Nuclear Factor-κB Contributes to Hedgehog Signaling Pathway Activation through Sonic Hedgehog Induction in Pancreatic Cancer

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Abstract

The hedgehog (Hh) signaling pathway, which functions as an organizer in embryonic development, is implicated in the development of various tumors. In pancreatic cancer, pathway activation is reported to result from aberrant expression of the ligand, sonic Hh (Shh). However, the details of the mechanisms regulating Shh expression are not yet known. We hypothesized that nuclear factor-κB (NF-κB), a hallmark transcription factor in inflammatory responses, contributes to the overexpression of Shh in pancreatic cancer. In the present study, we found a close positive correlation between NF-κB p65 and Shh expression in surgically resected pancreas specimens, including specimens of chronic pancreatitis and pancreatic adenocarcinoma. We showed that blockade of NF-κB suppressed constitutive expression of *Shh* mRNA in pancreatic cancer cells. Further activation of NF-κB by inflammatory stimuli, including interleukin-1β, tumor necrosis factor-α, and lipopolysaccharide, induced overexpression of Shh, resulting in activation of the Hh pathway. Overexpression of Shh induced by these stimuli was also suppressed by blockade of NF-κB. NF-κB-induced Shh expression actually activated the Hh pathway in a ligand-dependent manner and enhanced cell proliferation in pancreatic cancer cells. In addition, inhibition of the Hh pathway as well as NF-κB suppressed the enhanced cell proliferation. Our data suggest that NF-κB activation is one of the mechanisms underlying Shh overexpression in pancreatic cancer and that proliferation of pancreatic cancer cells is accelerated by NF-κB activation in part through Shh overexpression. (Cancer Res 2006; 66(14): 7041-9)

Introduction

Pancreatic cancer is one of the most lethal of all malignancies. Therapeutic options for patients with unresectable, metastatic, or recurrent disease are extremely limited, and few patients survive for 5 years, which underscores the need for new therapies (1, 2). A better understanding of the mechanisms that underlie development of pancreatic cancer would help to identify novel molecular targets for treatment.

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The hedgehog (Hh) signaling pathway is crucial to growth and patterning in a wide variety of tissues during embryonic development (3, 4). Of three Hh ligands, sonic Hh (Shh), Indian Hh (Ihh), and desert Hh (Dhh), Shh is reported to play an essential role in the development of pancreatic cancer as well as pancreatic organogenesis (5, 6). Shh undergoes extensive post-translational modifications to become biologically active (7). The signal peptide is cleaved from the precursor form of Shh to yield the 45-kDa full-length form. Further processing of this molecular form of Shh generates a 19-kDa amino and 26-kDa COOH-terminal peptides. The 19-kDa NH_2-terminal peptide undergoes COOH-terminal esterification to a cholesterol molecule before being secreted into the extracellular spaces where it mediates the physiologic actions of Shh (8). The response to Shh is mediated by two transmembrane proteins, Smoothened (Smo) and Patched (Ptc), and by downstream transcription factors that are members of the Gli family. In the absence of Shh, Ptc suppresses the signaling activity of Smo. When Shh binds Ptc, Ptc is inactivated, enabling signaling via Smo. Smo releases the transcription factor Gli from a large protein complex, and Gli translocates to the nucleus to activate Hh target genes, including *Ptc1* (3, 4). It has been reported that the plant-derived teratogenic steroidal alkaloid cyclopamine inhibits the Hh pathway by antagonizing Smo (9).

Recently, ligand-dependent activation of the Hh pathway, especially due to aberrant expression of its ligand, Shh, has been detected in pancreatic cancer, and cyclopamine suppresses the growth of pancreatic cancer cells both *in vitro* and *in vivo* (10). These findings suggest that the Hh pathway may be a viable therapeutic target for treatment of pancreatic cancer, but it remains unclear how Shh is regulated in pancreatic cancer (11). One clue toward identification of the factors regulating Shh is the similarity between cancer development and tissue repair, a late phase of inflammation. It has been reported that, during repair of bronchial epithelium, transient activation of the Hh pathway occurs within the normally quiescent bronchial epithelium, and such a process may well occur during adult gut epithelial turnover (12, 13).

Association between chronic inflammation and the development of cancer has been recognized for several years (14–18). Various cytokines, such as tumor necrosis factor-α (TNF-α) and interleukin-1 (IL-1), which seem to play roles in inflammatory responses and the fibrotic reaction, are overexpressed in pancreatic cancer (19–21). Most of these cytokines are potent activators of nuclear factor-κB (NF-κB), which has been reported to be also activated in pancreatic cancer (22, 23). NF-κB is a transcription factor that controls expression of numerous genes involved in inflammation and immune response processes, including proliferation, invasion

and adhesion, angiogenesis, and apoptosis (22). In most unstimulated, normal cells, NF-κB is present in the cytoplasm as an inactive heterodimer composed of the p50, p65, and IκBα subunits. After activation, IκBα undergoes phosphorylation and ubiquitination-dependent degradation by the proteasome. Consequently, nuclear localization signals on the p50-p65 heterodimer are exposed, leading to nuclear translocation and binding to a specific consensus sequence that activates gene transcription, including genes encoding inflammatory cytokines, chemokines, growth factors, cell adhesion molecules, and cytokine receptors (24).

In the present study, we hypothesized that NF-κB activation upregulates expression of Shh, resulting in activation of Hh signaling in pancreatic cancer. We first investigated the expression of both NF-κB and Shh in clinical samples of pancreas. We then examined the effect of NF-κB activity on Shh expression and Hh pathway activation in pancreatic cancer cells. Finally, we explored the biological significance of the correlation between NF-κB and activation of the Hh pathway in proliferation of pancreatic cancer cells.

Materials and Methods

Cell culture, reagents, and antibodies. Two human pancreatic ductal adenocarcinoma (PDAC) cell lines (AsPC-1 and SUIT-2; ref. 25) and Cos7, an SV40-immortalized monkey kidney cell line, were maintained in RPMI 1640 (Life Technologies, Grand Island, NY) supplemented with 10% FCS (Life Technologies) and antibiotics [100 units/mL penicillin (Meijiseika, Tokyo, Japan) and 100 μg/mL streptomycin (Meijiseika), referred to as complete culture medium] at 37°C. Lipopolysaccharide (LPS) from *Escherichia coli* (B4), pyrrolidine dithiocarbamate (PDTC), and recombinant human IL-1β were purchased from Sigma (Deisenhofen, Germany). Recombinant human TNF-α was purchased from Dainippon Pharmaceutical (Osaka, Japan). Cyclopamine, purchased from Toronto Research Chemicals (North York, Ontario, Canada), was diluted in 100% methanol. Rabbit anti-NF-κB p65 (sc-109) and anti-Shh (sc-9024) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit anti-β-actin antibody and rat anti-Shh NH_2-terminal peptide antibody were purchased from Biomedical Technologies (Stoughton, MA) and R&D Systems (Minneapolis, MN), respectively.

