Association of Increased Immunostaining for Inducible Nitric Oxide Synthase and Nitrotyrosine With Fibroblast Growth Factor Transformation in Pancreatic Cancer

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Background: Despite recognition of the devastating malignant potential of pancreatic cancer, the exact pathophysiological events contributing to tumor growth, vascular invasiveness, and hepatic metastasis remain to be elucidated.

Methods: Twelve human pancreatic adenocarcinomas were evaluated using immunohistochemical and in situ hybridization techniques for the appearance of the angiogenic and neurogenic growth factors, acidic fibroblast (FGF-1) and basic fibroblast growth factor (FGF-2), and their high-affinity receptors. Since FGF biological processes appear to be regulated by oxidant stress, tumors were examined further for the immunopappearance of inducible nitric oxide synthase (iNOS) and nitrotyrosine.

Results: Compared with normal human pancreatic tissue, tumor specimens exhibited varying levels of enhanced staining for FGF ligands and receptors. The increased appearance of FGF-1 and FGF-2 proteins was accompanied by increased detection of messenger RNA encoding each growth factor. In addition, these pancreatic tumors demonstrated the overexpression of iNOS and immunostaining of nitrotyrosine compared with normal pancreatic tissue.

Conclusions: The enhanced expression of FGF and FGF receptors suggests that these polypeptide mitogens may serve as important mediators of growth and of angiogenic and metastatic responses associated with pancreatic tumors, not seen in normal pancreatic tissue. Furthermore, we provide the first indication of increased expression of iNOS and protein tyrosine nitration, thereby predicting the potential involvement of oxidant stress during development and progression of pancreatic adenocarcinoma.

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Human pancreatic adenocarcinoma remains one of the most difficult cancers to treat, as evidenced by little improvement in survival even with the addition of chemotherapy or radiation therapy. During the past 15 years, significant progress has occurred in the clinical management of pancreatic cancer due to increased early diagnosis and improved statistics on operative mortality (25% in the 1960s vs ≤5% more recently). However, the 1997 cancer statistics show that little improvement in overall patient survival has transpired from such progress. Specifically, in 1997 there were 27,600 new cases of pancreatic cancer with 28,100 deaths due to pancreatic cancer, with an overall 5-year survival of less than 5%.1,2 The simple conclusion is that current detection and therapy are not satisfactory solutions to the unremitting problem of pancreatic cancer.

Recent advances in molecular biology have advanced our understanding of the pathogenesis of neoplasia. Collectively, these efforts have established potential molecular mechanisms associated with tumor development and progression that provide a rational basis for early detection techniques and interventional treatment. Proposed pathophysiological mechanisms associated with pancreatic adenocarcinoma have suggested an important role for numerous polypeptide growth factors and cytokines competent to induce pancreatic cell responses during development, physiologic processes, and tumorigenesis.3-5 For instance, several recent studies have suggested a role for acidic fibroblast (FGF-1) and basic fibroblast growth factor (FGF-2) during progression of pancreatic adenocarcinoma.6-9

Both growth factors are prototypical members of a family of 10 structurally related polypeptides that serve as potent mitogens for neuroectoderm-, ectoderm-, and mesoderm-derived cells in vitro and as hormonal inducers of neurogenic, angiogenic, and wound repair mechanisms in

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MATERIALS AND METHODS

TISSUE SPECIMENS

We analyzed pancreatic cancers from 12 patients who underwent pancreaticoduodenectomy for biliary obstruction or pancreatic adenocarcinoma. Three samples of healthy pancreas obtained from heart-beating donors deemed unsuitable for transplantation were used as control samples.

