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Induction and Expression of Amphiregulin in Human Pancreatic Cancer

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Abstract

The epidermal growth factor receptor is activated by a family of polypeptides that includes the growth factor amphiregulin (AR). Using Northern blot analysis and the polymerase chain reaction, we now report that AR mRNA is expressed in human pancreatic cancer cell lines, and that this expression is enhanced in several of these cell lines by tetradecanoyl phorbol acetate and transforming growth factor α. AR was also expressed in normal and malignant pancreatic tissues. However, in the normal pancreas, AR immunostaining was most evident in the nuclei of ductal cells. In contrast, in many carcinomas, AR was also present in the cytoplasm of the ductal-like cancer cells. Cytoplasmic localization of AR was associated with a more advanced clinical stage. These findings suggest that AR may contribute to aberrant activation of the epidermal growth factor receptor in human pancreatic cancer, and may enhance disease progression.

Introduction

A number of different polypeptide growth factors that are expressed in normal human tissues are overexpressed in human cancers. This excessive production of growth factors may allow cancer cells to escape physiological constraints on cell growth. Similarly, cancer cells may overexpress the receptors that are activated by specific growth factors, thereby leading to excessive activation of growth signaling pathways and aberrant cell growth (1). The EGFR (1) is a transmembrane protein whose overexpression has been associated with malignant transformation (2). EGFR belongs to a family of closely related transmembrane proteins that include c-erbB2 or human EGFR2 (HER2), HER3, and HER4. In addition to binding EGF, EGFR binds TGF-α, AR, betacellulin and heparin-binding EGF (3). HER2 also appears to bind a family of ligands, whereas the ligands that bind to HER3 and HER4 are yet to be identified (4).

Carcinoma of the pancreas is the fourth or fifth leading cause of cancer death in the United States, with an overall 1-year survival of only 12%. The diagnosis of pancreatic cancer is frequently established at an advanced stage, and both surgical and nonsurgical treatment modalities for pancreatic cancer have not been successful (5). Although the reasons for the aggressiveness of this disorder are not known, cultured human pancreatic carcinoma cells overexpress EGFR and produce TGF-α (6, 7). In addition, human pancreatic cancers overexpress EGFR, HER2, HER3, EGF, and TGF-α (8, 9), underscoring the importance of this receptor family in human pancreatic cancer. However, it is not known whether ligands other than EGF or TGF-α that bind to EGFR are also overexpressed in pancreatic cancer. AR is a positively charged Lys-Arg-rich peptide growth factor and possesses a hydrophilic region at its amino terminal end with homology to TGF-α (10). Besides binding to the cell surface EGFR, AR binds to single and double-stranded DNA, raising the possibility that it exerts direct regulatory effects on the expression of certain genes. This hypothesis is supported by the finding that AR may be present in the nuclei of certain cells (11). We now report that AR is expressed in human pancreatic cancer cells and that there is a differential distribution of AR immunostaining in the normal and cancerous human pancreas.

Materials and Methods

Fetal bovine serum, Dulbecco’s minimal essential medium, RPMI 1640 medium, trypsin EDTA solution, and penicillin-streptomycin solution were from Irvine Scientific (Santa Ana, CA); biotinylated goat IgG was from Kirkegaard & Perry Laboratories (Gaithersburg, MD); GeneScreen membranes were from New England Nuclear (Boston, MA); pBluescript IISK+ vector was from Stratagene (La Jolla, CA); pGEM3ZI vector was from Promega Biotech (Madison WI); SuperScript reverse transcriptase was from BRL (Gaithersburg, MD); Taq polymerase was from Perkin-Elmer Cetus Corp. (Norwalk, CT); [α-32P]dCTP (3000 Ci/mmol) was from Amersham, Inc. (Arlington Heights, IL). Human recombinant TGF-α and TGF-β, were gifts from Genentech, Inc. (San Francisco, CA).

Cell Culture. PANC-1 and AsPC-1 human pancreatic cancer cells and MCF-7 human breast cancer cells were obtained from the American Type Culture Collection (Rockville, MD). T3M4 and COLO-357 human pancreatic cancer cells were obtained from R. S. Metzger at Duke University. PANC-1, COLO-357, and MCF-7 cells were grown in Dulbecco’s minimal essential medium. T3M4 and AsPC-1 were grown in RPMI 1640. Media contained antibiotics and 10% fetal bovine serum. Argonists were added after culturing the cells in serum-free media, containing 0.1% bovine serum albumin, insulin, and transferrin (5 µg/ml), selenious acid (5 ng/ml), and antibiotics for 48 h.

Tissue Samples. Pancreatic cancer tissues (24 female, 24 male) were obtained from patients undergoing pancreatic surgery. Normal pancreatic tissues were obtained from 8 individuals (2 female, 6 male) through an organ donor program. The median age of the patients with pancreatic cancer was 63 years (range, 31–77). The median age of the organ donors was 41 years (range, 18–57). Immediately following surgical removal, tissue samples were either fixed in Bouin’s solution or frozen in liquid nitrogen. The tumor samples were classified as pancreatic ductal adenocarcinomas according to the tumor-node-metastasis classification for pancreatic tumors (12). All studies were approved by the Human Subjects Committee of the University of California, Irvine, CA, and the University of Berne, Berne, Switzerland.