Clinical samples and immunohistochemistry. Surgical specimens were obtained from six patients with a benign pancreatic endocrine cell tumor, eight with chronic pancreatitis, and six with PDAC, all of whom underwent resection at the Department of Surgery and Oncology, Kyushu University (Fukuoka, Japan). All patients gave informed consent. Samples were fixed in 10% formalin and embedded in paraffin. For immunostaining of clinical samples, single-antibody detection was accomplished as described previously (26). In brief, all primary antibodies were incubated overnight at 4°C. Secondary antibodies were applied for 1 hour at room temperature. Immune complexes were visualized with 3,3′-diaminobenzidine as chromogen. Slides were counterstained with hematoxylin. The intensity of p65 staining of the pancreatic duct epithelium or cancer cells was scored according to the predominant pattern as 0, negative (weak or similar to background); 1, weak (less intense than adjacent acini); 2, moderate (similar intensity to adjacent acini); and 3, strong (stronger than adjacent acini). Ten pancreatic ducts or cancer nests in each section were scored, and the average score for each section was taken as the p65 staining score. For immunostaining of Shh, numbers of cytoplasmic-positive cells in pancreatic duct epithelium or cancer cells were counted. If the intensity of staining was similar to that of background staining, the staining was judged as negative. Five hundred cells were counted, and the percentage of Shh-positive cells was calculated for each section.

Plasmids, oligodeoxynucleotides, and cell transduction. Phosphorothioated and double-stranded NF-κB decoy oligodeoxynucleotides, 5′-CCTTGAAGGGATTTCCCTCC-3′, and scramble oligodeoxynucleotides, 5′-TTGCCGTACCTGACTTAGCC-3′, were purchased from Hokkaido System Science (Sapporo, Japan). Cells were transfected with 0.5 μmol/L NF-κB decoy or scramble oligodeoxynucleotides with LipofectAMINE (Life Technologies, Inc., Gaithersburg, MD) according to the manufacturer's instructions. Transfected cells were used for experiments 48 hours after transfection. pIRES2-hSHH-EGFP (referred to as pSHH-GFP) and pcDNA3.1/His-hGli1 (referred to as pGli1) were kindly provided by Dr. Aubie Shaw (Division of Urology, Department of Surgery, University of Wisconsin, Madison, WI; ref. 27) and Dr. H. Sasaki (Center for Developmental Biology, RIKEN, Kobe, Japan; ref. 28), respectively. pCMV-IκBα wild-type (WT) and pCMV-IκBα mutant were purchased from BD Biosciences/Clontech (Palo Alto, CA). Cells seeded in six-well plates were transfected with 2 μg plasmid with TransFast reagent (Promega, Madison, WI) per manufacturer's protocol. Cells were used for experiments 48 hours after transfection. To collect control medium and Shh-rich medium, Cos7 cells were transfected with 2 μg pGFP and pSHH-GFP, respectively, with Superfect reagent (Qiagen, Valencia, CA) according to the manufacturer's instructions. Supernatant was collected 48 hours later and dissolved in complete culture medium at a 1:10 dilution. The supernatant from cells transfected with pSHH-GFP was called Shh-rich medium, whereas supernatants from cells transfected with pGFP were collected as control medium.

Luciferase assay. Cells in six-well plates were transfected with plasmids with Superfect transfection reagent according to the manufacturer's instructions. Cells on each well were cotransfected with 5 ng pRL-SV40 (Promega) and 2 μg pELAM-Luc (29), the NF-κB-dependent luciferase reporter (kindly provided by Dr. K. Takeda, Division of Embryonic and Genetic Engineering, Medical Institute of Bioregulation, Kyushu University). After 24 hours of pretreatment with PDTC or cyclopamine, IL-1β or TNF-α was added to each well, and luciferase assays were done 6 hours later with the dual luciferase assay kit (Promega) according to the manufacturer's instructions. The luciferase activities were normalized to the *Renilla* luciferase activity. For cotransfection of reporter plasmids and effector plasmids or oligodeoxynucleotidess, we transfected cells with 1 μg of each plasmid (cells were transfected with a total of 2 μg plasmid mixture). Luciferase assays were done 48 hours after transfection as described above.

Real-time reverse transcription-PCR. Total RNA was extracted by the guanidinium isothiocyanate-phenol-chloroform extraction method (30) and quantified by spectrophotometry (Ultrospec 2100 Pro, Amersham Pharmacia Biotech, Cambridge, United Kingdom). RNA (1 μg) was treated with DNase and reverse transcribed to cDNA with the Quantitect Reverse Transcription kit (Qiagen) according to the manufacturer's protocol. Reactions were run with iQ SYBR Green Supermix (Bio-Rad, Hercules, CA) on a DNA Engine Opticon 2 System (MJ Research, Waltham, MA). cDNA, prepared from AsPC-1 and SUIT-2 cells transfected with pSHH-GFP, were serially diluted in 10-fold increments and amplified in parallel with the various primer pairs to generate standard curves. Each sample was run in triplicate. All primer sets amplified fragments <200 bp long. Sequences of the primers used were β-actin forward, 5′-TTGCCGACAG-GATGCAGAAGGA-3′, and reverse, 5′-AGGTGGACAGCGAGGCCAGGAT-3′; Shh forward, 5′-GTGTACTACGAGTCCAAGGCAC-3′, and reverse, 5′-AGG-AAGTCGCTGTAGAGCAGC-3′; and Ptc1 forward, 5′-ATGCTGGCGGGATCT-GAGTTCGACT-3′, and reverse, 5′-GGGTGTGGGCAGGCGGTTCAAG-3′. The amount of each target gene in a given sample was normalized to the level of β-actin in that sample.

Immunoblotting. Whole-cell extraction was done with M-PER Reagents (Pierce Biotechnology, Rockford, IL) according to the manufacturer's instructions. Protein concentration was determined with a Bio-Rad Protein Assay (Bio-Rad). Whole-cell extract (100 μg) was separated by electrophoresis on 12.5% SDS-polyacrylamide gel and transferred to Protran nitrocellulose membranes (Schleicher & Schnell BioScience, Dassel, Germany). Blots were then incubated with anti-Shh (1:100) or anti-β-actin (1:500) primary antibody overnight at 4°C. Blots were incubated with horseradish peroxidase–linked anti rabbit IgG antibody (Amersham Biosciences, Piscataway, NJ) at room temperature for 1 hour. Immunocomplexes were detected with an enhanced chemiluminescence reagent (Amersham Biosciences) and visualized with a Molecular Imager FX (Bio-Rad).