IMMUNOLOCALIZATION

Thin sections (5 µm) of each formalin-fixed, paraffin-embedded tissue were cut onto commercially available slides (Superfrost/plus; Fisher Scientific, Pittsburgh, Pa), deparaffinized (45 minutes at 55°C), and cleansed through a series of xylene and alcohol washes. Dehydrated tissues were examined using light microscopy, after hematoxylin-eosin staining. After exhaustion (3 minutes) of endogenous peroxidase activity with 3% (volume-volume ratio) hydrogen peroxide in methanol and blocking (20 minutes) in 1% (weight-volume ratio) bovine serum albumin, tissues were incubated with polyclonal antibodies (Santa Cruz Labs, Santa Cruz, Calif) directed against FGFR-1 (1:50) and FGFR-2 (1:50). According to the manufacturer, both of these polyclonals were raised against a unique peptide sequence and demonstrated no crossreactivity with other members of the FGFR family. In addition, preincubation (16 hours at 4°C) of the individual antibodies with 100-fold molar excess of the specific peptides (Santa Cruz Labs) completely blocked immunostaining. Fixed sections were also incubated with affinity-purified anti–FGF-1 and anti–FGF-2 (5 µg/mL), as described previously.46-48 As a control for FGF-1 and FGF-2 monospecificity, the affinity-purified antibodies were preincubated (16 hours at 4°C) with 100-fold molar excess of recombinant FGF-1 or FGF-2, a process that completely abrogated immunostaining. The involvement of oxidant stress was characterized using commercially available monoclonal anti-iNOS (1:250) (Upstate Biotechnology, Inc, Lake Placid, NY) and polyclonal anti-nitrotyrosine (1:450) (a generous gift from J. Beckman, PhD, University of Alabama at Birmingham). The specificity of protein tyrosine nitration was demonstrated by preincubating (30 minutes at 25°C) the polyclonal antibody with 10 mmol/L 3-nitrotyrosine (Sigma-Aldrich Corp, St Louis, Mo), which prevented all immunostaining.

All primary antibodies were incubated (45 minutes at 37°C) in a humidified chamber. Negative controls included incubation of fixed sections with preimmune serum and deletion of the primary antibody, a process that completely prevented immunostaining. Antibody binding was detected with a Quick Staining Kit (Dako Corp, Carpenteria, Calif) using swine anti-rabbit or swine anti-mouse horseradish peroxidase–conjugated secondary antibodies. Peroxidase-stained sections were developed with 0.5 mg/mL 3,3′-diaminobenzidine and counterstained with Mayer hematoxylin stain (Sigma-Aldrich Corp). Following chromogenic development of stained samples, slides were dehydrated, coverslips were applied using Permount (Fisher Scientific, Fair Lawn, NJ), and positive brown staining was evaluated using light microscopy.

All sections were examined and immunohistochemical staining was assessed by 4 independent investigators (S.M.V., M.G., C.E., and J.A.T.) unaware of control vs cancer specimens. The following scores were assigned to each specimen according to the intensity of staining: 3+ (intense), 2+ (moderate), 1+ (minimal), and 0 (none). Five separate areas for each specimen were evaluated, and the total cumulative score was divided by 5 to obtain an average grade.

IN SITU HYBRIDIZATION

Detection of endogenous FGF-1 and FGF-2 messenger RNA (mRNA) was performed on routine paraffin sections essentially as described.46-48, 50 Briefly, DNA restriction fragments containing the complete human complementary DNA sequence encoding FGF-1 or FGF-2 were subcloned into the pBluescript SK+ phagemid (Stratagene, La Jolla, Calif). Endogenous labeling of sense and antisense riboprobes was achieved in the presence of digoxigenin-11-2′-deoxyuridine 5′-triphosphate during in vitro transcription reactions performed according to manufacturer’s recommendations (Boehringer Mannheim, Indianapolis, Ind). Fixed sections were permeabilized (30 minutes at 37°C) with proteinase K (10 µg/mL) and hybridized (16 hours at 50°C) with the digoxigenin-labeled antisense riboprobe (4 µg/mL). After extensive washing (20°C) and RNase A treatment (60 minutes at 37°C), digoxigenin was reacted immunologically (3 hours at 20°C) with a 1:500 dilution of alkaline phosphatase–conjugated sheep anti-digoxigenin antiserum (Boehringer Mannheim). Alkaline phosphatase was detected (18 hours at 4°C) using nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate. Phosphatase-stained sections were visualized for the appearance of a dark blue color using light microscopy. Specificity of the riboprobe and staining protocol included hybridization reactions performed in the absence of added riboprobe or in the presence of the sense riboprobe, 2 processes that failed to exhibit alkaline phosphatase activity.

