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Fibroblast growth factor-5 stimulates mitogenic signaling and is overexpressed in human pancreatic cancer: evidence for autocrine and paracrine actions

Marko Kornmann[1], Toshiyuki Ishiwata[1], Hans G Beger[2] and Murray Korc[1]

[1]*Department of Medicine, Biological Chemistry and Pharmacology, University of California, Irvine, Medical Science Institute C240, Irvine, California 92697, USA;* [2]*Department of General Surgery, University of Ulm, Steinhövelstr. 9, 89075 Ulm, Germany*

Fibroblast growth factor (FGF)-1 and -2 are overexpressed in human pancreatic cancer. In this study the role of FGF-5 in human pancreatic cancer was investigated, as FGF-5 has a classical signal sequence for secretion not found in FGF-1 or -2. Northern blot analysis with a 306 bp FGF-5 cDNA revealed the presence of 4.0 kb and 1.6 kb FGF-5 mRNA transcripts in both normal and cancerous pancreatic tissues. Densitometric analysis indicated that 4.0 kb and 1.6 kb FGF-5 mRNA transcripts levels were increased 2.4- and 2.7-fold in the cancers by comparison with normal tissues, respectively ($P<0.002$, $P<0.0001$). Immunohistochemistry and *in situ* hybridization demonstrated that FGF-5 localized in the cancer cells, stromal fibroblast and inflitrating macrophages. FGF-5 mRNA was also detected in COLO-357 human pancreatic cancer cells. Furthermore, secreted FGF-5 protein was present in conditioned medium of COLO-357 cells. Exogeneous FGF-5 (0.37 nM) increased the growth of COLO-357 cells by 48% ($P<0.0001$) and increased mitogen-activated protein kinase activity. COLO-357 cells expressed the IIIc isoform of the type I FGF receptor, the preferred FGF receptor for FGF-5. These observations suggest that FGF-5 may participate in autocrine and paracrine pathways promoting pancreatic cancer cell growth *in vivo*.

Keywords: fibroblast growth factor-5; fibroblast growth factor receptor splice variant; *in situ* hybridization; mitogenic signaling; pancreatic cancer; protein kinases

Introduction

Fibroblast growth factor-5 (FGF-5) belongs to a group of homologous heparin-binding polypeptides that are involved in various biological processes including development, morphogenesis, tissue growth, and repair (Fernig and Gallagher, 1994; Burgess and Winkles, 1996). FGFs are angiogenic and mitogenic, and have been implicated in a variety of human neoplasms (Basilico and Moscatelli, 1992; Burgess and Winkles, 1996). The FGF family consists presently of at least ten members, acidic FGF (aFGF or FGF-1), basic FGF (bFGF or FGF-2), FGF-3 (*int-2*), FGF-4 (*hst*/kFGF), FGF-5, FGF-6, keratinocyte growth factor (KGF or FGF-7), androgen-induced growth factor (AIGF or FGF-8), glia-activating factor (GAF or FGF-9), and FGF-10 (Yamasaki *et al*., 1996). The *FGF-5* gene, located on chromosome 4q11 (Nguyen *et al*., 1988), was originally discovered as a human oncogene which had acquired transforming potential by DNA rearrangement accompanying transfection of NIH3T3 cells with human tumor DNA (Zhan *et al*., 1987). Molecular cloning revealed that the oncogene encoded for a glycosylated 267 amino acid protein (Zhan *et al*., 1988). Unlike the prototypical FGFs, FGF-1 and FGF-2, FGF-5 contains a hydrophobic N-terminal leader sequence typical for efficiently secreted proteins (Zhan *et al*., 1988), suggesting that this factor may have an important role in autocrine and paracrine mediated events.

The signaling of the FGFs is mediated by a dual-receptor system, consisting of four high-affinity tyrosine kinase receptors, termed fibroblast growth factor receptors (FGFRs), and a number of low-affinity heparan sulfate proteoglycan receptors (Klagsburn and Baird, 1991; Givol and Yayon, 1992; Johnson and Williams, 1993; Fernig and Gallagher, 1994; Mason, 1994; Burgess and Winkles, 1996). FGFRs are glycoproteins composed of usually three extracellular immunoglobulin (Ig)-like domains, a hydrophobic transmembrane region and a cytoplasmic domain that contains a tyrosine kinase catalytic domain. Alternative mRNA splicing mechanisms result in receptor isoforms that display unique ligand binding properties (Givol and Yayon, 1992; Johnson and Williams, 1993). Ligand interaction with FGFRs also requires the presence of endogenous heparan sulfate proteoglycan or exogenous heparin (Givol and Yayon, 1992) and results in oligomerization, activation of the cytoplasmic receptor tyrosine kinase and receptor autophosphorylation (Jaye *et al*., 1992). Subsequent signaling is mediated through a number of substrates for tyrosine phosphorylation (Ullrich and Schlessinger, 1990), including mitogen-activated protein kinases (MAPKs) or ERK-1, -2 and -3 (Mason, 1994; Friesel and Maciag, 1995).

Previously, we have demonstrated that FGF-1, FGF-2 and FGF-7 are overexpressed in human pancreatic cancer and that overexpression of FGF-2 correlates with shorter post-operative survival (Yamanaka *et al*., 1993; Siddiqi *et al*., 1995). Moreover, the human pancreas is rich in heparan sulfate (Gambarini *et al*., 1993). Despite these observations, nothing is known about FGF-5 expression and localization in human pancreatic tissues. Furthermore, its mitogenic potential in pancreatic cancer cells has not been examined. Therefore, the aim of this study was to characterize FGF-5 expression in normal and cancerous pancreatic tissues and to investigate its effect on

Correspondence: M Korc
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growth and mitogenic signaling in cultured human pancreatic cancer cell line.