Proliferation assay. Cells (2×10^3 per well) seeded in 48-well plates in complete culture medium were incubated overnight. Medium was changed to RPMI 1640 with 0.8% FCS with or without 0.1 μg/mL LPS, 0.1 IU/mL TNF-α, or 0.1 ng/mL IL-1β in the presence or the absence of 0.3 μmol/L PDTC, 5 μg/mL anti-Shh NH_2-terminal peptide antibody, or 5 μmol/L cyclopamine. To test for the effects of Shh-rich medium on the cell proliferation, medium was changed to control or Shh-rich medium. After 4 days of incubation, cells were harvested by trypsinization, and cells were counted with a Coulter counter (Beckman Coulter, Fullerton, CA). When transfection was needed, cells (8×10^3 per well) seeded in 48-well plates were transfected with 0.8 μg pSHH-GFP or 0.4 μg pGli1 with Transfast reagent. Transfected cells were incubated in complete medium for 16 hours, and the medium was changed to RPMI 1640 with 0.8% FCS and manipulated as described above.

Electrophoretic mobility shift assay. Electrophoretic mobility shift assay (EMSA) was done as reported previously (30, 31). Nuclear extract (10 μg) was incubated for 30 minutes at 37°C with binding buffer [60 mmol/L HEPES (pH 7.5), 180 mmol/L KCl, 15 mmol/L MgCl2, 0.6 mmol/L EDTA, 24% glycerol], poly(deoxyinosinic-deoxycytidylic acid), and ^{32}P-labeled double-stranded oligonucleotide containing the binding motif of NF-κB (Promega). The sequence of the double-stranded oligomer used for EMSA was 5′-AGTTGAGGGGACTTTCCCAGGC-3′. The reaction mixtures were loaded onto a 4% polyacrylamide gel and electrophoresed with a running buffer of 0.25% Tris-borate EDTA. Gel being dried, the DNA-protein complexes were visualized by autoradiography.

Statistical analysis. Student's *t* test was used for statistical analysis. The relations between variables were assessed with Spearman rank correlation coefficient. A $P < 0.05$ was considered significant.

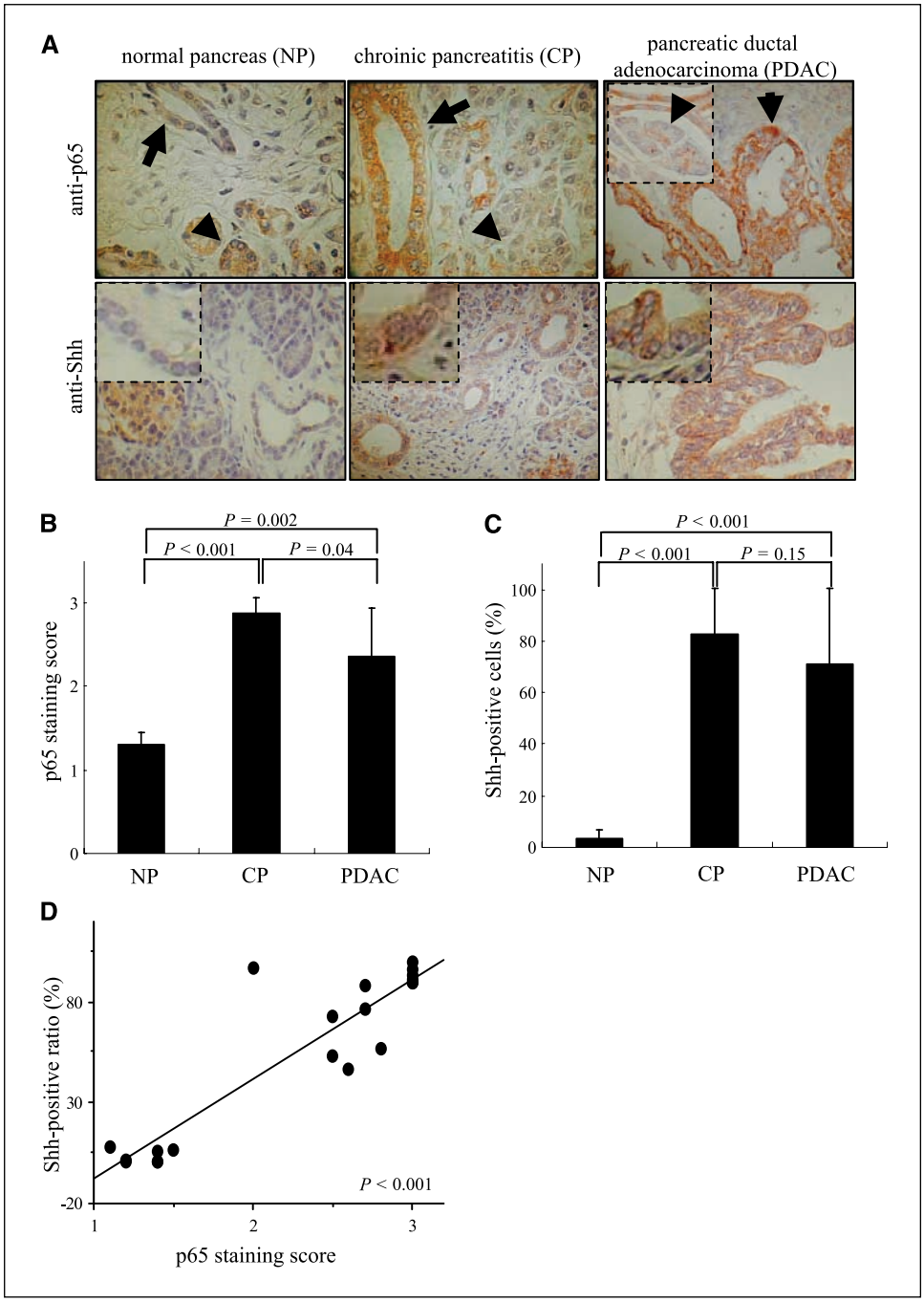

Figure 1. Expression of NF-κB p65 and Shh in human pancreatic tissues. *A, top,* p65 expression by duct epithelium (*arrow*) is less intense than that by adjacent acinar cells (*arrowhead*) in normal pancreatic tissue (*NP*). P65 expression by duct epithelium is stronger than that by adjacent acinar cells in chronic pancreatitis (*CP*). P65 expression by PDAC cells is stronger than that in acinar cells in normal pancreas in the same section. *Bottom,* few duct epithelium cells in normal pancreatic tissues showed immunoreactivity for Shh, whereas most duct epithelium cells in chronic pancreatitis and cancer cells in PDAC express Shh. Magnification, ×200. *Inset,* magnification, ×400. *B,* the intensity of p65 staining of the pancreatic duct epithelium (normal pancreas and chronic pancreatitis) or cancer cells (PDAC) was scored as described in Materials and Methods. *C,* the number of immunoreactive cells for Shh in pancreatic duct epithelium (normal pancreas and chronic pancreatitis) or cancer cells (PDAC) was counted. *D,* p65 staining score and Shh-positive cell ratio (%). The relations among the variables were assessed with the Spearman rank correlation coefficient.

