Advances in Brief

The Helix-Loop-Helix Protein Id2 Is Overexpressed in Human Pancreatic Cancer[1]

Jörg Kleeff, Toshiyuki Ishiwata, Helmut Friess, Markus W. Büchler, Mark A. Israel, and Murray Korc[2]

Departments of Medicine, Biological Chemistry, and Pharmacology, University of California, Irvine, California 92697 [J. K., T. I., M. K.]; Department of Visceral and Transplantation Surgery, University of Bern, 3010 Bern, Switzerland [H. F., M. W. B.]; and Preuss Laboratory, Department of Neurological Surgery, University of California, San Francisco, California 94143 [M. A. I.]

Abstract

Id2 belongs to the Id family of helix-loop-helix (HLH) proteins, which upon heterodimerization with basic HLH proteins prevent basic HLH proteins from DNA binding. Proteins of the Id family act as negative regulatory transcriptional factors, and their expression correlates with cell proliferation and arrested differentiation in many cell lineages. In this study, we characterized the expression of Id2 in normal and cancerous pancreatic tissues. Pancreatic cancers markedly overexpressed Id2 mRNA in comparison to the normal pancreas. Furthermore, there was abundant Id2 immunoreactivity in the cancer cells within the pancreatic tumor mass. In PANC-1 human pancreatic cancer cells, steady-state Id2 mRNA levels increased upon serum addition and decreased after induction of differentiation with either sodium butyrate or 12-*O*-tetradecanoylphorbol-13-acetate. Inhibition of Id2 expression with Id2 antisense oligonucleotides inhibited the growth of these cells, whereas random and sense oligonucleotides were without effect. These findings suggest that Id2 may have a role in human pancreatic cancer.

Introduction

Control and initiation of gene transcription requires the interaction of DNA-binding proteins with each other and with specific DNA sequence elements. One family of such DNA-binding proteins is characterized by the HLH[3] motif that consists of two conserved amphiphatic α-helices separated by a loop of variable length and sequence (1). Within each group, various HLH members are capable of forming hetero-oligomeric complexes with each other, leading to modulation of diverse regulatory functions (1). A subgroup of HLH proteins is characterized by a region of basic amino acids as the DNA binding domain (bHLH; Refs. 2 and 3) and is known to activate differentiation-specific genes, including neuronal-specific genes, muscle-specific genes, immunoglobulin genes, and insulin-related genes (1). There are two main categories of bHLH proteins. The A class of bHLH proteins, which are widely expressed, includes proteins that are encoded by the *E2A*, *E2-2/ITF2*, and *HEB/HTF4* genes (2, 4, 5), the family of *myc*-related genes, and other widely expressed transcription factors (1). The class B of bHLH proteins is composed of more cell-specific proteins such as MyoD, NeuroD, MASH, and TAL (6–9). Dimerization of the bHLH proteins is required for DNA binding and transcriptional activation *in vivo*, and in general, ubiquitously expressed bHLH proteins form heterodimers with tissue-specific bHLH proteins (10, 11).

Id proteins form another class of HLH proteins, which lack the DNA binding region, but which are still capable of heterodimerization with specific bHLH proteins. This complex formation prevents bHLH from binding to DNA (10, 12). Thus, Id proteins act as negative regulators of transcription factors. Expression of *Id* genes is down-regulated upon differentiation in many cell types, and it is thought that Id proteins prevent bHLH proteins from enhancing the expression of lineage-specific genes (10, 12–14). Furthermore, Id proteins might play a role in the G_0 to S phase cell cycle transition. In some cell types, such as murine fibroblasts, Id expression correlates with cell proliferation, and inhibiting Id protein synthesis prevents cell cycle reentry of arrested cells (15). Furthermore, Id2 can enhance S-phase progression and cellular proliferation by inactivating pRB (16).

