Vol. 215, No. 1, 1995 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
October 4, 1995 Pages 309-315

INCREASED EXPRESSION OF KERATINOCYTE GROWTH FACTOR IN HUMAN PANCREATIC CANCER

Imran Siddiqi, Hitoshi Funatomi, Michael S. Kobrin, Helmut Friess,[1] Markus W. Büchler,[1] and Murray Korc*

Departments of Medicine and Biological Chemistry, University of California, Irvine, CA 92717
[1]Department of Visceral and Transplantation-Surgery, University of Berne, Inselspital, CH-3010 Berne, Switzerland

Received August 25, 1995

Keratinocyte growth factor (KGF) is a member of the fibroblast growth factor (FGF) group of heparin-binding polypeptides. In the present study we sought to determine whether KGF is expressed in human pancreatic cancers. Using reverse transcriptase polymerase chain reaction (RT-PCR), a cDNA fragment of KGF was cloned and used to analyze Northern blots of RNA isolated from normal and cancerous human pancreatic tissues. Seven of 16 (44%) pancreatic cancer samples revealed significant overexpression of the 2.4 kilobase KGF mRNA transcript by comparison with the normal pancreas. Northern blot analysis failed to reveal the KGF transcript in several cultured human pancreatic cancer cell lines. However, by PCR analysis, some of the cell lines expressed KGF mRNA. Furthermore, 5 of 7 tested cell lines expressed the KGF receptor, and the growth of one cell line was enhanced by human recombinant KGF. These results suggest that KGF may participate in aberrant paracrine and autocrine pathways in human pancreatic cancer.

The fibroblast growth factor (FGF) family presently consists of nine homologous polypeptides that share 30-70% amino acid sequence homology (1,2). These factors are acidic FGF (aFGF or FGF-1), basic FGF (bFGF or FGF-2), int-2 (FGF-3), hst/K-FGF (FGF-4), FGF-5, FGF-6, keratinocyte growth factor (FGF-7), androgen-induced growth factor (FGF-8), and glia-activating factor (FGF-9). FGFs are mitogenic and angiogenic, and are involved in tissue development and repair (1,2). Members of the FGF family have also been implicated in a variety of human neoplasms. For example, aFGF and bFGF are overexpressed in human pancreatic cancers, and overexpression of bFGF has been correlated with shorter post-operative survival of pancreatic cancer patients (3). Furthermore, cultured human pancreatic cancer cell lines have been reported to express aFGF and bFGF, as well as FGF-3, FGF-4, and FGF-5 (4).

KGF was originally isolated from human embryonic lung fibroblasts (5,6). It is an important mitogen for a variety of epithelial cells, including cells of the liver and gastrointestinal tract (7), type II pneumocytes in the lungs (8), and mammary gland ductal epithelial cells (9). Although the

*To whom correspondence and reprint requests should be addressed.

Abbreviations: KGF, keratinocyte growth factor; bFGF, basic fibroblast growth factor; aFGF, acidic fibroblast growth factor.

0006-291X/95 $12.00

in vivo administration of KGF enhances pancreatic ductal cell proliferation in rats (10), it is not known whether human pancreatic cancers express KGF. Therefore, in the present study, we characterized the expression of KGF in normal and malignant pancreatic tissues and in cultured human carcinoma cell lines. We now report that KGF mRNA levels are increased in human pancreatic cancers, and that KGF is expressed in some human pancreatic cancer cell lines.

MATERIALS AND METHODS

Preparation of KGF and KGF Receptor cDNAs. A cDNA encoding sequences corresponding to the human KGF gene was produced by amplification with the polymerase chain reaction (PCR) of single-stranded cDNA that was reverse transcribed (RT) from human placenta RNA, using previously described conditions (11). The primers used for KGF cDNA preparation were: 5'-CTGACATGGATCCTGCCAAC-3' corresponding to nucleotides 461-480 of human KGF cDNA, and 5'-GAGAAGCTTCCAACTGCCACTGTCCTG-3' corresponding to nucleotides 745-764 of this cDNA (3). A cDNA encoding sequences corresponding to the human KGF receptor gene was similarly generated by RT-PCR. The primer pair used to amplify the KGF receptor was derived from sequences located on either side of the KGF receptor specific exon: 5'-GCGGATCCGTTCTCAAGCACTCGGGGA-3' corresponding to nucleotides 1349-1367, and 5'-GCAAGCTTCCAGGCGCTTGCTGT-3' corresponding to nucleotides 1498-1515 (12). The 311 bp KGF cDNA fragment and the 167 bp KGF receptor cDNA fragment were subcloned into pGEM3Zf vectors (Promega Biotechnology, Madison, WI), and their authenticity confirmed by sequencing.