Results

Shh expression correlated positively with NF-κB activation in specimens of human pancreas. To examine whether NF-κB activation was associated with expression of Hh pathway components in clinical samples, we stained a series of 20 paraffin-embedded specimens, including 6 specimens of normal pancreas from resected benign pancreatic tumors, 8 specimens of chronic pancreatitis, and 6 specimens of PDAC, which had normal pancreatic structures in the same section. Expression of the functional component of NF-κB, p65 (Fig. 1*A, top*), and Shh (Fig. 1*A, bottom*) was examined immunohistochemically. We then scored the intensity of p65 immunostaining of pancreatic duct epithelium (normal pancreas and chronic pancreatitis) or cancer cell (pancreatic cancer). The p65 staining scores (mean ± SE) of pancreatic duct epithelium in normal pancreas and chronic pancreatitis and of cancer cells in PDAC were 1.3 ± 0.15, 2.88 ± 0.19, and 2.37 ± 0.56, respectively (Fig. 1*B*). Chronic pancreatitis and PDAC showed significantly higher scores than these of normal pancreas. The percentage of cells with cytoplasmic staining of Shh (mean ± SE) was 3.4 ± 3.3% in normal pancreas, 82.5 ± 17.9% in chronic pancreatitis, and 70.8 ± 29.7% in PDAC (Fig. 1*C*). The Shh-positive staining ratios of chronic pancreatitis and PDAC were significantly higher than the ratio of normal pancreas. In addition, when all specimens were included, a strong positive correlation was detected between the p65 staining score and the Shh-positive staining ratio (Fig. 1*D*). These data suggested that there was a correlation between NF-κB activation and Shh expression in clinical specimens of pancreas.

Expression of *Shh* mRNA correlated positively with NF-κB activity in cultured pancreatic cancer cells. To clarify the relation between *Shh* mRNA expression and NF-κB activity observed in our clinical study, we did *in vitro* experiments with two human pancreatic cancer cell lines, AsPC-1 and SUIT-2 (25). We confirmed that both cell lines showed increased NF-κB DNA-binding activity by EMSA and that they constitutively expressed *Shh, Ptc1*, and *Gli1* at both mRNA and protein levels (data not

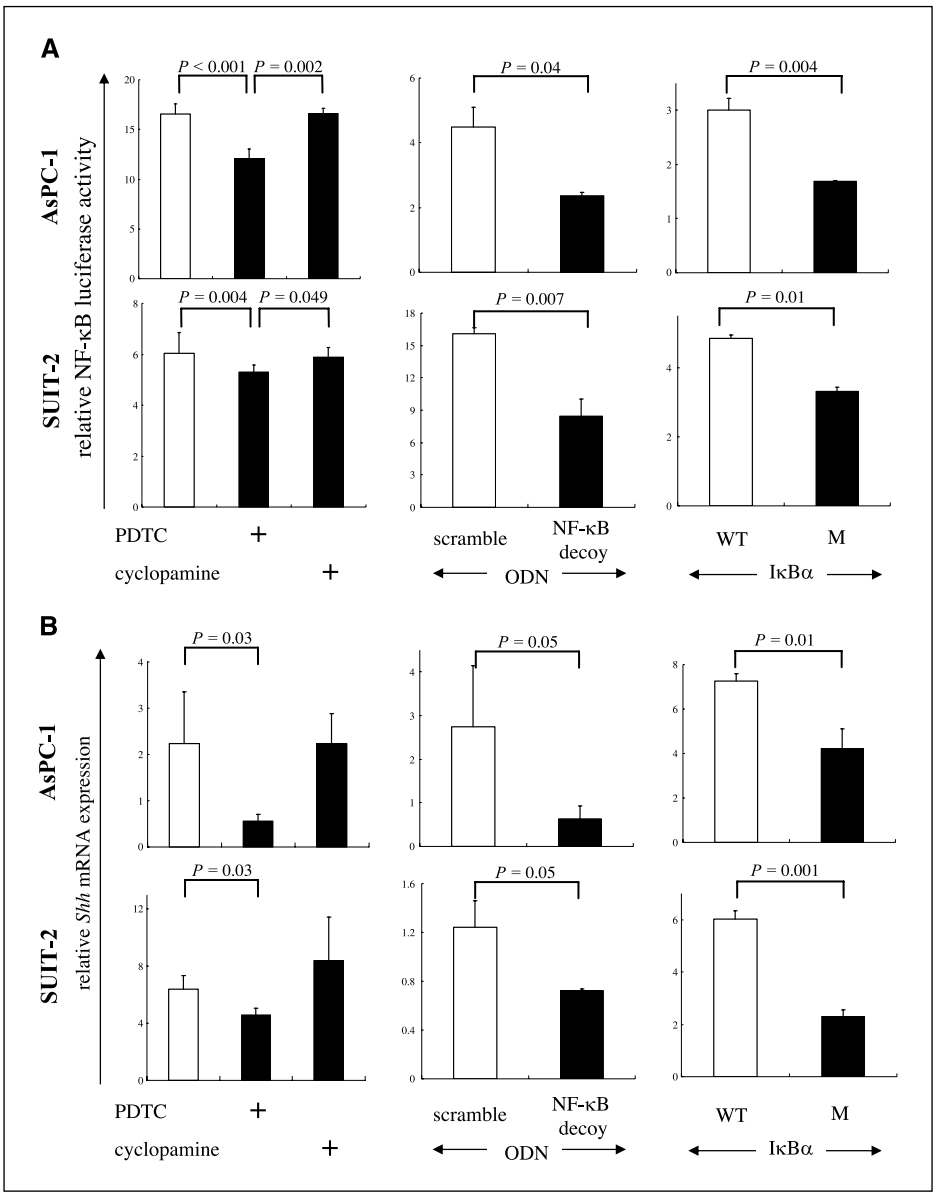

Figure 2. Effects of NF-κB inhibitors on NF-κB transcriptional activity and *Shh* mRNA expression in pancreatic cancer cells. *A,* dual luciferase assay was done 6 hours after adding 10 IU/mL of TNF-α. Relative NF-κB luciferase activity after normalization to *Renilla* luciferase activity. *Columns,* mean of triplicate experiments; *bars,* SD. *Left,* cells were pretreated for 24 hours with 10 μmol/L PDTC or cyclopamine 24 hours after transfection with reporter plasmids; *middle,* scramble or NF-κB decoy oligodeoxynucleotides and reporter plasmids were cotransfected for 48 hours; *right,* IκBα WT or IκBα mutant (*M*) plasmid and reporter plasmids were cotransfected for 48 hours. *B, Shh* mRNA expression by AsPC-1 and SUIT-2 cells was examined with real-time RT-PCR. Relative *Shh* mRNA level after normalization to the corresponding β-actin mRNA expression. *Columns,* mean of three independent experiments; *bars,* SD. *Left,* cells were treated with 10 μmol/L PDTC or cyclopamine for 24 hours; *middle,* relative *Shh* mRNA levels were assessed 48 hours after transfection of oligodeoxynucleotidess; *right,* relative *Shh* mRNA levels were assessed 48 hours after transfection of oligodeoxynucleotidess, IκBα WT, or IκBα mutant.

