 28S and 2.0 kb for 18S) are indicated on the left.

Statistical analysis

Results are expressed as the median $\pm$ mean (S.E.M.). For statistical analysis Student's *t*-test was used. Significance was defined as $P < 0.05$.

Results

First we examined the expression of IHH by Northern blotting using total RNA isolated from human pancreatic tissues. The 2.5 kb human IHH transcript was expressed at low to moderate levels in all normal ($n=8$) and CP ($n=8$) samples. There was a 1.7-fold increase in IHH mRNA expression in CP tissue samples compared with normal pancreatic tissue samples (Fig. 1).

Next, immunohistochemistry was carried out to determine the exact localization of IHH in the normal pancreas and CP tissues. In normal pancreatic tissue samples

obtained from organ donors, IHH displayed strong immunoreactivity in the islet cells (Fig. 2A), predominantly as intracytoplasmic punctate aggregations (Fig. 2B,C). Ductal cells and acini were devoid of IHH immunoreactivity (Fig. 2D). To determine which islet cells express IHH, double immunostaining using anti-insulin and anti-IHH antibodies was carried out. This analysis demonstrated that most of the islet cells were positive both for insulin and for IHH (Fig. 2E). Notably, however, some islet cells displayed immunoreactivity for IHH, but were devoid of insulin immunoreactivity (Fig. 2F), suggesting that IHH expression is not restricted to the insulin-producing islet cells.

In CP, IHH immunoreactivity was present homogeneously in the islet cells, with complete loss of the intracytoplasmic punctate aggregations that were observed in the normal specimens (Fig. 3A). Ductal cells, especially those forming tubular complexes, as well as degenerating

Figure 2 Immunohistochemical analysis of IHH expression in normal pancreatic tissue samples as described in the Materials and Methods section. IHH displayed strong immunoreactivity in the islet cells (A), predominantly as intracytoplasmic granular aggregations (B and C). Ductal cells (arrow) and acini were devoid of IHH immunoreactivity (D). Doublestaining using anti-insulin and anti-IHH antibodies demonstrated that most of the islet cells were positive both for insulin (brown) and for IHH (red) (E). Some islet cells (arrows) displayed immunoreactivity for IHH, but were devoid of insulin immunoreactivity (F).

Figure 3 Immunohistochemistry of IHH in chronic pancreatitis tissue samples as described in the Materials and Methods section. IHH immunoreactivity was present homogeneously in the islet cells, with complete loss of the intracytoplasmic granular aggregations that were observed in the normal specimens (A). Ductal cells - especially tubular complexes - as well as degenerating acini displayed IHH immunoreactivity (B and C), but to a lesser extent than observed in the islet cells (D).

acini displayed IHH immunoreactivity (Fig. 3B,C), but to a lesser extent than observed in the islet cells (Fig. 3D). Correlation between IHH immunoreactivity in CP samples and clinicopathological parameters revealed no difference in the staining pattern between non-diabetic and latent diabetic or manifest diabetic patients.

In the next set of experiments, we investigated the expression patterns of the two IHH receptors, PTC and SMO. PTC immunoreactivity was evident mainly in the islets of normal pancreatic tissue specimens, with a diffuse homogeneous signal (Fig. 4A). No PTC immunoreactivity was observed in the ductal or acinar cells, nerves or connective tissue. In CP specimens, PTC immunoreactivity was present in the islets (Fig. 4B), proliferating ductal cells, and degenerating acini (Fig. 4C). Islet cells clearly exhibited a more intense signal compared with

ducts and acini. No signal was present in the inflammatory cells. Similar to IHH immunostaining, there was no difference in PTC immunoreactivity between diabetic and non-diabetic CP patients. Also, no difference in the PTC signal distribution or intensity was evident between latent and manifest diabetic CP patients.

SMO immunoreactivity in normal pancreatic tissue specimens was present mainly in the islets (Fig. 4D), with a diffuse homogeneous cytoplasmic staining pattern. In CP patients the islets, tubular complexes, and degenerating acini displayed strong immunoreactivity (Fig. 4E,F). Again, there was no difference in SMO immunostaining between diabetic and non-diabetic CP patients. The specificity of the observed IHH, SMO and PTC immunostaining was confirmed by serial sections using isotype matched IgGs as controls instead of the respective primary antibodies (Fig. 5).

Figure 4 Immunohistochemistry of PTC (A-C) and SMO (D-F) in the normal pancreas and in chronic pancreatitis, as described in the Materials and Methods section. PTC immunoreactivity was evident mainly in the islets of normal pancreatic tissue specimens (A). In CP specimens, PTC immunoreactivity was present in the islets (B), tubular complexes, and degenerating acini (C). SMO immunoreactivity in normal pancreatic tissue specimens was present mainly in the islets (D). In CP patients the islets, tubular complexes, and degenerated acini displayed strong immunoreactivity (E and F).

Figure 5 Immunohistochemistry of IHH, SMO and PTC. To determine the specificity of the observed staining, consecutive slides were incubated with IHH (A), SMO (B) and PTC (C) antibodies respectively, and the corresponding IgG control (D-F). A and D: normal pancreas, B and E, C and F: chronic pancreatitis.

