Advances in Brief

Aberrant Expression of Type I Fibroblast Growth Factor Receptor in Human Pancreatic Adenocarcinomas

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Abstract

Acidic and basic fibroblast growth factors are mitogenic polypeptides that are overexpressed in pancreatic cancer. To determine whether fibroblast growth factors may exert direct effects on pancreatic cancer cells in vivo, we compared the expression of the high-affinity type I fibroblast growth factor receptor (FGFR-1) in human pancreatic tissues. In the normal pancreas, FGFR-1 immunostaining was seen mainly in acinar cells. In pancreatic cancers, FGFR-1 was abundant in ductal-like cancer cells which also exhibited many FGFR-1 mRNA in situ hybridization grains. Analysis by the polymerase chain reaction and RNase protection revealed that the 2-immunoglobulin-like and the 3-immunoglobulin-like forms of FGFR-1 were expressed in all tissue samples, and that the 2-immunoglobulin-like form was overexpressed in the cancer tissues by comparison with the normal tissues. These findings suggest that the 2-immunoglobulin-like form of FGFR-1 may contribute to aberrant autocrine and paracrine pathways in pancreatic cancer.

Introduction

The FGFR family consists of a number of homologous polypeptide growth factors that have an affinity for heparin and glycosaminoglycans and that participate in the regulation of biological processes in numerous cell types (1-4). This family currently includes aFGF, bFGF, keratinocyte growth factor, FGF-4 (Kaposi FGF), FGF-5, FGF-6, the gene product of int-2, androgen-induced growth factor, and FGF-9 (1-6). FGFs are mitogenic, promote angiogenesis and chemotaxis, and participate in the regulation of cellular differentiation and tissue repair. aFGF and bFGF are overexpressed in a significant proportion of pancreatic cancers, raising the possibility that FGFs may exert autocrine and paracrine effects in these cancers. It is not known, however, whether FGF receptors are expressed in these tumors.

To date, five distinct high-affinity FGF receptors, designated as FGFR-1, -2, -3, -4, and -5 (the latter is also known as flg-2) have been cloned and sequenced (7). All five receptors contain an intracellular domain that possesses intrinsic tyrosine kinase activity but which is separated into two contiguous regions, and an extracellular domain that has 3 immunoglobulin-like (3-Ig) regions (7). As a result of alternative splicing, in some cell types FGFR-1 and -2 exhibit a number of variants, including the loss of the first immunoglobulin region and the generation of a 2-Ig form (7). In the present study, we employed immunohistochemical and in situ hybridization techniques to examine FGFR-1 localization and expression in normal and malignant human pancreatic tissues. We also used a PCR-based strategy and a sensitive RNase protection assay to determine whether there is differential expression of the 3- and 2-Ig forms of FGFR-1 in the normal and cancerous tissues. We now report that human pancreatic adenocarcinomas express high levels of the 2-Ig form of FGFR-1.

Materials and Methods

Monoclonal anti-FGFR-1 antibody, previously shown to be useful for immunohistochemical analysis (8), was purchased from Chemicon Int., Inc. (Temecula, CA); biotinylated goat IgG from Kirkegaard & Perry Laboratories (Gaithersburg, MD); GeneScreen membranes from New England Nuclear (Boston, MA); pBluescript IIISK+ vector from Stratagene, LaJolla, CA; pGEM3ZF vector from Promega Biotech., Madison, WI); SuperScript reverse transcriptase from BRL, Gaithersburg, MD; Taq polymerase from Perkin-Elmer Cetus Corp., Norwalk, CT; intensifying screens from Eastman Kodak Co. (Rochester, NY); [α-32P]dCTP (3000 Ci/mmol) and [α-35S]UTP (1000 Ci/mmol) from Amersham, Inc. (Arlington Heights, IL). Human FGFR-1 pCD115 cDNA (9) was a gift from Dr. M. Jaye, Rorer Central Research, Inc. (King of Prussia, PA).

Pancreatic cancer tissues were obtained from 5 patients (2 female, 3 male) undergoing pancreatic cancer surgery. Normal pancreatic tissues were obtained from 5 individuals (2 female, 3 male) through an organ donor program. Immediately following surgical removal, all tissue samples were either frozen in liquid nitrogen. The tumor samples were classified as pancreatic ductal adenocarcinomas according to the TNM classification for pancreatic tumors (10). All studies were approved by the Human Subjects Committee of the University of California, Irvine, CA.

Immunohistochemistry. Paraffin-embedded tissue sections were subjected to immunostaining by using a streptavidin-peroxidase method. After blocking endogenous peroxidase activity with 0.3% hydrogen peroxide in methanol, the sections were incubated for 40 min at 23°C with 10% normal horse serum and overnight at 4°C with anti-FGFR-1 (1:50 dilution) antibodies (11). Bound antibody was detected with a biotinylated anti-mouse IgG secondary antibody and streptavidin-peroxidase complex, using diaminobenzidine tetrahydrochloride as the substrate. Counterstaining was performed with Mayer’s hematoxylin. Incubation of sections with nonimmunized mouse IgG instead of a primary antibody failed to reveal any immunostaining.