Immunohistochemistry. A highly specific polyclonal antibody against human AR was utilized for immunohistochemical analysis (13). The antibody was raised against a 19-amino acid synthetic peptide (VKPPQNKTESENTSVKDKPKR) which corresponds to amino acid residues 8–26 of the mature 84-amino acid AR protein. Antibody specificity was confirmed by immunoblotting and competition assays, as previously described (13). In addition, nonimmunized rabbit IgG or neutralized primary antibody failed to reveal any immunostaining. Paraffin-embedded tissue sections were subjected to immu-
nontesting by using a streptavidin-peroxidase method. After blocking endog-
ous peroxidase activity with 0.3% hydrogen peroxide in methanol, the
sections were incubated for 45 min (23°C) with 10% normal goat serum and
for 4 h (4°C) with the anti-AR antibody (10 μg/ml) (8). Bound antibody was
detected with a biotinylated anti-rabbit IgG secondary antibody and streptavi-
din-peroxidase complex, using diaminobenzidine tetrahydrochloride as the
substrate. Counterstaining was performed with Mayer’s hematoxylin. Positive
immunostaining was defined as the presence of AR immunoreactivity in at
least 10% of the cancer cells (14), as determined by 2 independent observers
blinded to patient status.

**PCR and Northern Blot Analysis.** Oligonucleotide primers were synthe-
sized on an Applied Biosystems Model 391 DNA synthesizer and purified by
electrophoresis (15). Two primer sequences, corresponding to nucleotides
5’-TCC-CCGAGCCGACFATOAC-3’; AR2, 5’-GGACFrlTCCCCACA
747—764, respectively, (11) were used to amplify AR: AR1,
435—455 and
primers corresponding to nucleotides
were amplified by PCR from reverse
transcribed human placenta RNA. The PCR primers (AR1, AR2) contained an
additional BamHI and HindIII restriction site. The PCR products were gener-
ated in 35 cycles (94°C, 1 min; 57°C, 1 min; and 72°C, 1 min), subcloned into
a pGEM3ZF vector and sequenced. A 190-base pair BamHI fragment of the
mouse 7S cDNA that cross-hybridizes with human 7S RNA was random
labeled with [α-32P]dCTP (8).

Total RNA was extracted from 5 cancer cell lines and blotted onto nylon
membranes (8). The blots were prehybridized, hybridized, and washed under
high stringency conditions for a cDNA probe (8). The blots were exposed at
−80°C to Kodak XAR-5 film with Kodak intensifying screens, and the intensity of the radiographic bands was determined by laser densitometry
(Ultrascan XL; Pharmacia LKB Biotechnology, Uppsala, Sweden).

**Statistical Analysis.** The relationship between the immunohistochemical
data and the clinicopathological parameters were analyzed by using χ² and
Fisher exact tests (17).

**Results**

PCR analysis with the use of AR-specific oligonucleotide primers
demonstrated the presence of AR mRNA in T3M4, AsPC-1, COLO-
357, and PANC-1 pancreatic cancer cells (Fig. 1A). Northern blot
analysis demonstrated the presence of the AR transcript (1.4 kilo-
bases) in T3M4, AsPC-1, and COLO-357 human pancreatic cancer
cells (Fig. 1B), but not in PANC-1 cells (not shown). However, the
basal level of AR mRNA in COLO-357 cells was very low, and only
readily visible on the original autoradiograph. As determined by laser
densitometry and standardization against the respective 7S signal,
TGF-α (10 ng/ml) caused a marked increase in AR mRNA levels in
T3M4 and COLO-357 cells, which was maximal at 4 h following
Fig. 2. AR immunostaining. In the normal pancreas (A–D), AR immunoreactivity (A, C) was present in many nuclei in the ductal cells (arrowheads) and in a few nuclei in islet cells (solid arrows). In the pancreatic adenocarcinomas (E–G), AR immunostaining (E, G) was present in the cytoplasm of many duct-like cancer cells. No immunoreactivity was evident when neutralized antibodies were used (B, D, F). Bar, 100 µm.

agonist addition. However, TGF-α did not alter AR mRNA levels in either AsPC-1 (Fig. 1B) or PANC-1 cells, and TGF-β (10 ng/ml) was without effect in any of the cell lines. In contrast, TPA, at a concentration of 100 ng/ml, markedly increased AR mRNA levels in AsPC-1, COLO-357, and T3M4 cells (Fig. 1C), but was also without effect in PANC-1 cells (not shown). The stimulatory effect of TPA in the pancreatic cancer cells was maximal at 3 h, whereas in the MCF-7 cells, it was maximal at 24 h following TPA addition (Fig. 1C).