Loss of growth control mechanisms and inhibited differentiation are hallmarks of tumorigenesis. Although Id proteins act as important regulators of cell growth and differentiation, their potential role in human malignancies is not well characterized. In the present study, we investigated the expression and localization of Id2, a ubiquitously expressed member of HLH proteins, in human pancreatic cancer. This malignancy is associated with a median survival of <6 months after diagnosis and an overall 5-year survival rate of <1% (17). It is often associated with the overexpression of a variety of mitogenic growth factors and their receptors and by oncogenic mutations of K-*ras* and inactivation of the *p53* tumor suppressor gene (18). We now show that Id2 is overexpressed in pancreatic cancer in comparison with the normal pancreas and that this overexpression is confined to the cancer cells within the tumor mass. We also show that serum addition increases Id2 steady-state mRNA levels in a pancreatic cancer cell line and that induction of differentiation in this cell line causes a decrease in Id2 mRNA levels.

Materials and Methods

The following materials were purchased: FBS, DMEM, and RPMI 1640, trypsin solution, and penicillin-streptomycin solution from Irvine Scientific (Santa Ana, CA); Genescreen membranes from New England Nuclear (Boston, MA); DECAprime II DNA labeling kit from Ambion (Austin, TX); and Id2 (C20) antibodies and the Id2 blocking peptide from Santa Cruz Biotechnology (Santa Cruz, CA); [α-^{32}P]dCTP from Amersham (Arlington Heights, IL). The following oligonucleotides were from Biosynthesis (Lewisville, TX): antisense, AAGGCTTTCATGCTGCTCGT (19); sense, ACGAGCAGCATGAAAGCCT; and random, AGGACGAGACAAGTGATCTC. All other reagents were from Sigma Chemical Co. (St. Louis, MO). PANC-1 human pancreatic cells were obtained from American Type Culture Collection (Rockville, MD).

Tissue Samples. Normal human pancreatic tissue samples (7 male and 5 female donors; median age, 41.8 years; range, 14–68 years) and pancreatic cancer tissues (10 males and 6 females; median age, 62.6 years; range, 53–83 years) were obtained through an organ donor program and from pancreatic cancer patients. According to the Tumor-Node-Metastasis classification of the Union Internationale Contre le Cancer, 6 tumors were stage 1, 1 tumor was stage 2, and 9 tumors were stage 3 ductal cell adenocarcinoma. Freshly removed tissue samples were fixed in 10% formaldehyde solution for 12–24 h and paraffin-embedded for histological analysis. In addition, tissue samples were frozen in liquid nitrogen immediately upon surgical removal and maintained in −80°C until use for RNA extraction. All studies were approved by

the Ethics Committee of the University of Bern and by the Human Subjects Committee at the University of California, Irvine.

RNA Extraction and Northern Blot Analysis. Total RNA was extracted by the single-step acid guanidinium thiocyanate-phenol-chloroform method. RNA was size fractioned on 1.2% agarose/1.8 M formaldehyde gels, electro-transferred onto nylon membranes, and cross-linked by UV irradiation. Blots were prehybridized and hybridized with cDNA probes and washed under high stringency conditions as reported previously. Blots were then exposed at −80°C to Kodak XAR-5 films, and the resulting autoradiographs were scanned to quantify the intensity of the radiographic bands. The following cDNA probes were used: a 440-bp human Id2 cDNA, covering the entire coding region of Id2; and a 190-bp fragment of mouse 7S cDNA, which hybridizes with human cytoplasmic RNA and confirms equal RNA loading and transfer (20).

Immunohistochemistry. A specific rabbit anti-human Id2 polyclonal antibody was used for immunohistochemistry. This affinity-purified rabbit polyclonal antibody specifically reacts with Id2 of human origin by Western blotting. Paraffin-embedded sections (4 μm) were subjected to immuno-staining using the streptavidin-peroxidase technique. Endogenous peroxidase activity was blocked by incubation for 30 min with 0.3% hydrogen peroxide in methanol. Tissue sections were incubated for 15 min (23°C) with 10% normal goat serum and then incubated for 16 h at 4°C with Id2 antibodies in PBS containing 1% BSA. Bound antibodies were detected with biotinylated goat anti-rabbit IgG secondary antibodies and streptavidin-peroxidase complex, using diaminobenzidine tetrahydrochloride as the substrate. Sections were counter-stained with Mayer's hematoxylin. Some sections were incubated with nonimmunized rabbit IgG or without primary antibodies, which did not yield positive immunoreactivity. Furthermore, preabsorption with an Id2-specific blocking peptide completely abolished immunoreactivity.