Since pancreatic ductal cells in CP expressed IHH and its receptors SMO and PTC, in the next sets of experiments, cyclopamine – a steroidal alkaloid known to act as an inhibitor of hedgehog signaling – was utilized in TAKA-1 cells. These cells are obtained from normal pancreatic ductal cells of the hamster and bear chromosome 3 alterations that induce immortalization, but do not involve genes related to pancreatic tumorigenesis (Takahashi *et al.* 1995a,b). Therefore, TAKA-1 cells can be used to study normal pancreatic ductal cells, although they may not exactly reproduce all the characteristics of normal human pancreatic ductal cells (Kleeff *et al.* 1999).

First, to determine whether a functional IHH pathway was present in TAKA-1 cells, protein extracts of these cells were subjected to polyacrylamide gel electrophoresis and probed with the anti-IHH, SMO and PTC antibodies used for immunohistochemistry. This analysis revealed that TAKA-1 cells expressed the 45 kDa IHH, 85 kDa SMO and 160 kDa PTC proteins (Fig. 6A). Next, TAKA-1 cells were incubated with cyclopamine at various concentrations for 48 h. At low concentrations (10 µg/ml), cyclopamine exerted a slight growth-stimulating effect (not significant). However, at higher doses there was strong dose-dependent growth inhibition, with maximal effects of – 76% ($P < 0.01$) occurring at a concentration of 100 µg/ml cyclopamine (Fig. 6B). Next, we investigated the effects of cyclopamine on the cell cycle in TAKA-1 cells. Flow cytometry analysis revealed that cyclopamine did not induce toxic cell death. In addition, no apoptotic changes could be demonstrated. In contrast, cyclopamine caused double blockage of the cell cycle at both the G2/M and G1/S phase transitions with consecutive S and G1 phase accumulation respectively (Fig. 6C). To further delineate the role of the IHH pathway in the proliferation of ductal cells, TAKA-1 cells were also incubated with the two hedgehog agonists Hh-Ag 1:2 and 1:3 (Frank-Kamenetsky *et al.* 2002) at various concentrations for 48 h. Hh-Ag 1:3 significantly stimulated the cell growth, a threshold effect occurring at a dose of 2 ng/ml (+35%) that increased only slightly at higher concentrations of up to 200 ng/ml (+39%). Hh-Ag 1:2 caused only modest and non-significant growth stimulatory effects at low concentrations of 2 ng/ml and 20 ng/ml (+10% and +13% respectively) (Fig. 6B).

Discussion

Indian hedgehog is involved in the regulation of the development and differentiation of many tissues (Vortkamp *et al.* 1996, Iwasaki *et al.* 1997, Hebrok *et al.* 2000, Ramalho-Santos *et al.* 2000, Thomas *et al.* 2000). Concerning its role in the developing pancreas, it has been demonstrated that islet cells, pancreatic mass and pancreatic morphogenesis are regulated by hedgehog signaling molecules expressed within and adjacent to the embryonic

pancreas itself. In addition, defects in hedgehog signaling may lead to congenital pancreatic malformations, such as annular pancreas, as well as to glucose intolerance (Roberts *et al.* 1995, Cooper *et al.* 1998, Kim & Melton 1998, Hebrok *et al.* 2000). Furthermore, Hh expression and signaling are not restricted to the developing pancreas but also continue to signal in differentiated beta cells of the endocrine pancreas thereby regulating insulin production. Thus, defective Hh signaling in the pancreas may act as a potential factor in the pathogenesis of type 2 diabetes mellitus (Thomas *et al.* 2000). The expression of IHH and its receptors in human pancreatic tissues has not been analyzed before. Since chronic pancreatitis is frequently associated with endocrine disturbances – i.e. latent or manifest diabetes mellitus – and since IHH signaling has a potential role in glucose intolerance, in the present study we investigated the expression of IHH and its receptors in the normal pancreas and in CP tissues. We now show that IHH and its receptors are present both in normal pancreatic and chronic pancreatitis tissues, yet with a markedly different cellular and subcellular distribution.

Our results first indicate that IHH expression and signaling are not only active during human pancreatic development; there is also a basal level of IHH expression in the post-developmental adult pancreas. IHH expression could be detected as granular staining within the islet cells of the normal pancreas, as has previously been described for the mouse pancreas (Thomas *et al.* 2000). Interestingly, this granular staining of IHH was absent in the islets of human CP tissues, where a diffuse staining pattern was observed. This indicates that IHH distribution and release might be different in CP compared with the normal pancreas. However, we could not detect a significant difference of IHH immunoreactivity among diabetic and non-diabetic CP patients. Nonetheless, the obviously different intracellular localization of IHH in islets of the normal pancreas and CP suggests that IHH might indeed have some role in islet cell dysfunction in this disease. We could also show that the IHH signaling receptors, PTC and SMO, are strongly expressed in the islets of normal pancreatic tissues as well as CP tissues, without there being any difference between normal and CP tissues or between diabetic and non-diabetic patients.