PCR Analysis and Preparation of cDNA. cDNAs were synthesized from total RNA (2 μg/sample) isolated from ASPC-1, CAPAN-1, COLO-357, HS766T, MIA-PaCa-2, PANC-1, or T3M4 human pancreatic cancer cells, using oligodeoxythymidylate and reverse transcriptase (11). The above primers were used to amplify KGF and KGF receptor sequences. For KGF amplification, 1 μl of the reaction mixture was incubated in buffer containing 1.23 mM concentrations of dATP, dCTP, dGTP, dTTP, 600 nM of the oligonucleotide primer pair, and 10% dimethyl sulfoxide in buffer consisting of 16.6 mM (NH4)SO4, 67 mM Tris-HCl (pH 8.0), 6.7 mM $MgCl_2$, 10 mM β-mercaptoethanol, and Taq polymerase (11). For KGF receptor amplification, 1 μl of the reaction mixture was incubated in buffer containing 100 mM Tris-HCl (pH 8.3), 500 mM KCl, 0.2 mM concentrations of dATP, dCTP, dGTP, dTTP, 250 nM of the oligonucleotide primer pair, 1.0 mM $MgCl_2$ and Taq polymerase (11). Internal controls for both amplification conditions were generated by using primers corresponding to human glyceraldehyde phosphate dehydrogenase (GAP) cDNA (11): 5'-GTATTGGGCGCCTGGTCACCA-3', and 5'-ACGTACTCAGCGCCAGCATCG-3'. There were 35 PCR cycles consisting of 1 min at 94°C, 1 min at 60°C, and 1 min at 72°C. PCR products were size fractionated on 0.9% agarose gels, and blotted onto nylon membranes. The membranes were then subjected to hybridization by the method of Southern (13). All oligonucleotide primers used in the present study were synthesized on an Applied Biosystem 391 DNA Synthesizer and purified by electrophoresis.

Cell Culture and Growth Assay. PANC-1, ASPC-1, HS766T, MIA-PaCa-2 and CAPAN-1 human pancreatic cancer cells were obtained from the American Type Culture Collection (Rockville, MD), whereas T3M4 and COLO-357 human pancreatic cancer cells were obtained from R.S. Metzgar at Duke University. Cells were grown in monolayer culture at 37°C in a humidified-air atmosphere. PANC-1, MIA-PaCa-2, HS766T and COLO-357 cells were grown in Dulbecco's modified Eagle's medium, and T3M4, ASPC-1 and CAPAN-1 cells were grown in RPMI 1640 medium. Media contained antibiotics and 10% fetal bovine serum. To assess the effects of KGF on pancreatic cancer cell growth, PANC-1, ASPC-1 and COLO-357 cells were plated at a density of 15,000 cells per well in 96 well plates and grown overnight in DMEM or RPMI supplemented with 10% fetal bovine serum. Following washing with Hank's buffered saline solution (HBSS), the cells were incubated in serum-free medium containing 0.1% bovine serum albumin, 5 mg/L transferrin, and 5 μg/L selenious acid, and incubated in the absence or presence of human recombinant KGF (Becton Dickinson, Inc., Bedford, MA). The following day, the medium was replaced with fresh medium containing KGF at the same concentrations. The cells were harvested 24 hours later, and incubated with 12.5 μl of 3-(4,5-methylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, 5 mg/ml) for 4 hours (14). The medium was aspirated

and MTT dye crystals were dissolved with acidified isopropanol (0.04N HCl). Optical density of the plates was measured using an ELISA plate reader (Molecular Devices) at 570 nm (14).