Results

Expression of FGF-5 mRNA in pancreatic tissues

FGF-5 mRNA expression in total RNA from 14 normal and 16 cancerous human pancreatic tissue samples was investigated by Northern blotting using a 306 bp *Bam*HI/*Hind*III human FGF-5 cDNA fragment generated by RT–PCR from human placenta RNA. The analysis indicated that FGF-5 mRNA levels were relatively low and hardly detectable in the normal pancreas, whereas two transcripts (approximately 4.0 and 1.6 kb) were detectable in many cancer samples (Figure 1). The 4.0 kb FGF-5 mRNA moiety was detectable on the original autoradiograph in 12 of 16 cancers (75%) and in seven of 14 normal tissues (50%). The 1.6 kb FGF-5 mRNA moiety was detectable in 10 of 16 cancers (63%) and in four of 14 normal tissues (29%). A larger band (approximately 6 kb) of unknown significance was visible in some of the lanes. FGF-5 mRNA transcripts were below the level of detection on the original autoradiographs in four cancers and eight normal tissues. The median values for FGF-5 expression in the normal tissues of the 4.0 kb and 1.6 kb moieties, determined by densitometry, were 0.117 and 0.073, respectively, and in the cancer tissues 0.280 and 0.195, respectively. Overall, there were 2.4-fold and 2.7-fold increases in the FGF-5 levels by comparison with the corresponding levels in normal tissues ($P<0.002$; $P<0.0001$). The relative values for all the samples with their corresponding median values are plotted in Figure 2.

Immunohistochemistry of FGF-5 in pancreatic tissues

To localize FGF-5 protein in pancreatic tissues, immunostaining with highly specific anti-human FGF-5 antibodies was carried out. In the normal pancreas (Figure 3a), moderate FGF-5 immunoreactivity was present in the ductal cells and faint immunoreactivity was present in the islet cells (outlined by arrowheads). In the cancer tissues, moderate FGF-5 immunoreactivity was observed in the cytoplasm of many cancer cells (Figure 4a) and faint immunoreactivity was present in the fibroblasts (Figure 4a) adjacent to the tumor cells and islet cells (not shown). The most intense staining was seen in infiltrating macrophages (arrowheads). Vascular smooth muscle cells were also faintly stained by FGF-5 (not shown). To exclude the possibility of non-specific staining by intrinsic peroxidase, we also used alkaline phosphatase labeled streptavidin and new fuchsin instead of the peroxidase labeled streptavidin and DAB system. Similar results for FGF-5 staining were obtained by both methods (not shown).

In situ *hybridization of FGF-5 in pancreatic tissues*

In view of the heterogenous populations of cells in the normal and cancerous tissues we next carried out *in situ* hybridization analysis. In the normal pancreas, a faint FGF-5 mRNA *in situ* hybridization signal was present in ductal and islet cells (data not shown). In the cancer tissues (Figure 4b), a moderate to strong FGF-5 mRNA *in situ* hybridization signal was evident in many cancer cells, macrophages (arrowheads) and some fibroblasts adjacent to the cancer cells. *In situ* hybridization with a sense FGF-5 probe did not produce any specific signal (Figure 4c).

Expression of FGF-5 and FGF receptors in COLO-357 cells

Northern blotting of total RNA prepared from COLO-357 cells revealed a 4.0 kb FGF-5 mRNA (Figure 5). A second 1.6 kb FGF-5 transcript was only detectable on

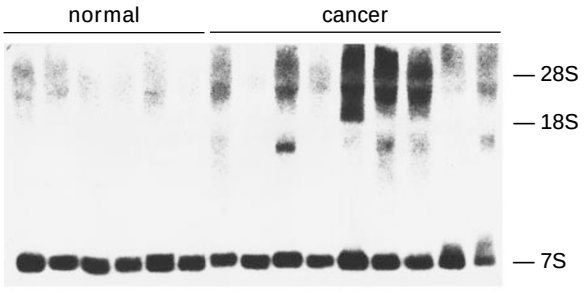

Figure 1 Expression of FGF-5 mRNA in six normal and nine cancerous human pancreatic tissues. Northern blotting of total RNA (20 μg/lane) was carried out using a human 306 bp *Bam*HI/*Hind*III FGF-5 cDNA fragment, randon-primed labeled with α-^{32}P-dCTP (750 000 c.p.m./ml; 14 day exposure). A mouse 7S cDNA probe, cross-reactive with human 7S, was used as a loading control (40 000 c.p.m./ml; 6 h exposure). 28S and 18S rRNA markers are shown on the right

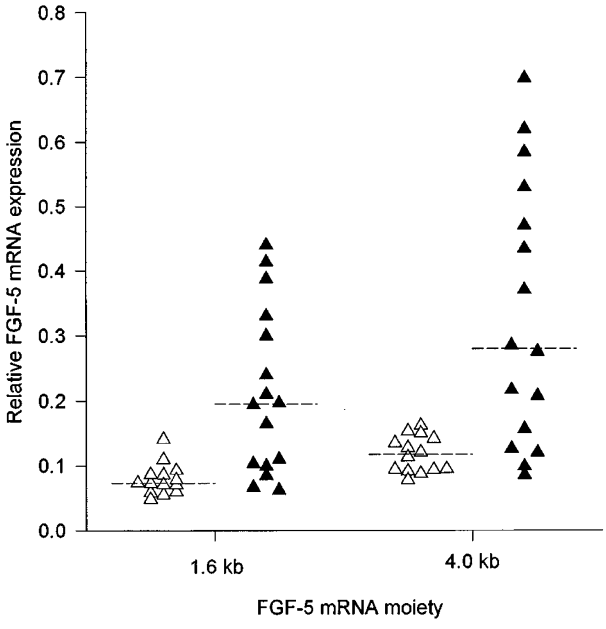

Figure 2 Relative FGF-5 mRNA expression in normal (open triangles, $n=14$) and cancerous (closed triangles, $n=16$) human pancreatic tissue samples. Autoradiographs of Northern blots for FGF-5 and 7S were analysed by densitometry and the level of FGF-5 expression was calculated as the ratio of FGF-5 and 7S, which served as loading control. The median FGF-5 scores (indicated as dotted lines) of normal and cancerous tissue differed significantly for the 1.6 and 4.0 kb moiety ($P<0.0001$ and $P<0.002$, respectively)

