Increased Glyceraldehyde-3-phosphate Dehydrogenase Gene Expression in Human Pancreatic Adenocarcinoma

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ABSTRACT

To identify and characterize genes, the products of which play a role in pancreatic adenocarcinoma, we constructed a complementary DNA (cDNA) library using mRNA from the pancreatic adenocarcinoma cell line HPAF, grown as a nude mouse tumor. Through differential screening, we identified a cDNA clone, pIIB5, that is homologous to an mRNA expressed at significantly higher levels in HPAF cells than in normal human pancreas. The pIIB5 cDNA was homologous to the 3′-untranslated region of glyceraldehyde-3-phosphate dehydrogenase (GAPDH, EC 1.2.1.12) mRNA. Partial sequencing of several HPAF tumor GAPDH cDNA clones revealed no significant differences from previously published GAPDH cDNA sequences. Increased levels of GAPDH mRNA, relative to actin mRNA levels, were found in six pancreatic adenocarcinoma cell lines and two nude mouse tumors, when compared to normal pancreas. Enolase and glucose transporter mRNA levels were also increased in HPAF cells and nude mouse tumor, suggesting a general increase in expression of genes associated with glycolysis in pancreatic adenocarcinoma. Levels of GAPDH protein were elevated in nude mouse tumors and fresh human pancreatic adenocarcinomas compared to normal pancreas. High GAPDH levels may be characteristic of human adenocarcinomas, since colon adenocarcinomas also exhibited high levels of GAPDH compared to normal colon.

INTRODUCTION

Tumor cells characteristically exhibit an increased rate of glycolysis (1). Specific alterations in the carbohydrate metabolic pathways of tumor cells have been described [reviewed by Eignerbrodt et al. (2)]; however, the molecular regulatory mechanisms responsible for these alterations have not yet been defined.

Recent evidence suggests that the activity of one enzyme of the glycolytic pathway, GAPDH, 3 is at least partially regulated at the mRNA level in accelerated growth states (3–6). GAPDH, a tetramer of identical subunits with a molecular weight of 37,000, catalyzes the oxidative phosphorylation of 3-d-glyceraldehyde-3-phosphate to 1,3-bisphosphoglycerate. In quiescent rat fibroblasts, the mRNA encoding GAPDH, as well as mRNAs encoding three other glycolytic enzymes, increases in level as a result of stimulation with EGF or serum (5). GAPDH mRNA and protein levels also increase when differentiated 3T3 adipocytes are exposed to insulin (3). A higher level of GAPDH mRNA was found in a human hepatocarcinoma cell line compared to the level in normal human liver (4). Recently, Tokunaga et al. (6) have shown that expression of both GAPDH mRNA and protein is highly elevated in human lung cancer tissues.

We are interested in studying genes the products of which play a role in the malignant transformation of exocrine secretory epithelial cells of the pancreas. To identify and characterize such genes, we have constructed a cDNA library using mRNA from the human pancreatic adenocarcinoma cell line HPAF (7), grown as a nude mouse tumor. These xenografts are histologically similar to the original tumor (7, 8). Through differential screening of the library, we have identified cDNA clones that represent mRNAs which are expressed at higher levels in HPAF nude mouse tumors than in normal pancreas. One of these cDNA clones was shown to contain a sequence that is homologous to a portion of GAPDH mRNA. We have therefore compared the expression of GAPDH mRNA and protein in pancreatic adenocarcinoma cell lines and nude mouse tumors to GAPDH mRNA and protein expression in the normal human pancreas. This study contributes additional evidence in another human tumor system that GAPDH protein levels increase in tumorigenesis as a result of elevated steady-state levels of GAPDH mRNA.

MATERIALS AND METHODS

Cell Lines and Tumors. The human pancreatic adenocarcinoma cell lines used in this study were HPAF (7), T3M4 (8), and CAPAN (9), obtained from R. S. Metzgar, Duke University, and PANC89 (8), SW979 (10), and PT-45-P1, obtained from Holger Kaithoff, University of Hamburg. The cells were maintained on RPMI 1640 supplemented with 10% fetal calf serum, 2 mM glutamine, penicillin (100 units/ml), and streptomycin (100 μg/ml). For tumor production, 1–5 × 10⁶ cells from various cell lines were injected into nude mice s.c. and tumors were harvested 2–3 weeks later. Cryopreserved human tumor samples were obtained through the Tumor Bank of the Duke University Comprehensive Cancer Center.

RNA Isolation. Total RNA was isolated from tissue (11) and from cultured cells (12), as described previously. Poly(A)⁺ RNA was isolated by chromatography on oligodeoxythymidylicate cellulose (13).