Cell Culture and Proliferation Assay. PANC-1 cells were routinely grown in DMEM supplemented with 10% FBS, 100 units/ml penicillin, and 100 μg/ml streptomycin (complete medium). To perform growth assays, cells were plated overnight at a density of 10,000 cells/well in 96-well plates and subsequently incubated in serum-free medium (DMEM containing 0.1% BSA, 5 μg/ml transferrin, 5 ng/ml sodium selenite, and antibiotics) in the absence or presence of the indicated oligonucleotides. Incubations were continued for the indicated times prior to adding MTT (62.5 μg/well) for 4 h. Cellular MTT was solubilized with acidic isopropanol, and absorbance was measured at 570 nm with an ELISA plate reader (Molecular Devices, Menlo Park, CA). In pancreatic cancer cells, the results of the MTT assay correlate with results obtained by cell counting with a hemocytometer and by monitoring [^{3}H]thymidine incorporation (21). Student's t test was used for statistical analysis of the experiments. Results are expressed as mean ± SE. $P < 0.05$ was taken as the level of significance.

Results

Expression of Id2 in Human Pancreatic Tissues. Northern blot analysis was performed on total RNA isolated from 12 normal pancreatic tissues and 16 pancreatic cancers. The 1.6-kb Id2 mRNA transcript was expressed at relatively high levels in 6 of 12 normal pancreatic tissue samples. Id2 expression was low to moderate or below the level of detection in the remaining samples. In the cancer tissues, high levels of Id2 mRNA were present in 14 of 16 samples. A representative Northern blot is shown in Fig. 1. Densitometric analysis of all of the autoradiograms indicated that by comparison with all of the normal pancreatic tissues, there was a 5-fold increase ($P < 0.01$) of Id2 mRNA levels in the pancreatic cancer samples.

Immunohistochemistry. To determine the site of localization of Id2, immunostaining was carried out using a highly specific Id2 antibody. In the normal pancreas, faint to moderate Id2 immunoreactivity was present in ductal cells of small and large pancreatic ducts, whereas the acinar and islet cells did not exhibit any Id2 immunoreactivity (Fig. 2A). In contrast, in the pancreatic cancer tissues, strong Id2 immunoreactivity was present in ductal-like cancer cells (Fig. 2B). Preabsorption with the Id2 specific blocking peptide completely abolished the immunoreactivity (Fig. 2C).
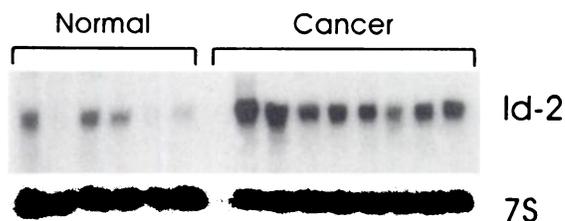

Fig. 1. Expression of Id2 in pancreatic tissues. Total RNA (20 μg/lane) from six normal and eight cancerous human pancreatic tissues were subjected to Northern blot analysis using a ^{32}P-labeled cDNA probe (500,000 cpm/ml) specific for Id2. A 7S cDNA probe (50,000 cpm/ml) was used as a loading and transfer control. Exposure times were 1 day for Id2 and 6 h for 7S.

Id2 Expression and Cell Proliferation and Differentiation in PANC-1 Pancreatic Cancer Cells. Id proteins are thought to play crucial roles in regulating cell growth and differentiation in many cell types. Therefore, we assessed the role of Id2 expression on cell proliferation and differentiation in PANC-1 cells. To this end, we first induced differentiation in this cell line with either sodium butyrate or the phorbol ester TPA. Both substances are known to act as differentiating agents. Treatment of PANC-1 cells with either sodium butyrate (2 mM) or TPA (20 nM) suppressed cell proliferation by −25% ± 8% and −32% ± 6%, respectively, after 48 h, without causing cell death as determined by the trypan blue exclusion test. In addition, both sodium butyrate and TPA induced morphological changes, which were apparent after 24 h of treatment. After 72 h, >90% of the cells exhibited marked morphological changes, including an abundant cytoplasm, a high cytoplasm:nucleus ratio, irregular shapes, and spindle-like structures (Fig. 3A). Northern blot analysis indicated that there was a decrease in Id2 mRNA expression, which paralleled the induction of the morphological changes by either sodium butyrate (Fig. 3B) or TPA (data not shown).