vivo.10-14 The biological potential of these FGF prototypes is consistent with the histological appearance of pancreatic tumors, characterized by atypical hyperplasia of ducal epithelium, periductal angiogenesis and neurogenesis, invasion of cellular infiltrate, and nonneoplastic stromal cell remodeling and fibrosis.15 Numerous studies have established that the biological activity of FGF requires extracellular heparin sulfate proteoglycan–dependent cell surface interactions with specific high-affinity receptors, including FGFR-1 (flg) and FGFR-2 (bek), to induce responding signal transduction cascades.16-22 However, these prototypical FGFs differ from the FGF oncoproteins by the absence of a signal peptide sequence to participate in conventional secretory processes.

Ongoing efforts16,23-25 have established that cellular release of the FGF prototypes involves an endoplasmic reticulum–independent pathway in response to biological stress. Tissue injury and biological stress in a variety of pathologic conditions (eg, tumorogenesis26-30) include excess production of reactive oxygen and nitrogen species, including superoxide and nitric oxide (NO).
The limited reactivity of superoxide with many biological molecules has raised questions about its toxic effects, which has led to more recent suggestions that NO is a critical target of superoxide. Nitric oxide has gained general acceptance as a necessary mediator of diverse physiologic functions, and a role for this reactive nitrogen species during pathophysiologic processes, such as tumorigenesis, atherosclerosis, arthritis, and transplant rejection, has been described. This paradoxical role for NO may be linked to local levels of NO generated by differential expression of NO synthase (NOS) isoforms. Production of NO is linked to a multitude of pathophysiological processes, such as physiologic functions, and a role for this reactive nitrooxide has raised questions about its toxic effects, including metal-catalyzed formation of nitrotyrosine, a permanent modification that can be detected immunologically using anti-nitrotyrosine antibodies. Nitrotyrosine and FGF have been detected immunologically in several human pathologic conditions, including atherosclerosis, arthritis, and renal transplant dysfunction. Whereas the involvement of the FGF prototypes and NO in tumorigenesis has been suggested, the participation of peroxynitrite during this pathophysiologic condition has not been reported. We examined this issue and designed our study to explore correlation between competent FGF-transforming pathways and the presence of NO production and reactivity in pancreatic adenocarcinoma. These experimental results provide a first indication that FGF transformation and oxidant stress induced by reactive nitrogen species participate in the pathophysiologic processes of human pancreatic cancer.

**RESULTS**

This retrospective study included analyses of 12 pancreatic adenocarcinomas, 6 from stage I and II lesions (less than 2 cm, no positive nodes, no metastasis) and 6 from stage III and IV lesions (greater than 2 cm, at least 1 positive node, 3 with metastasis) recovered from patients surviving longer than 3 years or less than 6 months, respectively. In each specimen, pancreatic tumor was located primarily in the head of the pancreas and, where evident (T3 and T4), metastasis was observed in the lymph node and liver. Results of routine histological analyses of control pancreas were unremarkable. In contrast, results of microscopic examination of pancreatic tumors under hematoxylin-eosin staining demonstrated the existence of irregularly shaped ductal structures with distinct lumens. Examination of the ductal epithelium revealed several layers of cells with marked atypical nuclei and general loss of polarity. Nonneoplastic periductal regions demonstrated the appearance of fibrosis, cellular infiltrate, perineural invasion, and angiogenesis.