Northern Blot Analysis. Total RNA was extracted from 7 pancreatic cancer cell lines, 16 pancreatic cancers and 13 normal pancreatic tissues, using the acid guanidinium thiocyanate-phenol-chloroform extraction method (15). In addition, poly(A)$^+$ RNA was prepared from all the cell lines using oligodeoxy-thymidine column chromatography. The median age of the 7 female and 9 male pancreatic cancer patients was 58.9 years, with a range of 32 to 78 years. All the tumor samples were classified as pancreatic ductal carcinomas according to the TNM classification for pancreatic tumor (16). The median age of the 4 female and 9 male organ donors was 37.3 years, with a range of 14 to 55 years. RNA samples were size-fractionated and blotted onto nylon membranes (GeneScreen; Dupont, Boston, MA), and the blots were prehybridized, hybridized and washed under high stringency conditions (17). The KGF cDNA and a 190-base pair BamHI fragment of the mouse 7S cDNA that cross-hybridizes with human 7S RNA (17) were labeled with [^{32}P]dCTP (3000 Ci/mmol) by random priming (18) prior to hybridization with the RNA blots. The blots were exposed at -80°C to Kodak XAR-5 film with intensifying screens. Densitometric analysis of the Northern blots was carried out with a Bio-Rad Imaging Densitometer and Molecular Analyst Software (version 1.1.1), allowing for an accurate determination of the optical density of the 2.4 kb KGF transcript in each lane on the autoradiograph, even when the signal was barely visible. The ratio of the optical densities of the RNA levels (KGF/7S) was calculated for each sample, and the data analyzed statistically by Student's t-test. All studies were approved by the Human Subjects Committee of the University of California, Irvine and the University of Berne, Switzerland.

RESULTS

Northern blot analysis of total RNA indicated that KGF mRNA levels were relatively low in the normal human pancreas (Fig. 1). Nonetheless, all the RNA samples derived form the normal human pancreas exhibited both the 2.4 kb and the approximately 5.0 kb KGF transcripts (Fig. 1, first 8 lanes). The levels of both KGF transcripts were increased in 3 of the 7 cancer samples seen

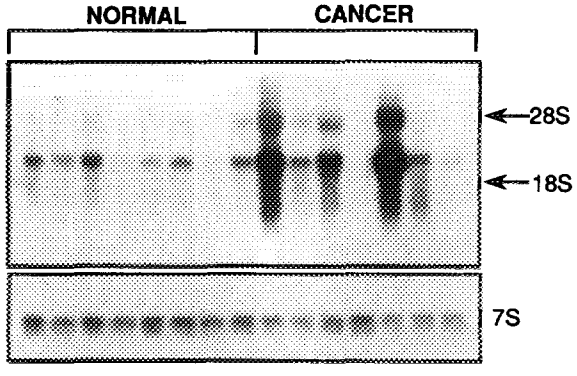

Figure 1. Northern blot analysis of pancreatic tissue RNA. Total RNA (20 μg/lane) from 7 normal and 8 pancreatic cancer samples was size-fractionated, transferred onto nylon membranes, and hybridized as described in the Methods section, using the labeled KGF cDNA probe (500,000 cpm/ml; 12 day exposure) and 7S cDNA probe (30.000 cpm/ml; 18 hours exposure). All normal samples exhibited both the 2.4 kb and 5.0 kb KGF mRNA transcripts. In two samples both bands were only visible on the original autoradiograph. Three of the 7 pancreatic cancer RNA samples expressed high levels of both KGF transcript, whereas in one sample (lane 12) both transcripts were below the levels of detection. Migration positions of 28S and 18S ribosomal subunits are indicated on the right.

on the same autoradiograph (Fig. 1). Overall, 7 of 16 (44%) pancreatic cancer samples overexpressed KGF mRNA. However, one cancer sample failed to exhibit either the 2.4 or 5.0 KGF transcripts (Fig. 1, lane 12). Densitometric analysis of the Northern blots for all the normal and cancer samples indicated that there was a 5-fold increase in KGF mRNA levels in the cancer samples relative to the normal samples. This increase was statistically significant ($p<0.002$).