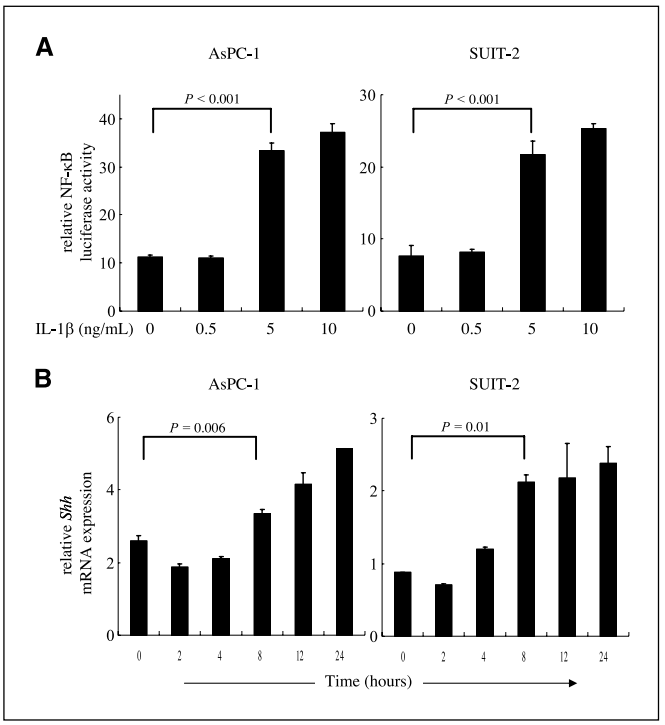

Figure 3. IL-1β enhances expression of *Shh* mRNA by pancreatic cancer cells. *A,* AsPC-1 and SUIT-2 cells were treated with IL-1β for 6 hours, and dual luciferase assay was done as described. *B, Shh* mRNA expression was examined by real-time RT-PCR after cells were exposed to 5 ng/mL IL-1β.

shown). We first examined whether inhibition of NF-κB activity altered Shh expression in these cell lines. We treated both cell lines with PDTC for 24 hours to inhibit NF-κB activity (30). Transcription activity of NF-κB and expression of *Shh* mRNA were examined by NF-κB reporter assay and real-time reverse transcription-PCR (RT-PCR), respectively. PDTC significantly inhibited NF-κB transcriptional activity in both cell lines (Fig. 2*A, left*). Constitutive expression of *Shh* mRNA in these cell lines was reduced significantly in parallel with NF-κB activity (Fig. 2*B, left*), suggesting that inhibition of NF-κB activity reduced constitutive expression of *Shh* mRNA in pancreatic cancer cells. In contrast, the Hh pathway inhibitor, cyclopamine, had no effect on NF-κB transcriptional activity or *Shh* mRNA expression in these cells (Fig. 2*A* and *B, left*).

To confirm these results, we transfected both cell lines with NF-κB decoy oligodeoxynucleotides to suppress NF-κB activity as reported previously (31). When transfected with NF-κB decoy oligodeoxynucleotides, NF-κB-dependent reporter activity of AsPC-1 and SUIT-2 cells decreased to 52.7% and 51.6%, respectively, that of the scramble oligodeoxynucleotides control (Fig. 2*A, middle*). Expression of *Shh* mRNA in AsPC-1 and SUIT-2 cells transfected with NF-κB decoy oligodeoxynucleotides was decreased to 23.2% and 58.3%, respectively, that of the scramble oligodeoxynucleotides (Fig. 2*B, middle*). Furthermore, we transfected both cell lines with pCMV-IκBα mutant, which was reported to have a dominant-negative effect on NF-κB activity (32). Transfection of IκBα mutant significantly suppressed NF-κB transcriptional activity in both cell lines (Fig. 2*A, right*). When cells were transfected with IκBα mutant, constitutive expression of *Shh* mRNA by AsPC-1 and SUIT-2 cells was reduced significantly to 58.1% and 37.7%, respectively, that of cells when transfected with IκBα WT (Fig. 2*B, right*). These data showed that inhibition of NF-κB activity resulted in reducing the constitutive expression of *Shh* mRNA in pancreatic cancer cells.

We next examined whether further activation of NF-κB could induce overexpression of *Shh* mRNA in pancreatic cancer cells. We used one of the proinflammatory cytokines, IL-1β, as a NF-κB activator because it has been reported that IL-1β causes constitutive NF-κB activation in pancreatic cancer cells (33). IL-1β (5 ng/mL) significantly enhanced NF-κB activity in both cell lines (Fig. 3*A*). When cells were treated with 5 ng/mL IL-1β, significantly enhanced expression of *Shh* mRNA was observed in these cell lines after 8 hours (Fig. 3*B*). Taken together, these data suggested that NF-κB activity affected *Shh* expression by pancreatic cancer cells.

Shh protein level depended on NF-κB activity in pancreatic cancer cells. To confirm that the Shh protein level is correlated with NF-κB activity in pancreatic cancer cells, we did immunoblotting. The Shh protein level was quantified with the 19-kDa fragment because it is the physiologic form of Shh (8, 34). We first treated pancreatic cancer cells with 5 ng/mL IL-1β and examined whether NF-κB activation altered the Shh protein level. As shown in Fig. 4*A*, IL-1β increased Shh levels in AsPC-1 and SUIT-2 cells. Furthermore, 1 IU/mL TNF-α and 10 μg/mL LPS, both of which are potent NF-κB activators, also enhanced expression of Shh protein (Fig. 4*A*). These findings indicated that NF-κB activation enhanced Shh protein production in these cell lines. We then investigated whether NF-κB inhibitors could reduce the enhanced protein level of Shh by IL-1β. When treated with IL-1β in