In Situ Hybridization. Pancreatic tissue sections were deparaffinized, treated with 1 μg/ml proteinase K, incubated for 3 h at 37°C in hybridization solution, and hybridized overnight at 50°C, using 200,000 cpm of the FGFR-1 riboprobe and 50 μg of yeast tRNA (11). The sections were then digested with RNase A, washed, dehydrated, coated with NTB2 autoradiography emulsion, and exposed for 6 days. After development the slides were counterstained with Mayer’s hematoxylin. Pretreatment of the slides with excess RNase abolished the hybridization signal. The antisense riboprobe consisted of a 435-base pair EcoRI/Pstl fragment of the human FGFR-1 cDNA (9) which was subcloned into the pGEM3ZF vector and labeled with [α-35S]UTP (11). The corresponding sense probe failed to produce a signal.

PCR Analysis. Oligonucleotide primers were synthesized on an Applied Biosystem 391 DNA synthesizer and purified by electrophoresis (12). Two primer sequences (EC1 and EC2) were used to amplify the extracellular domain of FGFR-1 (Fig. 1):

EC1 - 5'-CGCTCTAGAGCAGAACTGGGATGTGGGGCTG-3'
EC2 - 5'CTCGGATCCAGGGCTTCCAGGAACGGTCT-3'

cDNAs were synthesized from total RNA (2 μg/sample) isolated from 5 human pancreatic tissues.
pancreatic cancers and 5 normal pancreatic tissues, using oligo(dT) and reverse transcriptase (12). Following inactivation, 2 μl of the reaction mixture were incubated in buffer containing 1.25 mM concentrations each of dATP, dCTP, dGTP, and dTTP, 650 nM each of oligonucleotide primers, and 10% dimethyl sulfoxide in buffer consisting of 16.6 mM (NH₄)₂SO₄, 67 mM Tris-HCl (pH 8.8), 6.7 mM MgCl₂, 10 mM β-mercaptoethanol, and Taq polymerase (12). Reaction cycles consisting of 1 min at 94°C, 1 min at 61°C, and 1 min at 72°C were repeated 35 times. The PCR products were size fractionated on 1% agarose gels, blotted onto nylon membranes, and hybridized by the method of Southern (13). A 1.2-kilobase BamHI fragment of FGFR-1 cDNA was random labeled with [³²P]dCTP and used to detect all extracellular forms of FGFR-1. Identity of the PCR products representing the 3-lg and 2-lg forms of FGFR-1 was confirmed by sequencing after subcloning into pGEM3Zf.

**RNase Protection Assay.** A cDNA encoding sequence corresponding to a 277-base pair region (nucleotides -11 to 266) of the 5' end of FGFR-1 cDNA (9), was produced by PCR amplification of the pCD115 FGFR-1 cDNA (9). For this purpose, the EC1 primer (Fig. 1) was used with primer EC3: 5'-CGTAAGCTTCTGTGATGCGGGTGCG-3'. The cDNA was subcloned into a Bluescript vector and used to generate the antisense riboprobe. Authenticity of the PCR product was confirmed by sequencing. RNase protection was performed with 10 μg total RNA from each tissue sample. Following overnight hybridization at 42°C with the ³²P-labeled riboprobe, single-stranded RNA was digested for 30 min at 37°C (14). Samples were then subjected to denaturing polyacrylamide gel electrophoresis with the use of 6% polyacrylamide and 8 M urea, followed by autoradiography (14). RNase protection performed with yeast tRNA alone failed to produce a signal.

**Statistical Analysis.** Whenever indicated, data were analyzed by Student's t test.

**Results**

In the normal pancreas there were focal areas of FGFR-1 immunostaining in the cytoplasm of many acinar cells and weak or no immunostaining in the ductal cells (Fig. 2A). Similarly, FGFR-1 mRNA in situ hybridization grains were present infrequently and at low levels in the ductal cells, but were more abundant in many of the acinar cells (Fig. 3A). In the pancreatic cancers there was intense FGFR-1 immunostaining in many of the ductal-like cancer cells, especially in the supranuclear regions (Fig. 2B). Furthermore, FGFR-1 mRNA in situ hybridization grains were present at high levels in many of these cells (Fig. 3B). Although the stroma was devoid of any FGFR-1 immunoreactivity in both the normal and cancerous tissues, FGFR-1 in situ hybridization grains were seen occasionally in the stromal fibroblasts in both groups of tissues.