Northern blot analysis of total or poly(A)$^+$ RNA isolated from ASPC-1, CAPAN-1, COLO-357, HS766T, MIA-PaCa-2, PANC-1 and T3M4 cells failed to reveal the presence of a KGF transcript (data not shown). However, by PCR analysis, ASPC-1, COLO-357, HS766T and PANC-1 cells expressed KGF (Fig. 2A, upper panel), as confirmed by Southern blot analysis of the PCR products using the KGF cDNA (Fig. 2A, middle panel). The highest levels of KGF mRNA were observed in ASPC-1 cells, whereas CAPAN-1, MIA-PaCa-2 and T3M4 cells did not express KGF even with this sensitive PCR method.

By PCR analysis, ASPC-1, CAPAN-1, COLO-357, PANC-1, and T3M4 cells also expressed the KGF receptor (Fig. 2B, upper panel). The presence of this transcript was confirmed by Southern blot analysis of the PCR products, using the KGF receptor cDNA (Fig. 2B, middle panel). In contrast, HS766T and MIA-PaCa-2 cells did not express the KGF receptor (Fig. 2B). Placental RNA, used as a positive control, expressed relatively high levels of both KGF and the KGF receptor, whereas in the absence of any RNA, neither transcript was evident (Fig. 2).

Three of the pancreatic cancer cell lines were tested in a cell growth assay to determine whether KGF modulated pancreatic cancer cell growth (Fig. 3). KGF did not increase the growth of ASPC-1 or PANC-1 cells (data not shown). However, in COLO-357 cells, KGF exerted a dose-dependent increase on cell proliferation (Fig. 3). One-half maximal and maximal growth stimulation occurred at 18 pM and 36 pM KGF, respectively. At a maximally effective concentration, KGF enhanced the growth of COLO-357 cells by 35%.

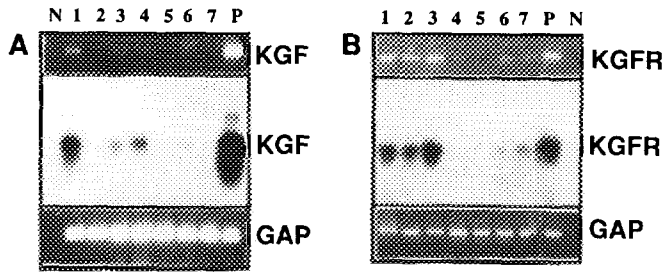

Figure 2. PCR analysis. Total RNA (2 μg/sample) from ASPC-1 (lane 1) CAPAN-1 (lane 2), COLO-357 (lane 3), HS766T (lane 4), MIA-PaCa-2 (lane 5), PANC-1 (lane 6) and T3M4 (lane 7) human pancreatic cancer cells, and from human placenta (P) was used to generate cDNAs. The cDNAs were amplified by using primers specific for KGF (A) and KGF receptor (B, KGFR). The PCR products were visualized by ethidium bromide staining (upper panels), and by hybridizing with the respective radiolabeled cDNAs (middle panels). Amplification products of the same cDNAs using primers for glyceraldehyde-3-phosphate dehydrogenase (GAP) were also visualized by ethidium bromide staining, and served as internal controls. N: negative control.

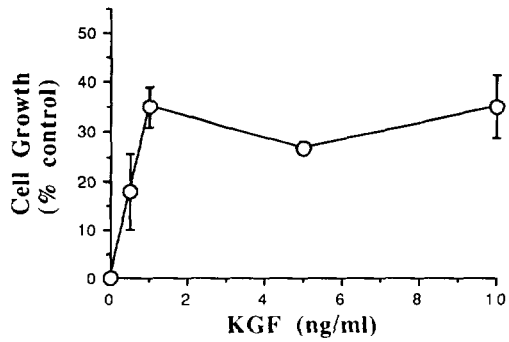

Figure 3. Effect of KGF on cell growth. COLO-357 cells were incubated for 48 h in the absence or presence of the indicated KGF concentrations. Cell growth was monitored with the MTT assay. Data are the means±SE of quadruplicate determinations from 3 separate experiments.