Immunohistochemical analysis of FGF-1 and FGF-2 in controls was not readily apparent (Figure 1, A and C). In contrast, increased immunostaining for FGF-1 and FGF-2 was observed in the hyperplastic ductal epithelium and the periductal cellular infiltrate surrounding the tumor (Figure 1, B and D). Moderate or intense staining for FGF-1 and FGF-2 was seen in 58% and 66%, respectively, when compared with controls (Table). In situ hybridization analysis of control tissue (Figure 2, A and D) failed to demonstrate significant expression of FGF-1 or FGF-2 mRNA. In contrast, detectable levels of FGF-1 and FGF-2 mRNA were readily apparent in tumor specimens (Figure 2, B and C) with immunostaining associated with hyperplastic ductal epithelium and surrounding cellular infiltrate. Hybridization reactions performed in the presence of sense riboprobe failed to exhibit alkaline phosphatase activity (data not shown). Of the 2 high-affinity FGF receptors analyzed in controls, staining for FGFR-1 and FGFR-2 was restricted to characteristic islands of islet cells (Figure 1, E and G). Moderate or intense staining was observed in 90% of the tumors for FGFR-1 and in 75% of the tumors for FGFR-2 in pancreatic tumors (when compared with controls), primarily in the epithelium lining of irregularly shaped ductal cells of the tumors (Figure 1, F and H) (Table).

Pancreatic tumor specimens routinely demonstrated exaggerated staining in greater than 90% of the tumors for iNOS and nitrotyrosine (when compared with controls) (Figure 3, B and D), primarily localized to hyperplastic ductal epithelium and acinar cells with minimal to no staining of the inflammatory infiltrate and surrounding connective tissue. In controls, no staining for iNOS was detected (Figure 3, A), whereas moderate staining for nitrotyrosine was restricted to islands of islet cells (Figure 3, C). Intense immunostaining for both of these markers of reactive nitrogen species was completely abolished in deletion controls. Blocking the polyclonal anti-nitrotyrosine with 10-nmol/L 3-nitrotyrosine totally abrogated immunostaining (data not shown).

A composite summary of immunohistochemical staining intensity for FGF ligands, FGFRs, and markers of reactive nitrogen species is presented in the Table. Differential levels of increased staining for these markers could not be attributed significantly to any aspect of clinical or pathological stage or growth of tumor associated with patients harboring pancreatic adenocarcinoma. However, the sample size within specific categories may not be sufficient to appreciate potential correlations. The principle consistent observation derived from these immunohistochemical analyses demonstrates that increased expression of FGF-1, FGF-2, and FGFRs in hyperplastic ductal epithelium is associated with an environment of oxidative stress, such as that provided by increased production of NO and one of its reaction products, peroxynitrite. Finally, serial sections prohibited direct comparison of tumor sample stains for markers of oxidant stress (iNOS and nitrotyrosine) and FGF-1, FGF-2, and FGFR, which is desirable. This often requires double staining with immunofluorescence. However, as the Table depicts, most tumors stained strongly (≥3+) for the receptor and markers of oxidant stress (iNOS and nitrotyrosine), giving credence to a strong association.
between this growth family of ligands and receptors and oxidant stress.

**COMMENT**

The in situ hybridization and immunohistochemical studies reported herein demonstrated minimal expression of FGF-1 and FGF-2 mRNA and protein in the ductal epithelium of normal human pancreas. The near absence of detectable FGF ligand expression predicts that normal pancreatic ductal epithelium contains a quiescent cell population, which is supported by the absence of immunostaining for FGFR-1 and FGFR-2. The absence of a local growth response would be consistent with the relatively low cellular turnover occurring in the pancreatic duct under normal conditions.\(^5\)\(^1\)-\(^3\)\(^3\) In contrast, periductal areas in pancreatic adenocarcinoma tissue demonstrated increased expression of FGF-1 and FGF-2 mRNA and protein. Immunostaining for the FGF proteins correlated well with the appearance of the FGF mRNAs, both of which were associated with resident cellular infiltrate. Since inflammatory cells are a rich source of FGF,\(^4\)\(^7\) and since the involvement of inflammation in pancreatic adenocarcinoma has been described,\(^1\)\(^5\) our observations provide a potential mechanism for delivery of FGF capable of inducing characteristic angiogenic, neurogenic, and stromal remodeling responses.\(^10\)\(^1\)\(^1\)

In addition to inflammatory-mediated synthesis and delivery of the prototypical FGFs, results provided herein also demonstrate the exaggerated appearance of intracellular FGF-1 and FGF-2 protein and mRNA in the ductal epithelium associated with pancreatic adenocarcinoma. This observation is consistent with a response to injury, wherein damaged ductal epithelial cells induce