Southern blot analysis of the PCR products obtained by using pancreas-derived cDNAs and FGFR-1-specific oligonucleotide primers revealed the presence of three anticipated bands (Fig. 4A). The upper and lower bands corresponded to the 3-lg and 2-lg forms of FGFR-1, respectively, whereas the intermediate band represented the 2-lg form containing an insert (2-lg + insert) in place of the deleted immunoglobulin loop (15). In the normal tissues, the 3-lg form of FGFR-1 was either present in relatively equal proportions with the 2-lg form or was slightly more prominent than the 2-lg form. In contrast, in the cancer tissues, the 2-lg form was clearly the most prominent moiety. Furthermore, densitometric analysis indicated that the intensity of the 2-lg form was 4-fold greater in the cancer tissues by comparison with the normal pancreas (P < 0.005), whereas the intensity of the bands representing the 3-lg and intermediate forms of FGFR-1 were similar in both groups.

All three PCR products were obtained in the same reaction vessels using the same RNA samples and primers. Therefore, it is unlikely that the results of the PCR analysis were due to variations in RNA purity and quality, buffer conditions, or enzyme potency. Nonetheless, to exclude the possibility that the abundance of the 2-lg form of FGFR-1 in the tumors was due to a PCR-associated artifact, an RNase protection assay was carried out next (Fig. 4B). Again, the transcript corresponding to the 2-lg form of FGFR-1 was more prominent in the cancer tissues, densitometric analysis revealing a 5-fold increase by comparison with the normal controls (P < 0.01). In contrast, the levels of the transcripts corresponding to the 3-lg form of FGFR-1 were similar in both groups.
Discussion

The human pancreas consists of an exocrine component represented by acinar cells that secrete digestive enzymes and ductal cells that produce bicarbonate-rich fluid, and an endocrine component represented by hormone-secreting cells that are grouped into islets which are dispersed throughout the exocrine tissue. Malignant neoplasms may arise from the islet, acinar, or ductal components of the pancreas. Although the acinar cell represents more than 90% of the pancreatic mass, the most common and deadliest of these neoplasms is the pancreatic ductal adenocarcinoma. The overall 1- and 5-year survival rates in this malignancy are less than 10 and 0.5%, respectively (16). Histologically, the cancer cells in these carcinomas form duct-like structures in association with an intense desmoplastic reaction and destruction of the normal pancreatic architecture (17).

In the present study we found that the pattern of distribution of FGFR-1 was dramatically different in the normal and malignant tissues. In the normal pancreas, FGFR-1 immunostaining was observed mainly in the acinar cells, whereas in the pancreatic cancers intense FGFR-1 immunostaining was evident in the ductal-like tumor cells. By in situ hybridization, there was a moderate level of expression of FGFR-1 mRNA in the acinar cells. In contrast, there was an abundance of this mRNA moiety in the ductal-like cancer cells. These cells were also recently shown to exhibit strong immunoreactivity with specific anti-aFGF and anti-bFGF antibodies, and to overexpress aFGF and bFGF mRNA by in situ hybridization (11). Thus, there is concomitant expression of high levels of FGFs and FGFR-1 in pancreatic cancer cells, which may allow for excessive autocrine and paracrine growth stimulation. Furthermore, the presence of intense FGFR-1 immunoreactivity in the cytoplasm of the cancer cells raises the possibility that FGFs may activate FGFR-1 without first being released from the cell, thereby acting through an intracrine mechanism.

In view of the differential splicing of the FGFR-1 gene and the generation of forms that have 2 or 3 immunoglobulin-like regions in the extracellular domain (9), we also used a PCR-based strategy and a sensitive RNase protection assay to analyze FGFR-1 expression. In both assays, there was a disproportionate abundance of the 2-Ig form of the receptor in the carcinomas. In contrast, both forms were either present in relatively equal proportions in the normal tissues, or there was a predominance of the 3-Ig form. Although expression of the 2-Ig form of FGFR-1 is not restricted to cancer cells in the pancreas, these differences raise the possibility of abnormal regulation of the splicing of FGFR-1 mRNA in the ductal-like cancer cells, and suggest that the 2-Ig form of the receptor is overexpressed in pancreatic cancers. aFGF and bFGF canbind and activate both forms of FGFR-1 (7). However, the presence of bFGF in the cancer cells, but not aFGF, is associated with shorter post-operative patient survival.4 It is conceivable, therefore, that the 2-Ig form of FGFR-1 in the cancer cells is preferentially activated by bFGF. FGFs have also been shown to exert additive and/or synergistic effects on the proliferative actions of EGF (18). Inasmuch as pancreatic cancer cells overexpress the EGF receptor, EGF, and transforming growth factor α (11), it is possible that the abundance of FGFR-1 and FGFs in these cells may also contribute to excessive activation of the EGF receptor autocrine cycle. Pancreatic cancer cells also overexpress c-erb-B2 and the type II transforming growth factor β receptor (19, 20). Therefore, there may also be potential interactions between the 2-Ig form of FGFR-1 and these other
receptors and their ligands, which may combine to give pancreatic cancer cells a distinct growth advantage.

References
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