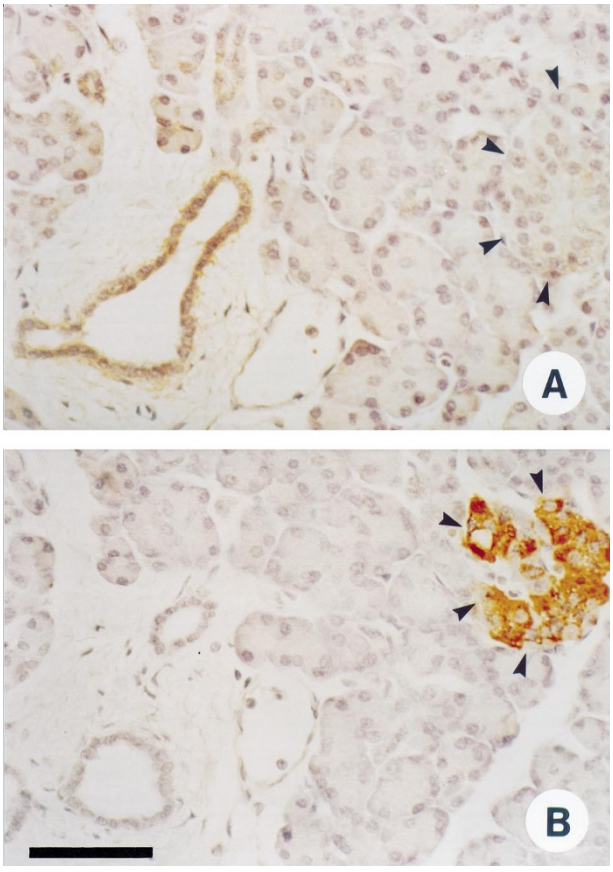

Figure 3 Immunohistochemistry of FGF-5 in normal pancreatic tissues. (**a**) FGF-5 protein was localized in ductal cells and islet cells (outlined by arrowheads). (**b**) localization of endocrine islets (outlined by arrowheads) in a serial section using an anti-porcine insulin antibody, cross-reactive with human insulin. Scale bar = 50 μm

the original autoradiograph after a long exposure (not shown). Northern blotting also revealed the presence of all four FGFR mRNA transcripts in COLO-357 cells (Figure 5). FGF-5 dependent signaling is mediated preferentially via specific splice variants termed FGFR-1 IIIc and FGFR-2 IIIc, but not via FGFR-3 IIIc or FGFR-4 (Ornitz *et al*., 1996; Kanai *et al*., 1997). Therefore, to further characterize FGFR-1 and -2 expression in COLO-357 cells, ribonuclease protection assays were performed for both receptors using antisense riboprobes that can distinguish between the IIIb and IIIc splice variants. The preponderant receptor expressed by COLO-357 cells was the IIIc splice variant of FGFR-1, shown as a 301 nt protected band in Figure 6 (lane 2). A faint 156 nt band, corresponding to the IIIb variant of FGFR-1 was also visible. In contrast, only the IIIb variant was present in the case of FGFR-2 (not shown).

Secretion of FGF-5 by COLO-357 cells

FGF-5 protein production and release into medium was assessed by Western blotting with the same specific anti-human FGF-5 antibodies used for immunostaining. Recombinant FGF-5 migrated as a 29.5 kDa band (Figure 7, lanes 1 and 4). Probably due to the fact that FGF-5 has a specific leader sequence for rapid secretion (Zhan *et al*., 1988) FGF-5 protein could not