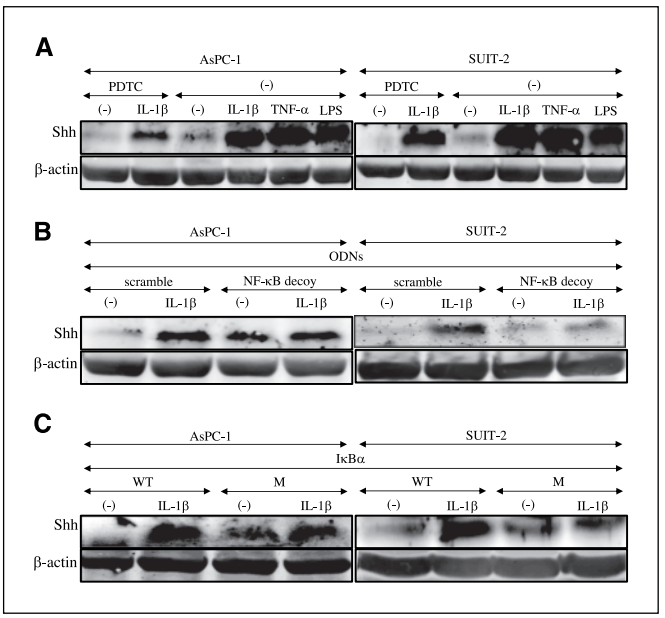

Figure 4. Level of Shh protein correlates with NF-κB activity in pancreatic cancer cells. Representative of three independent experiments. Whole-cell extract (100 μg) was resolved by electrophoresis on a 12.5% SDS-polyacrylamide gel and immunoblotted. *A,* AsPC-1 and SUIT-2 cells were treated with 5 ng/mL IL-1β, 10 μg/mL LPS, and 1 IU/mL TNF-α for 24 hours after they were pretreated with 10 μmol/L PDTC for 24 hours. *B,* AsPC-1 and SUIT-2 cells were transfected with 0.5 μmol/L of oligodeoxynucleotidess for 48 hours. After 24 hours of incubation with 5 ng/mL IL-1β, immunoblotting was done. *C,* AsPC-1 and SUIT-2 cells were transfected with IκBα WT or IκBα mutant plasmid for 24 hours. After 24 hours of incubation with 5 ng/mL IL-1β, immunoblotting was done.

the presence of NF-κB inhibitors, including PDTC (Fig. 4*A*), NF-κB decoy oligodeoxynucleotides (Fig. 4*B*), and IκBα mutant (Fig. 4*C*), protein levels of Shh were reduced. These data suggested that the Shh protein level was positively correlated with NF-κB activity in pancreatic cancer cells.

Overexpression of Shh induced by NF-κB activation led to enhanced Hh pathway activation in pancreatic cancer cells. We examined whether NF-κB-induced Shh could activate the Hh pathway because activation of this pathway was reported to be ligand dependent in pancreatic cancer, which means that the aberrant signaling must be mediated by Shh protein (35). To confirm that ligand-dependent Hh pathway activation occurs in AsPC-1 and SUIT-2 cells, the cells were transfected with pSHH-GFP to overexpress Shh, and the *Ptc1* mRNA level was then monitored by real-time RT-PCR. Because Ptc1 is a transcriptional target of the Hh pathway as well as a receptor in this pathway, the level of *Ptc1* mRNA reflects the degree of Hh pathway activity (11, 36). The transfection efficiency reached ~70% in both cell lines (data not shown). As shown in Fig. 5*A*, real-time RT-PCR revealed that cells transfected with pSHH-GFP expressed higher levels of *Shh* mRNA than were expressed by cells transfected with control plasmid (34- and 126-fold increases in AsPC-1 and SUIT-2 cells, respectively). Expression of *Ptc1* mRNA in pSHH-GFP-transfected cells was approximately 4.8- and 2.2 -fold higher in AsPC-1 and SUIT-2 cells, respectively, than on control-transfected cells (Fig. 5*A*). These data suggest that overexpression of Shh enhanced Hh pathway activation within the same cells.

We next examined whether IL-1β also activated the Hh pathway in these cell lines. When cells were treated with 5 ng/mL IL-1β, expression of *Ptc1* mRNA was increased 1.6-fold after 8 hours in AsPC-1 cells and 2.2-fold after 12 hours in SUIT-2 cells (Fig. 5*B*). These data suggested that IL-1β activated the Hh pathway through up-regulation of Shh in pancreatic cancer cells.

NF-κB activation up-regulated Shh and promoted proliferation of pancreatic cancer cells. We examined whether Shh and the Hh pathway could promote proliferation of pancreatic cancer cell lines. When AsPC-1 and SUIT-2 cells were transfected with vector expressing Gli1, a transcription factor in the Hh pathway, cell proliferation was 2.1- and 1.9-fold higher in pGli1-expressing cells, respectively, than in cells transfected with control plasmid, pcDNA3.1 (Fig. 6*A, top*). Proliferation of AsPC-1 and SUIT-2 cells transfected with pSHH-GFP was 1.9- and 1.8-fold higher, respectively, than that of cells transfected with pGFP (Fig. 6*A, bottom*). To confirm that the secreted Shh had autocrine effects on physiologic functions, we transfected Cos7 cells with pSHH-GFP and prepared Shh-rich medium. Immunoblotting confirmed that Shh-rich medium contained more Shh protein than control medium (data not shown). AsPC-1 and SUIT-2 cells incubated in Shh-rich medium proliferated more rapidly than those in control medium (Fig. 6*B*). In contrast, 10 μmol/L cyclopamine, a specific inhibitor of the Hh pathway, inhibited proliferation of these cell lines (Fig. 6*C*). Taken together, these findings indicated that Shh and the Hh pathway contributed to proliferation of these cell lines, which was consistent with previous reports (10).

Finally, we examined whether overexpression of Shh induced by inflammatory stimuli also stimulated proliferation of pancreatic cancer cells. When AsPC-1 and SUIT-2 cells were treated with 0.1 μg/mL LPS (Fig. 7*A*), 0.1 IU/mL TNF-α (Fig. 7*B*), and 1 ng/mL IL-1β (Fig. 7*C*), proliferation of both cell lines was significantly enhanced. As expected, the enhanced proliferation induced by each of these stimuli was blocked completely by PDTC (Fig. 7*A*; data not shown). Enhanced proliferation was also suppressed by anti-Shh neutralizing antibody (Fig. 7*B*; data not shown). Furthermore, enhanced proliferation was inhibited completely by cyclopamine (Fig. 7*C*; data not shown). These data suggest that inflammatory stimuli enhance proliferation of pancreatic cancer cells, at least in part, through overexpression of Shh, resulting in Hh pathway activation. To confirm these findings, both cell lines were cultured with PDTC. Proliferation of these cell lines was significantly inhibited by PDTC (Fig. 7*D*). However, the repressive effect of PDTC on cell proliferation was reduced when these cells were transfected with pSHH-GFP (Fig. 7*D*). These data suggested that NF-κB activation enhanced proliferation of pancreatic cancer cells, at least in part, through up-regulation of Shh expression. Taken together, these data suggested that inflammatory stimuli activated NF-κB, which led to overexpression of Shh, resulting in activation of the Hh pathway and enhanced proliferation of pancreatic cancer cells.