Construction and Screening of the HPAF cDNA Library. A cDNA library was constructed in pBR322, as described (14), from poly(A)⁺ RNA of HPAF cells grown as s.c. tumors in nude mice. The library of over 11,000 cDNA clones was screened by colony hybridization (15) with a 32P-labeled cDNA probe copied from peripheral blood leukocyte mRNA. Approximately 160 colonies which hybridized poorly or not at all with the peripheral blood leukocyte probe were then screened with cDNA probes made from normal human pancreas, HPAF cell line, or HPAF nude mouse tumor mRNA. Nineteen cDNA clones hybridized more intensely with the HPAF cell line or tumor cDNA probe than with the normal pancreas cDNA probe. One clone, pIIB5, that was barely detectable with the normal pancreas cDNA but hybridized very strongly with the HPAF probes, was chosen for further analysis.

Construction and Screening of a GAPDH-specific cDNA Library. The cDNA was prepared from HPAF nude mouse tumor poly(A)⁺ RNA template, using the GAPDH-specific primer synthesized for sequencing (see below), as described (16). Library screening, using the pIIB5 cDNA as a probe, was done as described (16). Of approximately 500 recombinant clones, 2.8% contained cDNA inserts homologous to the pIIB5 cDNA. A 1.3-kilobase GAPDH cDNA insert was ligated into the vector pUC19, and the resulting plasmid was denoted pA1-1.

Northern Blot Analysis. Northern analysis was performed as described previously (12), except that total yeast RNA was used as carrier in hybridization mixes. Hybridization was quantitated by scanning...
densitometry with a Zeineh soft laser scanning densitometer (model SL 504-XL).

DNA Probes. The human fibroblast γ-actin cDNA clone pHF-1 (17) was obtained from P. Gunning, P. Fonte, and L. Kedes (Stanford University School of Medicine and Veteran's Administration Center, Palo Alto, CA). The full-length human α-enolase cDNA clone pHAE (18) was a gift of L. C. Showe (The Wistar Institute, Philadelphia, PA). The glucose transporter cDNA clone pGT25L (19) was obtained from M. Mueckler (Washington University School of Medicine, St. Louis, MO).

Radioactive DNA probes were prepared by oligolabeling (20). Probes were purified by Sephadex G-50 column chromatography.

DNA Sequence Determination. Double-stranded DNA sequencing in pBR322 was performed as described by Bartlett et al. (21), except for using the Klenow fragment of Escherichia coli DNA polymerase I with appropriate adjustments to dideoxynucleotide solutions. Synthesis was primed by the pBR322 Pst primer P1 (Pharmacia). Dideoxynucleotide sequencing in M13 was done by the method of Sanger et al. (22), using [α-35S]dATP as the radioactive label and buffer gradient gels (23). Synthesis was primed with a 20-mer (5'-AGCACAGGGTACTTATTTGA-3') representing sequences from the 3' end of pIISB cDNA as determined from double-stranded DNA sequencing. Sequence homology searches were done in the GenBank database (24), through the Duke Comprehensive Cancer Center.

Protein Extraction. Protein extractions were carried out on Nonidet P-40 lysis buffer (0.5% Nonidet P-40-10 mm Tris-HCl, pH 7.6-1 mm MgCl2-100 mm iodoacetamide-0.2 trypsin inhibitor units/ml aprotinin). Cells were washed three times with phosphate buffered saline (137 mm NaCl-2.7 mm KCl-1.5 mm KH2PO4 monobasic-15 mm Na2HPO4 dibasic, pH 7.2-7.4), resuspended in lysis buffer at 107 cells/ml or greater, and kept on ice for 30 min. Lysates were then centrifuged for 60 min at 16,000 x g and 4°C. Supernatants were stored at -20°C. For tissue samples, approximately 0.5 g of frozen tissue was pulverized and resuspended in 1 ml of lysis buffer and lysis was carried out as described.

Protein determinations were performed according to the modification by Markwell et al. (25) of the Lowry technique, using bovine serum albumin as a standard.

Western Blot Analysis. Protein extracts (20 μg/lane) were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (26) and then transferred to nitrocellulose as described by Towbin et al. (27). Filters were probed with a polyclonal, anti-GAPDH, rabbit anti-

RESULTS

Identification of pIISB cDNA Insert as a GAPDH cDNA Fragment. Differential screening of the HPAF cDNA library yielded 19 cDNA clones that appeared to represent mRNAs expressed at higher levels in HPAF cells (grown in culture or as a nude mouse tumor) than in normal human pancreas. One of these clones, pIISB, was striking in that, upon colony hybridization, the concentration of homologous mRNA(s) appeared to be at least 20-fold higher in HPAF cell line and nude mouse tumor than in normal human pancreas or normal human kidney (not shown).