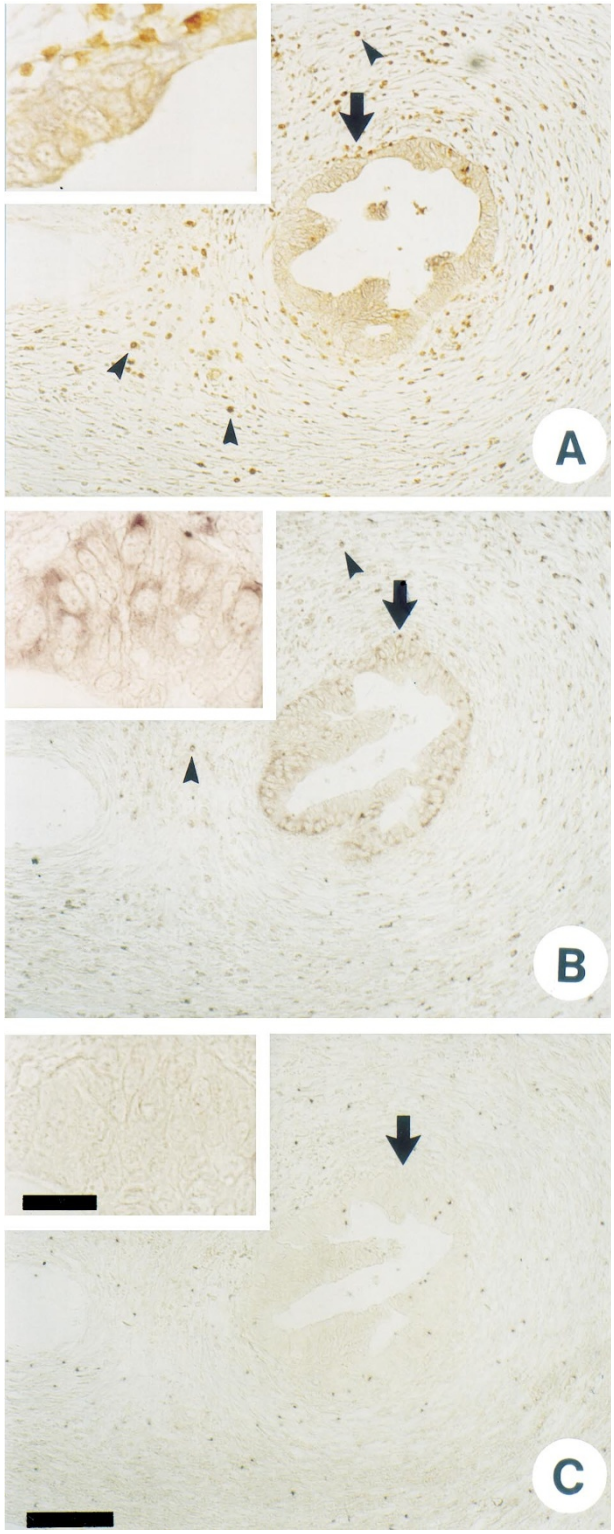

Figure 4 Localization and expression of FGF-5 in human pancreatic tissues. (**a**) immunostaining showed moderate to strong FGF-5 immunoreactivity in the cytoplasm of many cancer cells as well as in fibroblasts and infiltrating macrophages (arrowheads) adjacent to the tumor cells. (**b**) *in situ* hybridization analysis of serial sections revealed a moderate to strong FGF-5 mRNA signal in most of the cancer cells and in many fibroblasts and macrophages (arrowheads) adjacent to the cancer cells, while a sense of FGF-5 probe did not show any signal (**c**). The solid arrows denote the regions magnified in the insets, demonstrating cytoplasmic localization of FGF-5 (**a**) and expression of FGF-5 mRNA (**b**) in duct-like cancer cells and adjacent macrophages. Scale bar = 100 μm, scale bar of inset = 25 μm

be found in the total cell lysates. In contrast, Western blotting of heparin-sepharose precipitates from serum-free conditioned medium revealed two major bands of 34 and 33 kDa corresponding to secreted FGF-5 proteins (Figure 7, lane 2). As previously reported, FGF-5 protein species released into conditioned medium of NIH3T3 cells migrate as 32.5 to 38.5 kDa bands due to FGF-5 glycosylation within the cells (Bates *et al*., 1991). Assuming comparable efficiency for precipitation of secreted FGF-5 proteins of COLO-357 cells (lane 2) and recombinant FGF-5 protein (lane 4) the concentration of FGF-5 in the medium was estimated to be 10–15 ng/ml at the end of the 2 day incubation period.

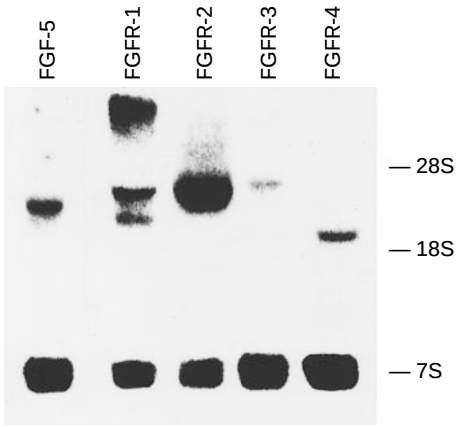

Figure 5 FGF-5 and FGFR mRNA expression in COLO-357 human pancreatic cancer cells. Northern blotting of total RNA (15 μg/lane) was carried out using α-^{32}P-dCTP labeled cDNA fragments (FGF-5: 750 000 c.p.m./ml, 10 day exposure; FGFR-1: 700 000 c.p.m./ml, 1 day; FGFR-2: 700 000 c.p.m./ml, 4 day; FGFR-3: 700 000, 1 day c.p.m./ml; FGFR-4: 700 000 c.p.m./ml, 7 day). A mouse 7S cDNA probe, cross-reactive with human 7S, was used as a loading control (40 000 c.p.m./ml; 6 h exposure). 28S and 18S rRNA markers are shown on the right

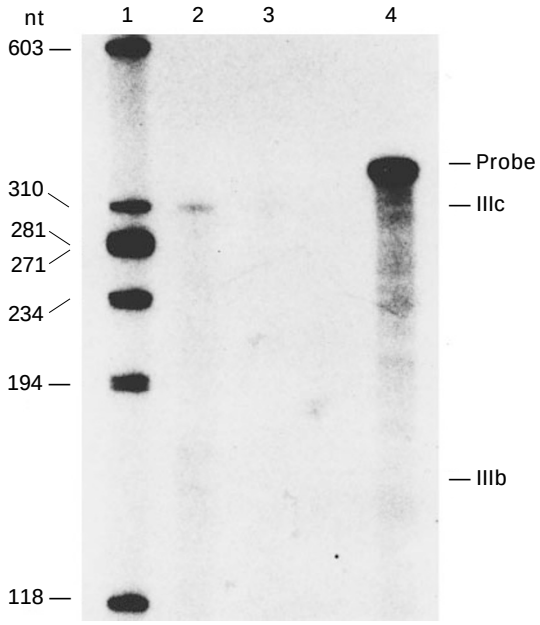

Figure 6 Ribonuclease protection assay for the IIIc and IIIb isoforms of FGFR-1. RNA samples were hybridized at 42°C overnight with α-^{32}P-CTP labeled antisense riboprobes (100 000 c.p.m./sample). After digestion of unprotected single-stranded RNA with Rnase for 30 min at 37°C, samples were separated on a 6% polyacrylamid/8 M urea gel and visualized by autoradiography (1 day exposure). Hybridization with the FGFR-1 probe (330 nt) revealed in protected bands of 301 nt for the IIIc and of 156 nt for the IIIb isoform. DNA ladder (lane 1), total RNA (20 μg) from COLO-357 cells (lane 2), yeast tRNA (20 μg; negative control; lane 3), yeast tRNA and probe without RNase (positive control; lane 4). The sizes of the DNA markers are indicated on the left

Biological actions of FGF-5 in COLO-357 cells

We next sought to determine whether FGF-5 modulated the growth of COLO-357 cells. FGF-5 exerted a dose-dependent increase on cell proliferation in the presence of 1 μg/ml heparin (Figure 8). In comparison to untreated controls, one-half maximal stimulation occurred at 19 pM and maximal stimulation at 111 pM enhancing the growth of COLO-357 cells by 52% ($P<0.0001$). In the absence of heparin FGF-5 was less effective and enhanced growth by 25% ($\pm$6% s.e.; $P=0.16$) at 5 nM. Heparin alone exerted a slight effect on cell growth (10%$\pm$7% s.e.; $P=0.67$).