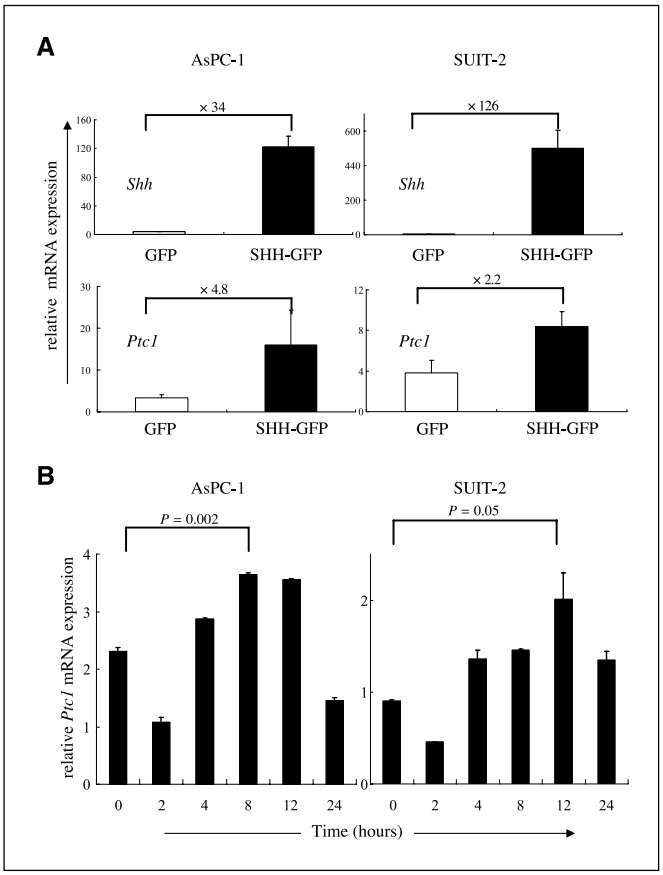

Figure 5. NF-κB activation and overexpression of Shh induce Hh pathway activation in pancreatic cancer cells. mRNA expression relative to that of corresponding β-*actin* mRNA expression. *Columns,* mean of three independent wells; *bars,* SD. *A,* cells were transfected with pGFP or pSHH-GFP for 24 hours, and then expression of *Shh* and *Ptc1* mRNAs was assessed by real-time RT-PCR. *B,* after adding 5 ng/mL IL-1β, relative *Ptc1* mRNA expression was determined with real-time RT-PCR.

Discussion

Here, we report for the first time that NF-κB contributes to Hh pathway activation through up-regulation of Shh expression in pancreatic cancer cells. There was a positive correlation between

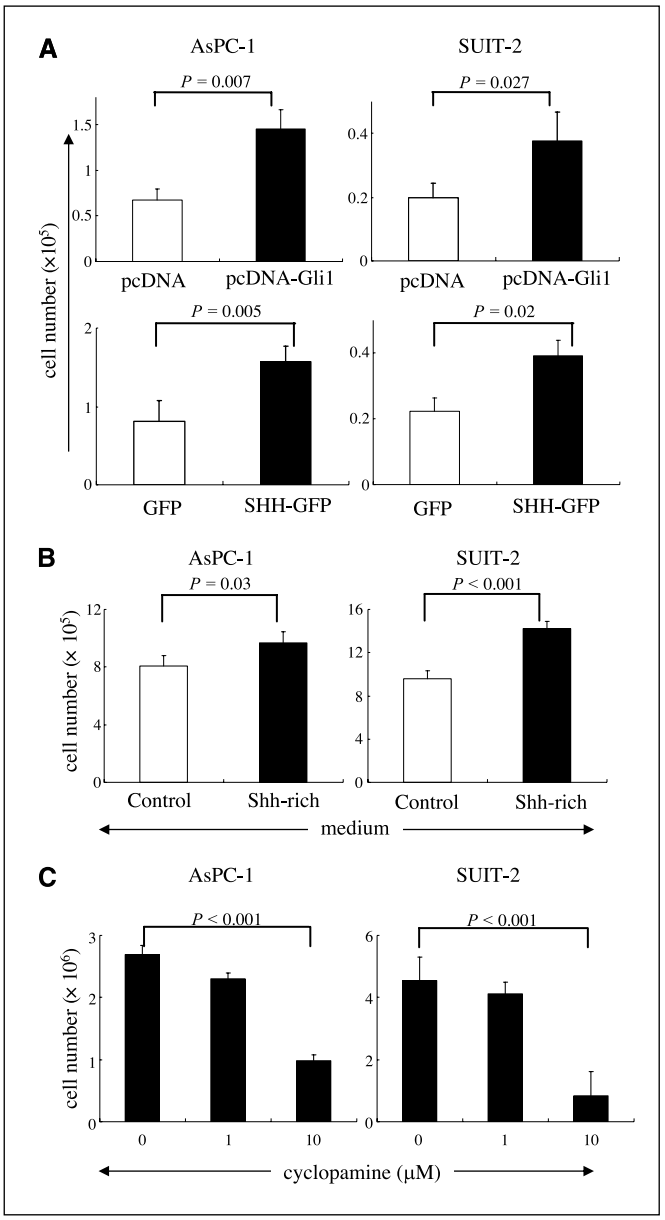

Figure 6. Ligand-dependent activation of the Hh pathway enhanced proliferation of pancreatic cancer cells. Proliferation assay was carried out in triplicate. *Columns,* mean of three independent experiments; *bars,* SD. *A,* cells in 48-well plates were transfected with 0.5 μg pcDNA3.1 or pcDNA3.1/Gli1 or 2 μg pGFP or pSHH-GFP, and cell numbers were determined after 4 days. *B,* numbers of AsPC-1 and SUIT-2 cells incubated in control medium or Shh-rich medium (as described in Materials and Methods) were counted after 4 days of incubation. *C,* cells were treated with 1 or 10 μmol/L cyclopamine for 4 days, and cell numbers were determined.

NF-κB and Shh expression in clinical tissue samples. Blockade of NF-κB suppressed constitutive *Shh* mRNA expression, whereas further activation of NF-κB induced overexpression of Shh *in vitro*. NF-κB-induced overexpression of Shh activated the Hh pathway in pancreatic cancer cells in a ligand-dependent manner, which resulted in enhanced cell proliferation.