The sequence of the 197-base insert of pIISB (Fig. 1A) was compared to sequences in the GenBank database and found to be homologous with the 3'-untranslated region of several GAPDH mRNA sequences, including two derived from human liver cDNAs (4, 30) and one from an SV40 transformed human fibroblast cDNA (28). While this study was in progress, Tokunaga et al. (6) published a sequence for a human lung cancer GAPDH cDNA, which contains no nucleotide differences from our sequence.

To compare the expression of GAPDH mRNA in HPAF cells grown in culture or as a nude mouse tumor with its expression in normal human pancreatic tissue, Northern analysis of poly(A)+ RNA was done using pIISB cDNA as a probe (Fig. 2A). Both normal and tumor cells contained a single hybridizing mRNA of approximately 1.3 kilobases, as previously reported for human cytoplasmic GAPDH mRNA (31). The level of GAPDH mRNA in each sample was quantitated by densitometry and normalized to the level of actin mRNA (Fig. 2B). Levels of GAPDH mRNA in HPAF cells grown as a nude mouse tumor and cells grown in culture were higher by 8- and 20-fold, respectively, than in normal human pancreas.

Analysis of GAPDH-specific cDNAs Synthesized from HPAF Poly(A)+ RNA. To demonstrate that the sequence represented by pIISB cDNA is contiguous with GAPDH coding sequence in HPAF tumor cells, we attempted to isolate a longer cDNA containing the pIISB cDNA sequence. No additional GAPDH cDNA-containing clones were identified from the original HPAF cDNA library. Therefore, a 20-mer from the 3' end of pIISB cDNA (Fig. 1A) was used to prime cDNA synthesis for...
Expression of GAPDH in Pancreatic Adenocarcinoma Cell Lines, Grown in Culture or as Nude Mouse Tumors, and in Fresh Tumor Samples. Western blotting analysis with a polyclonal anti-GAPDH antiserum was performed in order to analyze GAPDH protein expression in the pancreatic adenocarcinoma cell lines HPAF, CAPAN, and T3M4 (Fig. 3C). A M, 37,000 polypeptide corresponding to the GAPDH monomer was detected in total protein extracts of all three cell lines. The monomer was also present, at similar levels, in a T-cell leukemia and an Epstein Barr virus immortalized B-cell line (not shown).

The expression of this polypeptide was not significantly different between cells growing exponentially and cells at confluence (data not shown). A M, 37,000 polypeptide, identified as the GAPDH monomer, was previously reported to be expressed on the outer cell surface of B- and T-lymphocytic cell lines and the K562 erythroleukemic cell line, but restricted to the construction of a cDNA library enriched in sequences homologous to the pIISB cDNA.

Fourteen clones containing pIISB-specific cDNA were isolated and partially sequenced. Nine of these clones (not shown) carried inserts of 80-150 bases, representing fragments of the 3'-untranslated region of GAPDH mRNA. No differences from the sequence of pIISB were found (Fig. 1A). Five of these inserts, along with pIISB, are depicted in Fig. 1B.

The longest cDNA clone isolated, A1, contained an insert of approximately 1.3 kilobases. The sequence of 166 nucleotides from the 3' end of this cDNA was again identical to that of pIISB cDNA and a portion of the GAPDH mRNA 3'-untranslated sequence reported by Tokunaga et al. (6). Sequencing at the 5' end covered 203 nucleotides. Sixty nucleotides were identical to a previously published sequence for the 5'-untranslated region of GAPDH mRNA (6, 30, 32), while 143 nucleotides of coding sequence were identical to the sequence previously published for GAPDH mRNAs (6, 28, 30, 32).

Expression of GAPDH mRNA in HPAF Pancreatic Adenocarcinoma Cell Lines and Nude Mouse Tumors. To determine whether the increase in GAPDH mRNA levels relative to actin mRNA levels is a common feature of human pancreatic adenocarcinoma cells, we compared the levels of these mRNAs in several pancreatic adenocarcinoma cell lines and in another nude mouse tumor, to the levels in normal human pancreas. Pancreatic RNA preparations are often damaged by the high concentration of nucleolytic enzymes in pancreatic tissue. Therefore, an internal standard is required when comparing the concentration of a particular mRNA in the pancreas to its concentration in other tissues or cells. Actin mRNA level was a more relevant internal control for the GAPDH mRNA level than the 18 S or 28 S rRNA level, because rRNA appeared more resistant than mRNA to attack by pancreatic RNases (data not shown). While actin mRNA level can be affected by accelerated growth states, it is usually increased rather than decreased (5, 33). Thus, the use of actin mRNA level as an internal control results, at worst, in an underestimate of change for an mRNA the level of which increases during tumorigenesis.