To determine whether FGF-5 activated the mitogenic signaling cascade in COLO-357 cells, the effects of FGF-5 on mitogen-activated protein kinase (MAPK) activity was examined. To this end, an assay that specifically detected activated MAPK was performed (Figure 9). Activated MAPK (ERK-2) was already present in quiescent cells. Incubation with heparin alone (1 μg/ml) for 15 min or with FGF-5 (370 pM) for 5 min in the presence of heparin (1 μg/ml for 15 min) resulted in a slight increase in the levels of activated ERK-2. When the incubation period with FGF-5 was increased to 15 min there was an additional increase in the level of activated ERK-2 and activated ERK-1 proteins.

Discussion

FGF-5 is a heparin-binding glycoprotein with mitogenic activity toward fibroblasts and endothelial cells (Zhan *et al*., 1988; Bates *et al*., 1991). It is expressed in several cancer cell lines (Zhan *et al*., 1988; Altorki *et al*., 1993; Yoshimura *et al*., 1996), cultured teratocarcinoma cell aggregates (Herbert *et al*., 1991), human fibroblasts (Werner *et al*., 1991) and skeletal muscle

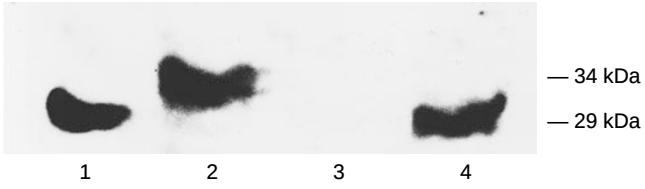

Figure 7 FGF-5 protein secretion of COLO-357 human pancreatic cancer cells. Two day conditioned serum-free medium and DMEM with/without FGF-5 were incubated with heparin sepharose (1 μl/ml) at 4°C for 24 h and submitted to 12% SDS–PAGE after washing and resuspension in 2$\times$ Laemmli buffer. Western blotting was carried out with specific human FGF-5 antibodies (0.8 μg/ml, exposure 60 min). Recombinant FGF-5 (100 ng; Santa Cruz Biotechnology) for Western blotting (lane 1); conditioned serum-free DMEM medium of COLO-357 cells (lane 2); serum-free DMEM medium (lane 3); serum-free DMEM medium with 750 ng recombinant FGF-5 (lane 4)

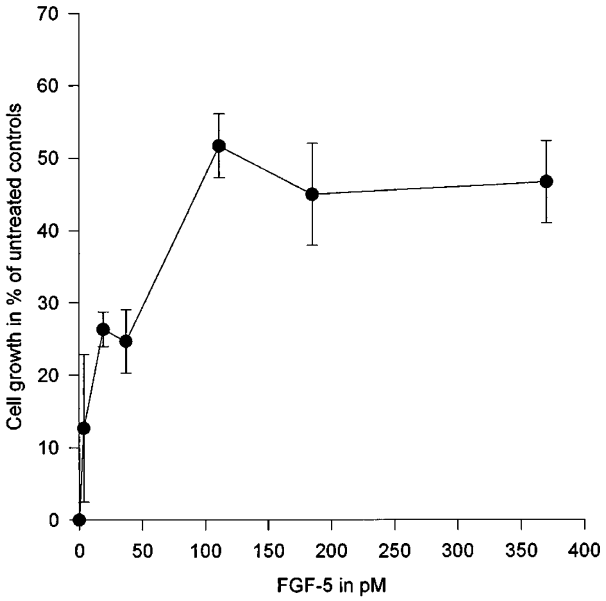

Figure 8 Effects of FGF-5 on pancreatic cancer cell growth. COLO-357 cells were grown in serum-free medium in the presence or absence of increasing concentrations of FGF-5, supplemented with 1 μg/ml Heparin. Data are expressed as percent increase above untreated controls and are the means $\pm$ s.e. of quadruplicate determinations from three separate experiments

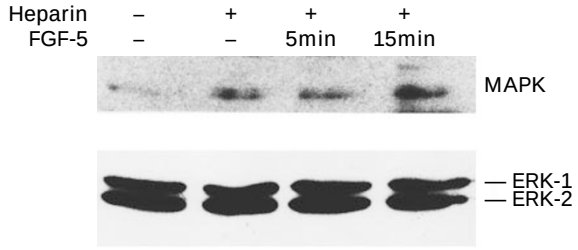

Figure 9 FGF-5 effect on mitogen-activated protein kinase (MAPK) in COLO-357 cells. After plating for 48 h in serum-free medium cells were incubated with or without 370 nM FGF-5 for the indicated times and in the absence or presence of 1 μg/ml Heparin for 15 min. Western blotting was carried out with specific anti-active MAPK antibodies (25 ng/ml, 60 min exposure, upper panel) following cell lysis. The membrane was stripped and reprobed with anti-ERK-1 antibodies cross-reactive with ERK-2 (0.1 μg/ml, 15 min exposure, lower panel) to confirm specificity and equal loading

cells (Hannon *et al*., 1996). However, its expression is limited at times during development (Goldfarb *et al*., 1991) and could so far only be detected in adult neurotropic (Goldfarb *et al*., 1991; Kitaoka *et al*., 1994) and myotropic tissues (Hughes *et al*., 1993). In the present study we found that FGF-5 is relatively abundant in the ductal cells of the normal pancreas, raising the possibility that FGF-5 may have a role in the regulation of pancreatic duct cell function. To our knowledge, this is the first report that describes FGF-5 expression in normal pancreatic tissues.