Next, we determined the effects of serum and growth factor addition on Id2 expression levels in PANC-1 cells. Cells were serum starved for 12 h and then incubated for 12 h in the absence or presence of serum or 1 nM epidermal growth factor, fibroblast growth factor 2, or insulin-like growth factor 1. There was a marked increase of Id2 mRNA after serum addition (Fig. 3C). In contrast, none of the growth factors caused a significant increase in Id2 expression levels (data not shown), although they are known to be mitogenic in PANC-1 cells (18).

To further investigate the effects of Id expression on cell proliferation, PANC-1 cells were treated with Id2 antisense oligonucleotides as well as Id2 sense and random sense oligonucleotides, which served as controls. Immunoblot analysis was carried out to determine the effects of Id2 antisense on Id2 protein levels. Compared with the control cells, there was a slight decrease in Id2 levels in cells incubated for 24 h with Id2-sense or random-sense oligonucleotides (1 μM). In contrast, Id2 expression decreased dramatically in the cells treated with Id2 antisense oligonucleotides (1 μM; Fig. 4A). Growth assays revealed a slight decrease in the growth of PANC-1 cells treated with Id2-sense or random-sense oligonucleotides for 24 h, when compared with the untreated cells. In contrast, there was a significant growth inhibition of −39% ± 2% ($P < 0.01$) in PANC-1 cells that were incubated for 24 h in the presence of Id2 antisense oligonucleotides (Fig. 4B).

Discussion

Id2 encodes a protein that, like other Id family members, negatively regulates bHLH transcription factors (1). Four members of the human Id family have been identified. They are expressed in numerous cell types, and their expression in several different cell types can be

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retinoblastoma protein, thus abolishing its growth-suppressing activity (26), and by antagonizing the growth-suppressive activities of the cyclin-dependent kinase inhibitors p16 and p21 (16). In addition, Id expression decreases with differentiation of neuroblasts (27), hepatocytes (28), osteogenic cells (29), and B lymphocytes (30).
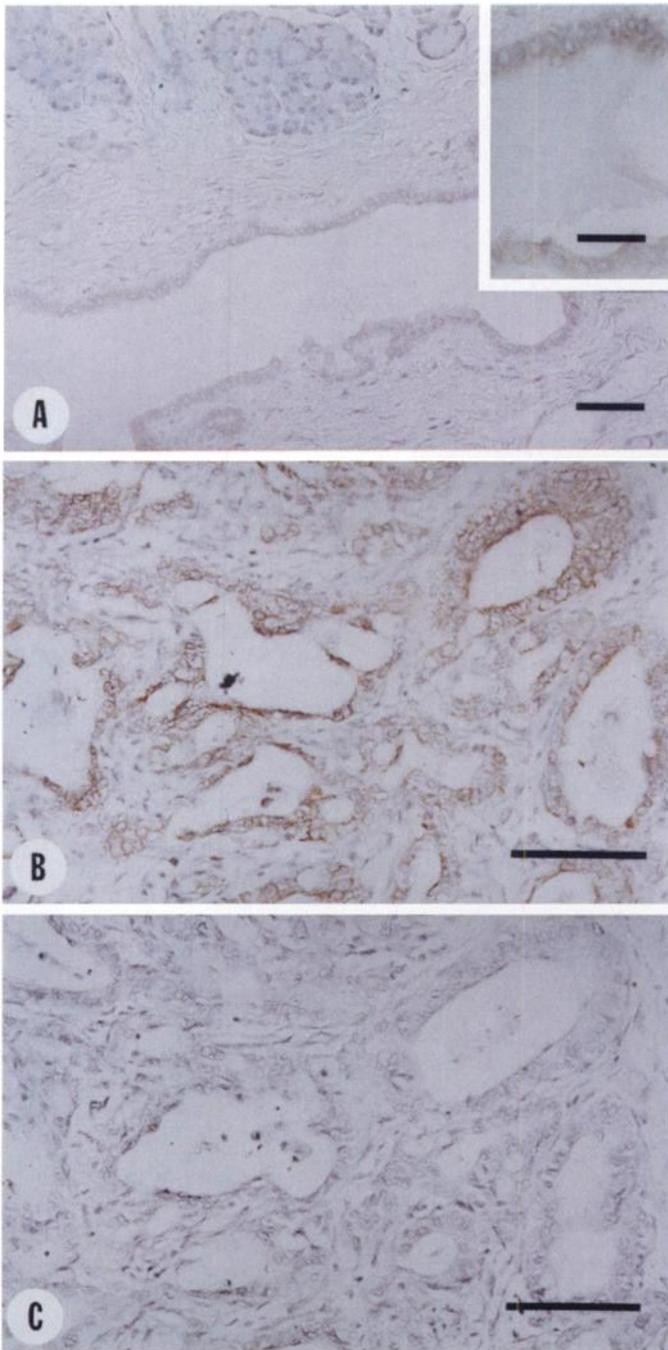