Table 1  Densitometric analysis of GAPDH and actin mRNA levels

<table>
<thead>
<tr>
<th>Sample</th>
<th>GAPDH</th>
<th>Actin</th>
<th>Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal human pancreas</td>
<td>0.46</td>
<td>0.68</td>
<td>0.68</td>
</tr>
<tr>
<td>Pancreatic adenocarcinoma cell lines</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HPAF</td>
<td>8.5</td>
<td>0.90</td>
<td>9.4</td>
</tr>
<tr>
<td>T3M4</td>
<td>8.3</td>
<td>3.6</td>
<td>2.3</td>
</tr>
<tr>
<td>CAPAN</td>
<td>8.5</td>
<td>5.3</td>
<td>1.6</td>
</tr>
<tr>
<td>PANc89</td>
<td>13</td>
<td>2.2</td>
<td>5.9</td>
</tr>
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<td>SW979</td>
<td>5.5</td>
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<tr>
<td>PT-45-P1</td>
<td>15</td>
<td>5.9</td>
<td>2.5</td>
</tr>
<tr>
<td>Nude mouse tumors</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HPAF</td>
<td>2.8</td>
<td>0.87</td>
<td>3.2</td>
</tr>
<tr>
<td>T3M4</td>
<td>11</td>
<td>1.1</td>
<td>10</td>
</tr>
</tbody>
</table>

*Expressed in arbitrary units.

Table 1  Densitometric analysis of GAPDH and actin mRNA levels

One µg of total cytoplasmic RNA from pancreatic adenocarcinoma cell lines, 1 µg of total RNA from nude mouse tumors, and 20 µg of total RNA from normal human pancreas were fractionated on a formaldehyde-agarose gel. Northern blot hybridization was done using the GAPDH (from pAl-1) and actin (from pHF-1) cDNA probes simultaneously, and the blot was exposed to Kodak XAR-5 film. The level of hybridization to individual bands was determined by scanning densitometry of the autoradiograph.
the cytoplasmic face of the cell membrane in mature erythrocytes (28, 34, 35). However, when we examined B-lymphocytic, T-lymphocytic, K562, CAPAN, HPAF, and T3M4 cell lines by surface immunofluorescence and by surface iodination followed by immunoprecipitation, all of the cell lines were negative for surface reactivity with the anti-GAPDH antiserum (data not shown).

In addition to the M, 37,000 species, the anti-GAPDH antiserum reacted with a M, 68,000 polypeptide in CAPAN and T3M4 cells. Although not obvious in the same exposure, this species was also detected in HPAF cells at lower levels (not shown). The M, 68,000 polypeptide could not be detected in either of the two lymphocytic cell lines and was therefore specific to pancreatic adenocarcinoma cells. We have not, as of yet, fully characterized this polypeptide.

The anti-GAPDH antiserum was used to analyze GAPDH expression in total protein extracted from a nude mouse tumor grown from a cloned HPAF subline (Fig. 3B). A M, 37,000 polypeptide was detected, as well as a species of lower molecular weight, which we presume to be a degradation product of GAPDH. In addition, a M, 68,000 species reacted with the antiserum. Several nude mouse tumors grown from different HPAF subclones showed a similar pattern of reactivity with the antiserum (not shown).

The level of GAPDH subunit in one nude mouse tumor, grown from T3M4 cells, was compared with that in normal human pancreas (Fig. 3B). The M, 37,000 GAPDH subunit was present at low levels in normal pancreatic tissue; no M, 68,000 species was detected. The level of GAPDH in the T3M4 nude mouse tumor was 50-fold higher than that in normal human pancreas. A polypeptide with a molecular weight of 68,000 was also detected in the nude mouse tumor.

Although we were unable to recover intact mRNA from any fresh human pancreatic adenocarcinoma tissue, undergraded protein could be extracted from these tumors, as assessed by Coomassie blue staining of gel fractionated samples (not shown). Levels of the GAPDH monomer were higher in the two different pancreatic adenocarcinoma samples examined than in normal human pancreas (Fig. 3C). The M, 68,000 polypeptide was also detected in both tumors. However, in PACA2, the level of M, 68,000 polypeptide was lower compared to that of the GAPDH subunit, while in PACA1 the reverse was the case. Several other bands which reacted with the antiserum appeared in all samples and were therefore not considered significant.