FGF-5 mRNA was overexpressed in a significant number of human pancreatic adenocarcinomas. Both the 4.0 and 1.6 kb FGF-5 transcripts, previously reported to be present in fibroblasts and cultured hepatoma and bladder carcinoma cell lines (Werner *et al*., 1991; Zhan *et al*., 1988), were overexpressed in the cancers. Immunostaining and *in situ* hybridization revealed the presence of FGF-5 protein and mRNA in duct-like cancer cells, as well as in adjacent fibroblasts and macrophages. In contrast, FGF-1 and FGF-2 are localized principally in the cancer cells (Yamanaka *et al*., 1993). This difference may be due, in part, to the fact that, unlike FGF-1 and FGF-2, FGF-5 is efficiently secreted (Zhan *et al*., 1988). However, the *in situ* hybridization results indicate that the presence of FGF-5 is the cancer-associated fibroblasts and infiltrating macrophages is also due to the synthesis of FGF-5 in these cell types. It is possible that FGF-5 synthesis in these cells is induced by the adjacent tumor cells inasmuch as FGF-5 was not detectable in fibroblasts from normal pancreatic tissues. In support of this hypothesis, FGF-5 mRNA expression in human fibroblasts is known to be induced by epidermal growth factor, transforming growth factor-α, and platelet-derived growth factor (Werner *et al*., 1991) and all these growth factors are overexpressed in human pancreatic cancer (Korc *et al*., 1992; Ebert *et al*., 1995). In view of the fact that the pancreas is rich in heparan sulfates (Gambarini *et al*., 1993), which are known to enhance FGF-5 activity (Kaplow *et al*., 1990; Clements *et al*., 1993), our observations raise the possibility that FGF-5 secreted by fibroblasts and macrophages adjacent to the cancer cells may participate in a paracrine manner to promote pancreatic cancer cell growth, and that cancer cell derived FGF-5 may act in an autocrine manner to directly enhance tumor growth.

Several observations in the present study support the hypothesis that there is a potential for FGF-5-dependent autocrine activation in pancreatic cancer. Thus, COLO-357 cells expressed and released FGF-5 into the medium, and exogenous FGF-5 promoted mitogenic signaling in these cells as evident by its actions on cell proliferation and MAPK activation. In addition, COLO-357 cells expressed variable levels of all four high-affinity FGF receptors. It has been established that the presence of an intron-exon boundary in the third Ig-like domain (domain III) allows for the generation of three alternative variants having domains termed IIIa, IIIb and IIIc. The IIIa splice variant yields a secreted FGF receptor that is derived of any signaling capacity. The expression of the IIIb splice variant is believed to be restricted to epithelial cell types whereas the expression of the IIIc splice variant is believed to be restricted expressed to mesenchymal cell types (Avivi *et al*., 1993; Gilbert *et al*., 1993; Orr-Urtreger *et al*., 1993; Yan *et al*., 1993; Alarid *et al*., 1994). Surprisingly, in the present study we found that COLO-357 cells express the IIIc splice variant of FGFR-1, but the IIIb variant of FGFR-2. The presence of the IIIc isoform of FGFR-1 in COLO-357 cells, which are of epithelial lineage, raises the possibility that malignant transformation in pancreatic cancer leads to dedifferentiation and subsequent aberrant expression of the IIIc isoform. Inasmuch as FGF-5 preferentially activates the IIIc variant form of FGFR-1 (Ornitz *et al*., 1996; Kanai *et al*., 1997), these observations suggest that the mitogenic effects of FGF-5 in COLO-357 cells are mediated via the IIIc variant of FGFR-1 and strengthen the concept that FGF-5 may contribute to the growth of pancreatic cancer cells *in vivo*.

Heparin enhanced the mitogenic activity of FGF-5 in COLO-357 cells. It is possible that heparin exerts this effect by increasing the affinity of FGF-5 to its receptors (Kaplow *et al*., 1990) or by attenuating FGF-5 degradation (Bates *et al*., 1991). In addition, heparin alone exerted a slight but not significant stimulatory effect on the growth of COLO-357 cells. This slight stimulatory effect heparin alone could be due to activation of FGFR-4, whose kinase activity is known to be stimulated by heparin (Gao and Goldfarb, 1995).

In summary, FGF-5 is overexpressed in pancreatic cancer, both in the cancer cells and surrounding macrophages and fibroblasts. Together with the observation that FGF-5 is produced and released by a cultured human pancreatic cancer cell line, and activates mitogenic signaling in these cells, these findings suggest that FGF-5 participates in autocrine and paracrine pathways promoting pancreatic cancer cell growth *in vivo*. It is possible, therefore, that modalities aimed at abrogating FGF-5 dependent pathways may ultimately have a role in pancreatic cancer therapy.

Materials and methods

Tissue samples

Normal human pancreatic tissue samples of six female and eight male organ donors with a median age of 26 years (range: 2–54) were obtained through a donor program. Pancreatic adenocarcinoma tissues were obtained from ten female and six male patients with a median age of 64 years (range: 44–77) undergoing surgery. Samples were either immediately frozen upon surgical removal in liquid nitrogen and stored at −80°C until RNA extraction or fixed in Bouin's solution for 18–20 h and embedded in paraffin for histological analysis. Studies involving human tissues were approved by the Ethics Committee of the University of Ulm, Germany and the Human Subjects Committee of the University of California, Irvine, CA.

Preparation of the FGF-5 cDNA

A 306 bp *Bam*HI/*Hind*III cDNA fragment, corresponding to nt 128 to 433 of the human FGF-5 cDNA sequence (Haub *et al*., 1990), was generated by RT–PCR from human placenta RNA. The primers used for the FGF-5 cDNA preparation were: 5′-CTC-GGATCCGCGGCTG-GAAGAATGA corresponding to nt 128 to 143 of the human FGF-5 cDNA, and 5′-GCG-AAGCTTACC-GATGCCCACTCTGCA corresponding to nt 416 to 433. The 306 bp *Bam*HI/*Hind*III FGF-5 cDNA fragment was subcloned into pGEM-7Zf (Promega, Madison, WI) and its authenticity confirmed by sequencing.