An increased proportion of ductular-like structures or tubular complexes occurs in association with chronic pancreatitis. These changes are either interpreted as ductular proliferation, implying their origin from ductal cells or they might result from dedifferentiating acinar cells, thus originating from acinar instead of ductal cells (Bockman *et al.* 1983). Irrespective of the origin of these tubular complexes, while in the normal pancreas proliferation is mainly confined to the acinar cells and does not involve ductal cells, in CP the percentage of proliferating acinar cells is increased significantly and proliferating cells in tubular complexes are also observed (Slater *et al.* 1998). We could demonstrate that in CP tissues, but not in the

normal pancreas, cells forming tubular complexes and to a lesser extent dedifferentiating acinar cells expressed IHH. This may indicate that the IHH signaling pathway is activated in ductal cells during the pathogenesis of CP. There was, however, only a slight increase in IHH mRNA expression levels in CP compared with normal pancreatic tissues, yet there was a strong up-regulation of IHH in the tubular complexes in CP. This discrepancy might be due to a difference in the ratio of these tubular complexes per tissue volume used for RNA extraction. Alternatively, but less likely, this effect might be due to enhanced translation of this mRNA moiety. The two IHH signaling receptors, PTC and SMO, displayed weak expression in ductal cells in the normal pancreas. In contrast, PTC and SMO expression was readily apparent in the cells forming tubular complex in CP, and to a lesser extent in dedifferentiating acinar cells. This co-localization of IHH, SMO and PTC in CP further suggests that activation of the IHH signaling pathway has a potential role in the pathogenesis of CP.

Supporting this hypothesis, cyclopamine – an IHH pathway inhibitor – slightly stimulated the growth of TAKA-1 cells at low doses (not significant), and exerted a strong growth-inhibitory effect at higher doses, suggesting that blockage of hedgehog signaling suppresses the growth of pancreatic ductal cells. Two lines of evidence suggest that the effects of the hedgehog inhibition by cyclopamine in TAKA-1 cells were specific. First, flow cytometry revealed that there was cell cycle blockage in response to cyclopamine and neither apoptotic nor necrotic cell death. Secondly, the hedgehog agonist Hh-Ag 1:3 significantly stimulated the growth of TAKA-1 cells. Although TAKA-1 cells, which are immortalized pancreatic ductal cells of the hamster, may not exactly reproduce all the characteristics of normal ductal cells, they can still be used as a model cell line for normal pancreatic ducts (Kleeff *et al.* 1999). Our data collectively suggest that in CP, expression of IHH and its receptors may have the capacity to modulate the growth of ductal cells, most likely through autocrine (ductal cells) or paracrine (ductal cells, acinar cells, islets) effects. It is currently not known, however, whether other hedgehog family members such as sonic hedgehog or desert hedgehog, which are thought to utilize the same receptors and signaling pathway, and whose signaling is also blocked by cyclopamine and stimulated by the used hedgehog agonists (Frank-Kamenetsky *et al.* 2002) are also responsible for the observed effects. Further studies are required to address this point. Inasmuch as

many growth factors are involved in the pathogenesis of CP (Kleeff *et al.* 2000), it is suggested that IHH may also act as a growth factor for ductal cells in this disease, as has been described for other cell types such as chondrocytes (Long *et al.* 2001). Furthermore, IHH expression has been found to be regulated by other growth factors such as transforming growth factor beta (TGF- β) (Murakami *et al.* 1997) and TGF- β is highly expressed in CP, suggesting that IHH expression might be regulated by TGF- β in this disease (van Laethem *et al.* 1995, Slater *et al.* 1995).

In conclusion, IHH and its receptors, SMO and PTC, are expressed in the normal pancreas as well as in CP tissues. The difference in the staining patterns between CP and the normal pancreas, as well as the effect of inhibition and stimulation of IHH signaling on the growth of a normal pancreatic ductal cell line suggests a growth-promoting function of the IHH signaling pathway in ductal cells in CP, as well as a role in the endocrine disturbances frequently observed in this disease.

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Figure 6 (A) Western blot analysis of IHH, PTC and SMO, as described in the Materials and Methods section. TAKA-1 cells expressed the 45 kDa IHH, 85 kDa SMO and 160 kDa PTC proteins. Molecular size markers (in kDa) are indicated on the left. (B) MTT cell proliferation assay, as described in the Materials and Methods section. TAKA-1 cells were incubated with the indicated concentrations of cyclopamine (in $\mu\text{g/ml}$; left panel) and Hh-Ag 1:2 (white triangles) and 1:3 (black circles, both in ng/ml ; right panel) for 48 h. Results are expressed as the mean $\pm$ standard error of mean of three separate experiments. (C) Flow cytometry analysis was carried out as described in the Materials and Methods section. TAKA-1 cells were incubated with 25 $\mu\text{g/ml}$ cyclopamine (black line and right lower corner) or left untreated (grey filling and upper right corner). The Figure is representative of three independent experiments.

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