DISCUSSION

KGF is often expressed by stromal fibroblasts and other types of mesenchymal cells (5). In contrast to aFGF and bFGF, the KGF precursor contains a signal sequence, allowing for its efficient secretion from its cell of origin (5). Following release, KGF stimulates epithelial cell growth in a paracrine manner, thereby contributing to epithelial-mesenchymal interactions (5). KGF may have a particularly important role among the FGF family of ligands in wound healing, inasmuch as there is a 160-fold increase in KGF mRNA expression following wounding in rats (19). Furthermore, inhibition of KGF receptor signaling in epidermal keratinocytes of a dominant-negative KGF receptor transgene delays the process of wound healing (20). The KGF receptor is a transmembrane protein generated as the result of alternative splicing of the gene encoding FGF receptor type II, or FGFR-2 (12). It contains 3 extracellular immunoglobulin-like (Ig) domains and an intracellular tyrosine kinase domain exhibiting a non-kinase intervening sequence (12). The KGF receptor binds aFGF and KGF, but not bFGF, with high affinity (12). In contrast, FGFR-2 binds aFGF and bFGF with high affinity but does not bind KGF. This difference in ligand binding is due principally to variations in amino acid sequences within the carboxy-terminal half of the third Ig domain of these receptors (21). However, other extracellular regions within the second Ig domain appear to be important as well (22).

In the present study, we determined that KGF is overexpressed in a significant number of human pancreatic ductal adenocarcinomas. There was increased expression of both the 2.4 kb and 5.0 kb KGF RNA species. These moieties arise as a result of transcription of the same gene from 2 alternate initiation sites (5). The relatively low abundance of the larger KGF transcript in both normal and cancerous pancreatic tissues is most likely due to its enhanced degradation, ostensibly as a result of the presence of numerous ATTTA sequences at its 3' end (5).

Previously, we found that aFGF and bFGF are overexpressed in human pancreatic cancers (3). Both factors have been shown by immunostaining and *in situ* hybridization to be expressed in the cancer cells within the pancreatic tumor mass (3). In contrast, the exact site of expression of KGF in the pancreatic cancer is not known. In view of the fact that KGF is most often produced by

stromal cells and then acts on epithelial cells (5), it is likely that KGF is expressed by the stromal elements within the tumor mass, and that it exerts paracrine effects on the neighboring cancer cells. It is also possible that some pancreatic cancer cells express KGF, which can then act in a paracrine manner on stromal cells, thereby enhancing aberrant epithelial-mesenchymal interactions. In addition, cancer cell derived KGF may act in an autocrine manner to directly enhance the growth of these cells. Three observations support this hypothesis. First, 4 of 7 pancreatic cancer cell lines expressed the KGF transcript, and 5 of these 7 cell lines expressed the KGF receptor transcript. Second, ASPC-1, COLO-357, and PANC-1 cells co-expressed both KGF and its receptor. Third, COLO-357 were exquisitely sensitive to the growth stimulatory effects of KGF. In this context, the failure of KGF to enhance the growth of ASPC-1 and PANC-1 cells may be due to a masking effect by endogenously produced KGF.

KGF mRNA expression is rapidly induced in cultured fibroblasts by a number of growth factors and cytokines, including platelet-derived growth factor (PDGF), interleukin-1-β, interleukin-6, and tumor necrosis factor-α (TNF-α) (23,24). KGF has also been found to induce transforming growth factor alpha (TGF-α) expression, thus suggesting that KGF may, in certain cases, stimulate cell growth by acting through the TGF-α/EGF receptor pathway (25). TGF-α and the EGF receptor are overexpressed in human pancreatic cancers (17). These cancers also express high levels of PDGF A and B, and overexpress both types of PDGF receptors (26). Together with the current findings, these observations suggest that KGF may participate in complex autocrine and paracrine pathways to promote pancreatic cancer cell growth *in vivo*. Therefore, pharmacological modalities aimed at abrogating KGF-dependent pathways may have a role in the therapy of patients with pancreatic cancer.

ACKNOWLEDGMENT

This study was supported by NIH grant DK-44948.