Northern blot analysis

Northen blotting was carried out as previously described (Korc *et al*., 1992). Total RNA was extracted, size-fractionated, electrotransferred, and hybridized under high stringency conditions. A 190 bp *Bam*HI mouse 7S cDNA was used to confirm equivalent loading of lanes (Korc *et al*., 1992). cDNAs were labeled with α-^{32}P-dCTP (3000 Ci/mmol) by random priming prior to hybridization. The resulting autoradiographs were scanned and the ratio of the optical densities of the RNA levels (FGF-5 : 7S) was calculated for each sample. In addition to the FGF-5 cDNA, a 435 bp *Eco*RI/*Pst*I human FGFR-1 cDNA (Dionne *et al*., 1990), a 1.2 kb *Sac*I/*Bam*HI human FGFR-2 cDNA (Dionne *et al*., 1990), a 2.5 kb *Eco*RI human FGFR-3 cDNA (ATTC), and a 2.7 kb *Eco*RI human FGFR-4 cDNA (ATTC) were used for hybridization.

Ribonuclease protection assay

Total RNA (20 μg) was incubated overnight at 42°C with α-^{32}P-CTP-labeled FGFR-1 and FGFR-2 riboprobes (100 000 c.p.m. sample) using an RPA II kit (Ambion, Austin, TX). After hybridization, single-stranded RNA was digested for 30 min at 37°C. Samples were then separated on a 6% polyacrylamide-8 M urea gel and subjected to autoradiography. The antisense riboprobes were synthesized with T7 RNA polymerase (Boehringer, Mannheim, Germany) using *Hind*III linearized plasmids. A *Hind*III/*Bam*HI cDNA fragment of the FGFR-1 (Dionne *et al*., 1990) that was subcloned into pGEM-3Z and included the entire Ig-domain III (nt 868 to nt 1177) was used to generate the antisense riboprobe of FGFR-1). The 330 nt *Hind*III antisense riboprobe results in protected fragments of 301 nt for the IIIc (nt 868 to nt 1168) and of 156 nt for the IIIb (nt 868 to nt 1023) isoform, respectively. A *Hind*III/*Bam*HI cDNA fragment of the FGFR-2 (Dionne *et al*., 1990) that was subcloned into pGEM-3Z and included the entire Ig-domain III (nt 937 to nt 1270) was used to generate the antisense riboprobe for FGFR-2. The 364 nt *Hind*III antisense riboprobe results in protected fragments of 334 nt of the IIIc (nt 937 to nt 1270) and of 182 nt for the IIIb (nt 937 to nt 1118) isoform, respectively. These probes were a gift of Prizm Pharm., San Diego, CA. Yeast tRNA was used as a negative control.

Immunohistochemistry

Paraffin-embedded 4 μm tissue sections were immunostained using the streptavidin-peroxidase technique (Kirkegaard and Perry Laboratories Inc, Gaithersburg, MD). After deparaffinization, endogenous peroxidase activity was blocked by incubation for 30 min with 0.3% hydrogen peroxide in methanol. Tissue sections were incubated for 15 min at 23°C with 10% normal rabbit serum and then incubated with a highly specific goat polyclonal anti-human FGF-5 antibodies (1 : 100 in PBS containing 1% BSA) at 4°C for 16 h. This antibody specifically detects an epitope corresponding to amino acids 249–267 mapping at the carboxy terminus of the FGF-5 precursor and is not cross-reactive with any other member of the FGF family as determined by immunoblotting (Santa Cruz Biotechnology, Santa Cruz, CA). Bound antibodies were detected with biotinylated rabbit anti-goat IgG secondary antibodies and streptavidin-peroxidase complex (Kirkegaard and Perry), using diaminobenzidine tetrahydrochloride (DAB) as the substrate. For insulin staining guinea pig polyclonal anti-porcine insulin antibodies (1 : 1000 in PBS containing 1% BSA; DAKO Corp, Carpinteria, CA), cross-reactive with human insulin, and biotinylated goat anti-guinea pig IgG secondary antibodies were used after incubation with 10% normal goat serum. Sections were counter-stained with Mayer's hematoxylin. Omission of primary antibodies or incubation in the presence of non-immunized goat serum instead of primary antibodies did not yield any immunoreactivity.

In situ *hybridization*

Tissue sections were placed on 3-aminopropylmethoxysilane-coated slides, deparaffinized, incubated sequentially at 23°C for 20 min with 0.2 N HCl and for 15 min with 20 μg/ml proteinase K at 37°C. The sections were then post-fixed for 5 min in PBS containing 4% paraformaldehyde, and quenched twice with glycine (2 mg/ml) in PBS.