Fig. 2. Normal (A) and cancerous pancreatic tissues (B and C) were subjected to immunostaining using a highly specific anti-Id2 antibody as described in "Materials and Methods." Faint to moderate Id2 immunoreactivity was present in the ductal cells (A and inset), whereas the acinar cells (upper portion of A) were completely devoid of Id2 immunostaining. In the pancreatic cancers, strong Id2 immunoreactivity was present in the duct-like cancer cells (B). Preabsorption with the Id2-specific blocking peptide abolished Id2 immunoreactivity (C). Bar: A–C, 50 μm; inset, 25 μm.

associated with either enhanced growth or decreased differentiation (15, 22–24). For example in human fibroblasts, Id2 expression is induced by mitogens and is essential for the onset of DNA replication and declines with replicative senescence (15). In mammary epithelial cells, Id1 expression is associated with increased proliferation (25), and in NIH 3T3 fibroblasts, reduction of Id mRNA levels by antisense oligonucleotides leads to a delayed reentry of arrested cells into the cell cycle after serum or growth factor stimulation (19). Conversely, enhanced Id2 expression promotes cell proliferation by binding to the
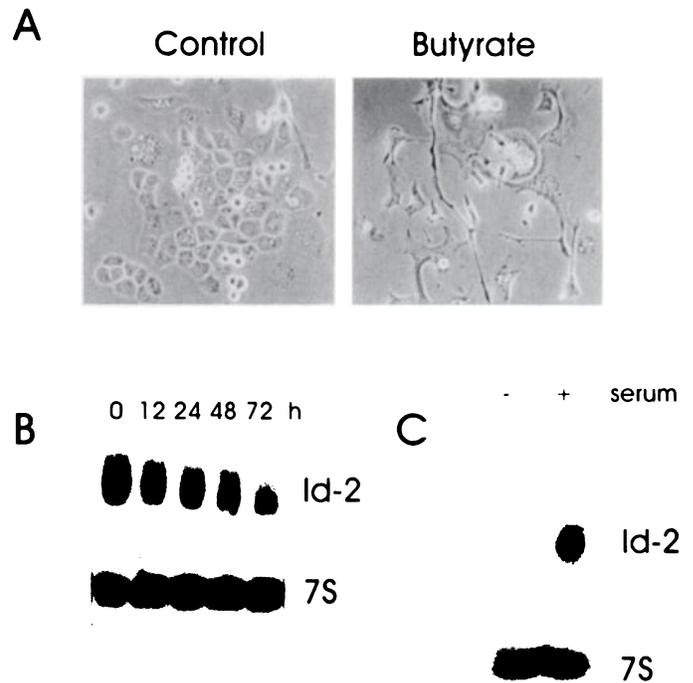