AR is not readily evident on Northern blot analysis by using RNA extracted from a variety of tissues (11). In the present study, using 20 µg of total RNA in each lane, Northern blot analysis failed to reveal an AR transcript in either the normal or cancerous pancreatic tissues. Therefore, PCR analysis was performed by using the conditions established to study the cell lines. This analysis revealed the presence of one band specific for AR in all cases of pancreatic cancer (8 of 8) and in 7 of 8 normal pancreatic tissues (Fig. 1D).

To determine whether the low levels of AR mRNA detected by the PCR technique were associated with the presence of AR proteins in these tissues, immunohistochemical analysis of 8 normal pancreas and 48 pancreatic cancer samples was performed next. In the normal pancreas, AR immunoreactivity was readily evident in the nuclei of many ductal cells and was occasionally faintly present in the nuclei of acinar and islet cells (Fig. 2, A–D). The only normal tissue sample that did not express AR by PCR analysis also failed to exhibit AR immunoreactivity (Fig. 1D). Seventy-one % (34 of 48) of the pancreatic cancer samples showed positive immunoreactivity in the cancer cells. AR was present in the nuclei, cytoplasm, or in both subcellular locations in 14, 17, and 3 cases, respectively (Fig. 2, E–G). There was a significant association between advanced tumor stage and cytoplasmic AR immunoreactivity, as determined by χ² (P < 0.01) and Fisher exact test (P < 0.05) (Table 1). However, there was no correlation between the immunostaining pattern and the histological grade of the tumors.

**Discussion**

AR was initially isolated from the serum-free conditioned medium of TPA-treated MCF-7 human breast cancer cells (18). Its 1.4-kilobase transcript encodes a 252-amino acid precursor, containing the 78- or 84-amino acid mature protein. Relatively high levels of AR are

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**Table 1** Correlation between distribution of AR immunostaining and tumor stage

<table>
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<th>Stage</th>
<th>I, II</th>
<th>III, IV</th>
<th>P</th>
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<td>Cytoplasmic or cytoplasmic/nuclear</td>
<td>3</td>
<td>17</td>
<td>0.01* 0.05c</td>
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<td>9</td>
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<td>Cytoplasmic or cytoplasmic/nuclear</td>
<td>3</td>
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<tr>
<td>Nuclear or negative</td>
<td>17</td>
<td>11</td>
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* According to the tumor-node-metastasis classification of pancreatic cancer (12).
* Analyzed by the χ² test.
* Analyzed by the Fisher exact test (17).
found in the normal ovary and placenta, whereas low levels have been reported in other tissues (11). AR expression is increased in human colorectal, gastric, and mammary carcinomas (19–21), but is decreased in most lung tumors (22).

In the present study we used the highly sensitive PCR technique to demonstrate the presence of AR mRNA in several human pancreatic cancer cell lines, most normal human pancreatic tissues and all the pancreatic tumors. We also found that TPA increases the steady-state levels of AR mRNA in AsPC-1, COLO-357, and T3M4 cells, as previously reported for MCF-7 breast cancer cells (18). However, the TPA-mediated effect was more rapid in the pancreatic cancer cells than in the MCF-7 cells. TGF-α, which enhances TGF-α expression in pancreatic cancer cells, also increased AR mRNA levels, but only in COLO-357 and T3M4 cells. In contrast, TGF-β did not alter AR expression in any of the cells. These findings are consistent with previous findings that signaling through the EGF receptor leads to activation of growth-promoting pathways (7), whereas signaling through the TGF-β receptor is associated with decreased pancreatic cancer cell growth in culture (23).

AR was also expressed in the normal and malignant pancreas. However, in the normal pancreas, AR immunoreactivity was most abundant in the nuclei of ductal cells, and was never observed in the cytoplasm of any of these cells. In contrast, in a significant number of pancreatic cancers, AR immunostaining was readily evident in the cytoplasm of the duct-like cancer cells. Inasmuch as the amino terminal portion of AR contains two putative nuclear targeting sequences, these observations suggest that the physiological actions of AR in the normal pancreas are exerted, in part, at the level of the nucleus. Similar nuclear localization of AR has been reported in epithelial cells of the colonic mucosa (13), raising the possibility that this may be a common phenomenon in different types of cells.

AR may exert either stimulatory or inhibitory effects on the growth of cells in culture, depending on the particular cell types that are examined (13, 18). Furthermore, in contrast to EGF and TGF-α, AR cannot act in conjunction with TGF-β to induce the anchorage-independent growth of NRK-SA6 rat kidney cells (24). Therefore, its potential role on the tumor growth in vivo is not readily evident. Recently, however, the presence of AR in breast cancer cells has been correlated with cancer spread to lymph nodes, indicating that it may act to enhance tumor metastasis (21). In the present study we found that the presence of AR in the cytoplasm of pancreatic cancer cells was associated with a more advanced clinical stage. Thus, while it is not clear whether the cytoplasmic localization of AR reflects excessive AR production or inefficient nuclear translocation, our findings suggest that AR may act to excessively activate the EGF receptor in human pancreatic cancer and may have the potential to contribute to disease progression.

References

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