The sections were then incubated in 50% (v/v) formamide/ 2 × SSC for 1 h. The hybridization buffer contained 0.6 M NaCl, 1 mM EDTA, 10 mM Tris-HCl (pH 7.6), 0.25% SDS, 200 μg/ml yeast tRNA, 1 × Denhart's solution, 10% dextran sulfate, 40% formamide and 50 ng/ml digoxigenin-labeled FGF-5 riboprobe. The riboprobe was prepared by digesting the 306 bp *Bam*HI/*Hind*III FGF-5 cDNA fragment subcloned into pGEM-7Zf and labeled with Genius 4 DIG RNA labeling kit (Boehringer, Mannheim). To initiate the hybridization reaction, 100 μl of the hybridization buffer was applied to each section, followed by incubation in a moist chamber for 16 h at 42°C. The sections were then washed sequentially with 50% formamide/2 × SSC for 30 min at 50°C, 2 × SSC for 20 min at 50°C, 0.2 × SSC for 20 min at 50°C, and 0.1 × SSC for 30 min at 50°C. For immunological detection, Genius 3, non-radioactive nucleic acid detection kit was used. The sections were washed briefly with buffer 1 solution (100 mM Tris-HCl and 150 mM NaCl, pH 7.6) and incubated with 1% (w/v) blocking reagents in buffer 1 solution for 60 min at 23°C. After washing briefly with buffer 1, the sections were incubated for 30 min at 23°C with a 1/2000 dilution of alkaline phosphatase conjugated polyclonal sheep anti-digoxigenin Fab fragment containing 0.2% Tween 20. The sections were then washed twice for 15 min at 23°C with buffer 1 solution containing 0.2% Tween 20 and equilibrated with buffer 3 (100 mM Tris-HCl, 100 mM NaCl, 50 mM $MgCl_2$, pH 9.5) for 2 min. The sections were then incubated with color solution containing nitroblue tetrazolium and X-phosphate in a dark box for 2–3 h. After the reaction was stopped with TE buffer, the sections were mounted in aqueous mounting medium.

Purification and immunoblotting of FGF-5 protein

FGF-5 protein purification was carried out as previously described (Bates *et al*., 1991). COLO-357 cells were grown in complete medium containing 10% FBS to 70% confluency in T75 flasks. After washing twice with Hank's buffered saline solution, cells were incubated for 48 h in 18 ml of serum-free medium containing 50 μg/ml Aprotinin, 10 μg/ml Leupeptin, 10 μg/ml Pepstatin A, and 10 μg/ml Benzamidine. The conditioned serum-free medium of three flasks was collected (50 ml) and incubated at 4°C overnight after adjusting the pH to 7.4 and adding 50 μl slurry heparin sepharose CL-6B (Pharmacia Biotech, Piscataway, NJ). Serum-free medium (50 ml) with inhibitors served as negative control and serum-free medium (50 ml) with inhibitors and 750 ng recombinant FGF-5 (Sigma) as positive control. The beds were collected by centrifugation, washed 3 × with 0.45 M NaCl/20 mM Tris-HCl (pH 7.4) and resuspended in 2 × Laemmli buffer. Samples were boiled for 5 min and then subjected to 12% SDS–PAGE and Western blotting. After blocking with 5% milk in TTBS for 60 min at 37°C the membranes were blotted with primary goat anti-human FGF-5 polyclonal antibodies overnight at 4°C (Santa Cruz Biotechnology, Santa Cruz, CA) and secondary rabbit anti-goat horseradish conjugated antibodies for 60 min at 25°C (Sigma, St Louis, MO). Bound antibodies were visualized using enhanced chemoluminescence substrate (Pierce, Rockford, IL).

Cell culture and growth assay

COLO-357 human pancreatic cancer cell line was obtained from RS Metzgar at Duke University and grown in Dulbecco's modified Eagle's medium at 37°C in humidified air with 5% CO_2. Media contained 100 U/ml penicillin, 100 μg/ml streptomycin and 10% fetal bovine serum, termed as complete medium. To assess the effects of FGF-5 on pancreatic cancer cell growth, the 3-(4,5-methylthiazol-2-yl)-2,5-diaphenyltetrazolium bromide (MTT) colorimetric assay was used (Mosmann, 1983). Cells were plated at a density of 15 000 cells per well in 96 well plates and incubated for 24 h in complete medium. After washing with Hank's buffered saline solution, cells were incubated overnight for 48 h in the absence or presence of human recombinant FGF-5 (Sigma, St Louis, MO) in medium containing 0.1% bovine serum albumin, 5 mg/l transferrin, 5 μg/l selenious acid, and antibiotics, termed serum-free medium. Medium was replaced after 24 h using the same concentrations and 62.5 μg/well of MTT was added 4 h prior to termination. After the aspiration of the medium the MTT dye crystals were dissolved with acidified isopropanol and the optical density was measured using an ELISA plate reader (Molecular Devices Corp, Menlo Park, CA). Data were expressed as percent stimulation of untreated control cell growth. Results are plotted as means ± s.e. of quadruplicate determinations of each test point from three separate experiments.

Mitogen-activated protein kinases (MAPK) assay

A specific and sensitive assay for the detection of activated MAPK with anti-active MAPK rabbit polyclonal antibodies (Promega, Madison, WI), raised against the dually-phosphorylated Thr/Glu/Tyr region within the catalytic core of the active form of the MAPK enzymes, was used. Cells were plated into 6-well plates and incubated 24 h in complete medium and 48 h in serum-free medium with changes every 24 h. The cells were then stimulated with FGF-5, washed twice with ice-cold PBS, and lysed in buffer containing 1% NP-40, 0.5% Sodium-Deoxycholat, 0.1% SDS, 150 mM NaCl, 10 mM Sodium-Phosphate, 2 mM EDTA, 50 mM Sodium-Fluoride, 5 mM Sodium-Orthovanadate, 50 μg/ml Aprotinin, 10 μg/ml Leupeptin, 10 μg/ml Pepstatin A, 10 μg/ml Benzamidine and 1 mM PMSF. Cell extract protein samples (60 μg) were subjected to 10% SDS–PAGE. Western blot analysis was carried out according to the protocol of the manufacturer (Promega, Madison, WI). Bound antibodies were visualized using enhanced chemoluminescence substrate (Pierce, Rockford, IL). To confirm equal loading membranes were stripped for 30 min at 50°C in buffer containing 2% SDS, 62.5 mM Tris (pH 6.7) and 100 mM 2-mercaptoethanol and blotted with anti-ERK1 (cross-reactive with ERK-2; Santa Cruz Biotechnology, Santa Cruz, CA).

Statistics

Data are expressed as median and range (Northern blotting) and mean ± s.e. (MTT assay). The Mann-Whitney Rank Sum Test was used to investigate the differences in median mRNA expression and the Student's *t*-test to assess the effect of FGF-5 on cell growth. A P value < 0.05 was taken as level of significance.

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