REFERENCES

1. Basilico, C., and Moscatelli, D. (1992) Adv. Cancer Res. 59, 115-165.
2. Miyamoto, M., Naruo, K.I., Seko, C., Matsumoto, S., Kondo, T., and Kurokawa, T. (1993) Mol. Cell. Biol. 13, 4251-4259.
3. Yamanaka, Y., Friess, H., Büchler, M., Beger, H.G., Uchida, E., Onda, M., Kobrin, M.S., and Korc, M. (1993) Cancer Res. 53, 5289-5296.
4. Leung, H.Y., Gullick, W.J., and Lemoine, N.R. (1994) Int. J. Cancer 59, 667-675.
5. Finch, P., Rubin, J., Miki, T., Ron, D., and Aaronson, S. (1989) Science 245, 752-755.
6. Rubin, J., Osada, H., Finch, P., Taylor, W., Rudikoff, S., and Aaronson, S. (1989) Proc. Natl. Acad. Sci. U.S.A. 86, 802-806.
7. Housley, R., Morris, C., Boyle, W., Ring, B., Blitz, R., Tarpley, J., Aukerman, S., Devine, P., Whitehead, R., and Pierce, G. (1994) J. Clin. Invest. 94, 1764-1777.
8. Ulich, T., Yi, E., Longmuir, K., Yin, S., Blitz, R., Morris, C., Housley, R., and Pierce, G. (1994) J. Clin. Invest. 93:1298-1306.
9. Imagawa, W., Cunha, G., Young, P., and Nandi, S. (1994) Biochem. Biophys. Res. Commun. 204, 1165-1169.
10. Yi, E., Yin, S., Harclerode, D., Bedoya, A., Bikhazi, N., Housley, R., Aukerman, S., Morris, C., Pierce, G., and Ulich, T. (1994) Am. J. Pathol. 145, 80-85.

11. Ebert, M., Yokoyama, M., Kobrin, M.S., Friess, H., Lopez, M.E., Büchler, M.W., Johnson,G.R., and Korc, M. (1994) Cancer Res. 54, 3959-3962.
12. Miki, T., Bottaro, D., Fleming, T., Smith, C., Burgess, W., Chan, A., and Aaronson, S. (1992) Proc. Natl. Acad. Sci. U.S.A. 89, 246-250.
13. Southern, E.M. (1975) J. Mol. Biol. 98, 503-517.
14. Raitano, A.B., and Korc, M. (1990) J. Biol. Chem. 265, 10466-10472.
15. Chomczynski, P., and Sacchi, N. (1987) Anal. Biochem. 162, 156-159.
16. Hermanek, P., and Sorbin L.H. (1987) UICC: TNM Classsification of Malignant Tumours, 198 pp. New York: Springer-Verlag.
17. Korc, M., Chandrasekar, B., Yamanaka, Y., Friess, H., Büchler, M., and Beger, H.G. (1992) J. Clin. Invest. 90, 1352-1360.
18. Feinberg, A.P., and Vogelstein, B. (1983) Anal. Biochem. 132, 6-13.
19. Werner, S., Peters, K., Longaker, M., Fuller-Pace, F., Banda, M., and Williams, L. (1992) Proc. Natl. Acad. Sci. U.S.A. 89, 6896-8900.
20. Werner, S., Smola, H., Liao, X., Longaker, M., Krieg, T., Hofschneider, P., Williams, L. (1994) Science 266, 819-822.
21. Bottaro, D., Rubin, J., Ron, D., Finch, P., Florio, C., and Aaronson, S. (1990) J. Biol. Chem. 265, 12767-12770.
22. Zimmer, Y., Givol, D., Yayon, A. (1993) J. Biol. Chem. 268, 7899-7903.
23. Brauchle, M., Angermeyer, K., Hubner, G., and Werner, S. (1994) Oncogene 9, 3199-3204.
24. Chedid, M., Rubin, J., Csaky, K., Aaronson, S. (1994) J. Biol. Chem. 269, 10753-10757.
25. Dlugosz, A., Cheng, C., Denning, M., Dempsey, P., Coffey, R., Yuspa, S. (1994) Cell Growth and Diff. 5:1283-1292.
26. Ebert, M., Yokoyama, M., Friess, H., Kobrin, M.S.,Büchler, M.W., and Korc, M. (1995) Int. J. Cancer, *In Press*.