Fig. 3. Effects of cell proliferation and differentiation on Id2 expression in PANC-1 cells. In A, cells were incubated in the absence (control) or presence of sodium butyrate (2 mM) for 72 h. In B, cells were incubated in complete medium (8% FBS) in the absence (0) or presence of 2 mM sodium butyrate for the indicated time. In C, cells were incubated for 12 h in the absence (−) or presence (+) of 10% FBS. Total RNA (20 μg/lane) was subjected to Northern blot analysis using a ^{32}P-labeled Id2 cDNA probe (500,000 cpm/ml) and a 7S cDNA probe (50,000 cpm/ml) as a loading and transfer control. Exposure times were 1 day for Id2 and 6 h for 7S.
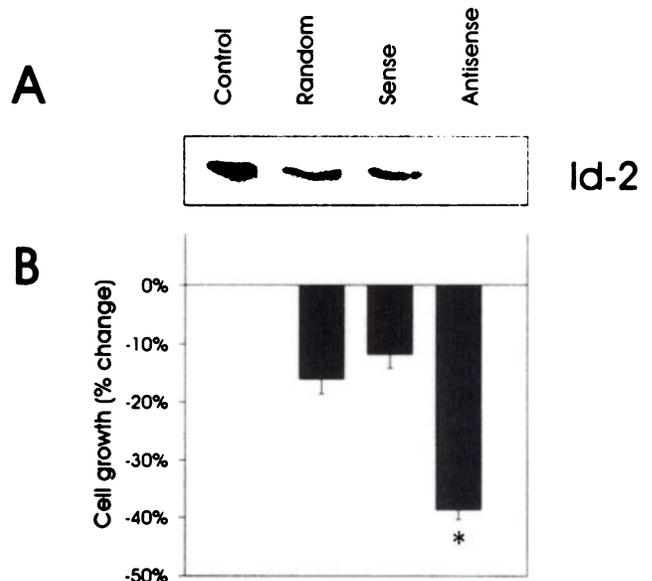

Fig. 4. Effects of Id2 antisense oligonucleotides. Cells were incubated for 24 h in complete medium (8% FBS) in the absence (control) or presence of the indicated oligonucleotides (1 μM). A, immunoblotting. Cell lysates were subjected to SDS-PAGE. Membranes were probed with a highly specific Id2 antibody. B, MTT cell growth assays. Data are expressed as the percentage of decrease by comparison with untreated controls and are the means of eight determinations/experiment from three separate experiments; bars, SE. *, P < 0.01.

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In this study, we determined by Northern blot analysis that Id2 expression was up-regulated in human pancreatic cancer. Immunohistochemistry localized this overexpression to the cancer cells within the pancreatic tumor mass. This observation suggests that Id2 may be involved in the perturbations of cell growth and differentiation in pancreatic tumorigenesis. In support of this hypothesis, serum increased Id2 expression in PANC-1 human pancreatic cancer cells, whereas induction of differentiation led to a decrease in Id2 expression in these cells. Furthermore, Id2 antisense oligonucleotides significantly inhibited basal growth in this cell lines. Thus, decreased Id2 expression suppresses cell proliferation *in vitro*, suggesting that Id2 either directly stimulates growth or acts to maintain the growth potential of pancreatic cancer cells. In this context, the presence of Id2 in the pancreatic ductal cells in the normal pancreas raises the possibility that Id2 may participate in the physiological regulation of duct cell proliferation.

Although the potential role of Id2 has not been systematically studied in human tumors, its expression has been observed in cell lines derived from different tumors such as lung cancer, neuroblastoma, neuroepithelioma, and astrocytic tumors (23, 31, 32). Pancreatic cancer is characterized by its aggressive growth behavior and its propensity to metastasize. This is, at least in part, due to the aberrant activation of growth-stimulatory pathways in conjunction with defective growth-inhibitory pathways. Thus, pancreatic cancers often overexpress a variety of mitogenic tyrosine kinase receptors and their respective ligands (18). The activation of those mitogenic pathways is enhanced as a consequence of mutations in the K-*ras* oncogene and cell cycle-associated kinases such as p16. Inasmuch as forced constitutive expression of *Id* genes may act to enhance immortalization functions under the appropriate conditions (33, 34) and *Id* genes may function in cell cycle pathways that regulate G_1 progression and oncogenesis (12, 26, 35), the overexpressed *Id2* gene in pancreatic cancer may allow for the enhanced expression of growth-stimulatory genes, thereby contributing to the malignant properties of pancreatic cancer *in vivo*.

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