**Figure 1.** Immunohistochemical analysis of human pancreas for prototypical fibroblast growth factors (FGFs). Thin sections (5 µm) of formalin-fixed, paraffin-embedded pancreatic tissue from nontransplant donors (controls) (A, C, E, and G) or patients with pancreatic adenocarcinoma were prepared and examined using microscopy after staining with specific antibodies against acidic FGFs (FGF-1; A and B), basic FGFs (FGF-2; C and D), and their high-affinity receptors (FGFR-1 [E and F] and FGFR-2 [G and H], respectively) (original magnification ×200). Arrows on panels B, D, F, and H identify staining of pancreatic ductal cells. Arrows on panels E and G identify staining of islet cells.
expression of FGF-1 and FGF-2 competent to function as a survival factor and promote local growth responses.\textsuperscript{54,55} Completion of this potential autocrine-paracrine transforming loop demands expression of specific high-affinity receptors to activate signal transduction processes. Indeed, compared with control specimens, immunostaining for FGFR-1 and FGFR-2 was detected readily in the ductal epithelium of pancreatic adenocarcinoma. Consequently, a local growth response is anticipated by receptor-positive cells exposed to the presence of competent mitogens, a prediction consistent with the appearance of hyperplastic ductal structures.

Several observations in our study provide insight into a potential mechanism regulating the pathologic consequences of increased intrinsic FGF expression beyond that previously reported.\textsuperscript{6,8,56} For instance, intracellularly sequestered FGF-1, which lacks a classic signal sequence for secretion, is not available to mediate its bio-

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<th>Summary of Immunohistochemical Staining Intensity for Specific Markers Associated With Healthy Pancreatic Tissue and Pancreatic Ductal Adenocarcinomas*</th>
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<td><strong>Healthy Tissue</strong> (n = 3)</td>
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*Data represent the total number of samples in this study that received the representative average grade of staining. Levels of staining intensity are described in the "Immunolocalization" subsection of the "Materials and Methods" section. FGF-1 indicates acidic fibroblast growth factor; FGF-2, basic FGF; FGFR-1 and FGFR-2, their respective high-affinity receptors; iNOS, inducible nitric oxide synthase; and NT, nitrotyrosine.

**Figure 2.** In situ hybridization analysis of human pancreas for acidic fibroblast (FGF-1) and basic fibroblast growth factor (FGF-2) messenger RNA (mRNA). Thin sections (5 mm) of formalin-fixed, paraffin-embedded pancreatic tissue obtained from nontransplant donors (controls) (A and D) or patients with pancreatic tumors (B and C) were prepared and examined using light microscopy after hybridization with antisense probes against FGF-1 (A and B) and FGF-2 (C and D) mRNA (original magnification ×200). Arrows on panels B and C identify staining of pancreatic ductal cells. Abbreviations are given in the legend to Figure 1.
logical effects on cell growth. Previous in vitro studies have demonstrated that FGF-1 is released from cells in response to oxidative stress, a pathologic condition that may involve inflammatory-mediated production of reactive oxygen-nitrogen species. Our results demonstrate the increased immunoappearance of iNOS and nitrotyrosine in the ductal epithelium of pancreatic tumor tissue. This observation predicts that endogenous production of reactive nitrogen species, as evidenced by markers for NO and peroxynitrite, induces oxidative stress in the ductal epithelium of pancreatic adenocarcinoma. The secretion of FGF-1 in response to this biological stress may be responsible for inducing an extracellular autocrine-paracrine transforming loop in pancreatic tumors. Consequently, hyperplasia of the ductal epithelium during pancreatic adenocarcinoma is consistent with an extracellular FGF pathway and may represent a normal regenerative response of these structures to oxidant stress. Although the enhanced and correlated appearance of FGF and oxidant stress in the ductal epithelium supports a mechanistic transforming role for these agents during development of pancreatic lesions associated with tumorigenesis, additional efforts will be required to establish a direct cause-and-effect relationship beyond that suggested in a limited retrospective analysis.

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