It was recently reported that Hh pathway is constitutively activated in a variety of tumors and may play a crucial role during tumor development (5, 10, 11, 35). In some tumors, such as basal cell carcinomas and central nervous system tumors, active mutations of Shh, Smo, and Gli1 and loss-of-function mutations of Ptc1 can activate the Hh pathway (13, 37). In contrast, in cancers of the lung, breast, gastrointestinal tracts, and pancreas, activation of the Hh pathway is mediated by aberrant expression of its ligand, Shh (10, 11, 26). If so, it is important to elucidate the identities of the molecules that regulate expression of Shh. However, to our

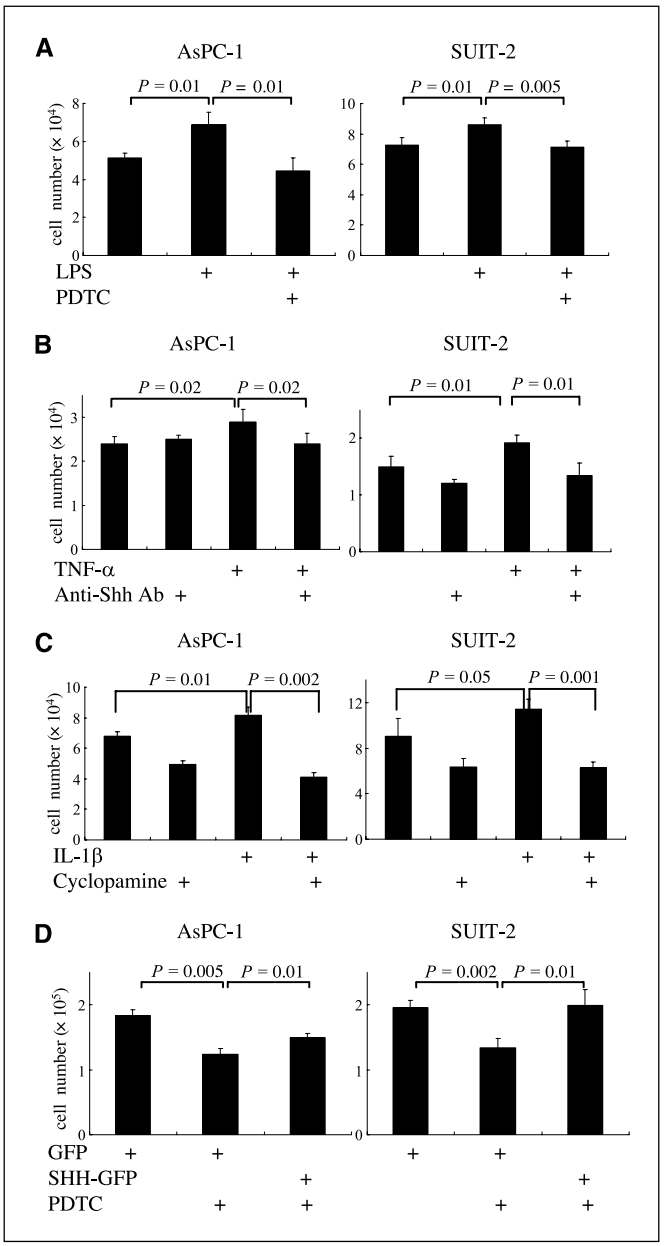

Figure 7. NF-κB activation induces proliferation of pancreatic cancer cells through overexpression of Shh and activation of the Hh pathway. Representative of three different experiments. Proliferation assay was carried out in triplicate. *Bars,* represent SD. *A,* cells were incubated in the presence of 0.1 μg/mL LPS with or without 0.3 μmol/L PDTC, and cell numbers were determined after 4 days. *B,* cells were incubated in the presence of 0.1 IU/mL TNF-α with or without 5 μg/mL anti-Shh neutralizing antibody, and cell numbers were determined after 4 days. *C,* cells were incubated in the presence of 0.1 ng/mL IL-1β with or without 5 μmol/L cyclopamine, and cell numbers were determined after 4 days. *D,* cells in 48-well plates were transfected with 2 μg pGFP or pSHH-GFP and incubated in complete medium overnight. Medium was changed to RPMI 1640 with 0.8% FCS with or without 0.3 μmol/L PDTC, and cell numbers were determined after 4 days.

knowledge, the detailed mechanisms that underlie up-regulation of expression of Shh in these tumors remain unclear (5, 10, 11, 35). Our present findings that NF-κB activation is one of the mechanisms responsible for Shh overexpression seem to have broad significance.

We focused on the role of NF-κB in Shh expression in pancreatic cancer for the following reasons. First, a close relationship between chronic inflammation and cancer development has been proposed (38). Second, NF-κB is one of the molecules responsible for the correlation between inflammation and cancer (22). Third, the Hh pathway has recently been regarded in adult tissue as a tissue repairing signal (13), and in general, tissue repairing signals seemed to be activated continuously in chronic inflammation.

Based on these findings, we hypothesized that NF-κB plays an important role in Hh pathway activation through induction of Shh expression in pancreatic cancer. As expected, a very close positive correlation between p65 and Shh expression was observed in clinical samples (Fig. 1*D*). We also observed that chronic pancreatitis specimens expressed high levels of Shh as well as NF-κB (Fig. 1*A-C*). Some reports have suggested that continuous Hh pathway activation could contribute to or promote carcinogenesis (10). Together with these results, our data raise a possibility that chronic pancreatitis provides such a setting via overexpression of NF-κB.

Using two PDAC cell lines, both of which showed constitutive NF-κB and Hh pathway activation (data not shown), we found that NF-κB contributes to Hh pathway activation in an autocrine manner through Shh induction. Three NF-κB inhibitors, which act at different points in the NF-κB pathway, all reduced Shh expression at both mRNA and protein levels (Figs. 2 and 4). Three inflammatory stimuli used as NF-κB activators, IL-1β, TNF-α, and LPS, all induced further activation of NF-κB in both cells (Figs. 2 and 3; data not shown). IL-1β and TNF-α have been reported to be causative molecules responsible for constitutive NF-κB activation in pancreatic cancer (38). Interestingly, LPS also induced NF-κB activation in these cells (data not shown). Although the detailed mechanism of NF-κB activation by LPS is unclear, both cells lines tested expressed Toll-like receptor 4, a main component of the LPS receptor complex (ref. 39; data not shown). These stimuli all increased expression of Shh protein (Fig. 4*A*). Increased expression of Shh was suppressed significantly by all three NF-κB inhibitors (Fig. 4; data not shown). We were also able to show that Shh induced by NF-κB activation is biologically active. NF-κB activation increased levels of the 19-kDa physiologically active form of Shh (Fig. 4), which activated the Hh pathway in an autocrine manner (Fig. 5) and enhanced cell proliferation (Fig. 7). Our data indicate a significant contribution of Shh induced by NF-κB activation to cell proliferation. Because cyclopamine is not specific inhibitor for Shh-induced Hh pathway activation, however, participation of other ligands, such as Ihh and Dhh in Hh pathway activation and cell proliferation cannot be excluded. In our system, an Hh pathway inhibitor, cyclopamine, had no effect on the transcriptional activity of NF-κB (Fig. 2*A*). We therefore believe that the Hh pathway has little effect on NF-κB activation.

In conclusion, in pancreatic cancer, overexpression of Shh results, at least in part, from constitutive activation of NF-κB; however, it remains to be determined whether NF-κB regulates Shh expression through a direct or indirect interaction. This novel finding that NF-κB influences Shh expression improves our understanding of the mechanism of Hh signaling activation in pancreatic cancer.

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