Expression of Other Glycolysis-associated mRNAs in HPAF Cells and Nude Mouse Tumor. We considered that the high GAPDH mRNA expression observed in HPAF cells grown in culture or as nude mouse tumor could be related to a general increase in expression of glycolysis associated genes at the mRNA level. Therefore, we examined the steady-state levels in these same cells of mRNAs for a-enolase, another glycolytic enzyme, and glucose transporter protein, responsible for glucose uptake. The levels of enolase mRNA (Fig. 5A), when normalized to levels of actin mRNA, were 6-fold higher in HPAF nude mouse tumor and HPAF cell cytoplasmic poly(A)+

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RNA compared to normal human pancreatic poly(A)+ RNA. Similarly, high levels of glucose transporter mRNA (Fig. 5B) were found in HPAF cells and in the HPAF nude mouse tumor, while in normal human pancreas this mRNA was barely detectable.

DISCUSSION

We have demonstrated that GAPDH mRNA levels are elevated in human pancreatic adenocarcinoma cells and that GAPDH protein levels are increased in nude mouse tumors and in fresh human pancreatic adenocarcinoma samples. In sequencing cDNAs of mRNAs isolated from pancreatic adenocarcinoma tissue, we found no evidence for a tumor specific GAPDH mRNA. While a multigene family homologous to GAPDH cDNA is present in the human genome (4, 30, 36, 37), most of the human GAPDH cDNA sequences obtained to date indicate there is only one functional human GAPDH gene (6, 28, 30, 32). One exception, not noted previously, is a published sequence for human liver GAPDH cDNA (4). Nucleotides 1–38 of this sequence bear little homology to the corresponding sequence of the 5'-untranslated region determined for four other cDNAs (6, 30, 32), while the remainder of the 5' leader sequence, nucleotides 39–60, is identical to the others. This discrepancy might be a cloning artifact or due to variability in processing of the GAPDH gene at the 5' terminus. The same group (4) has presented a 65-base pair sequence of a GAPDH coding region cDNA fragment, from a human fetal liver cDNA library, which contains several significant nucleotide differences from other GAPDH coding sequences.

The Mr 37,000 GAPDH monomer was readily detected by Western blotting analysis in several pancreatic adenocarcinoma cell lines. Analyses of human pancreatic adenocarcinoma tissues revealed higher levels of GAPDH monomer in tumors than in normal pancreatic tissue. Since these tumors are thought to be of ductal cell origin, we considered that overexpression of GAPDH might simply reflect an abundance of this protein in pancreatic ductal cells. However, when the same anti-GAPDH antiserum was used to stain frozen tissue sections, normal human pancreas sections stained negative or uniformly faintly positive, with no specific staining of the ductal cells (data not shown).

We consistently observed an Mr 68,000 polypeptide in Western blotting analyses of pancreatic adenocarcinoma cells, grown in culture or as nude mouse tumors, and in human pancreatic adenocarcinoma tissues. This polypeptide was never detected in lymphocytic cell lines or normal human pancreatic tissue. Because a polyclonal antiserum was used for these studies, we are not certain of the relationship of this polypeptide to GAPDH or the significance of its apparently exclusive expression in pancreatic adenocarcinoma cells. Peptide mapping studies will be required to resolve the relationship between these two species.

Ohkubo et al. (38) found no significant increase in the amount of Mr 37,000 polypeptide when normal colon and colon tumor tissues were compared by Coomassie blue staining, a less sensitive and less specific method than the Western blotting analysis we have used for assaying GAPDH expression. We consistently found very low or undetectable levels of GAPDH in normal colon tissues, but relatively high levels in two colon adenocarcinomas. An increased level of GAPDH expression, therefore, is characteristic not only of various histological types of human lung cancer (6) but also of pancreatic and colon adenocarcinomas.

The increases in GAPDH mRNA and protein expression demonstrated here for pancreatic adenocarcinoma might be associated with the increased rate of glycolysis generally found in tumor cells (1, 2). Elevation of GAPDH mRNA levels was accompanied by an increase in the levels of enolase and glucose transporter mRNAs. Regulation at the level of mRNA expression has previously been shown for both the enolase and glucose transporter genes (5, 39, 40). The level of enolase mRNA, like GAPDH mRNA, increases after EGF or serum stimulation of rat fibroblasts (5). Interestingly, Korc et al. (41) have shown...
that several pancreatic adenocarcinoma cell lines, particularly T3M4, overexpress the EGF receptor. An accelerated rate of glucose transport has long been known to accompany cellular transformation (42). Recently, glucose transporter mRNA levels were shown to increase as a result of transformation of rat fibroblasts with either Fujinami sarcoma virus (39) activated ras or src oncogenes (40). Experiments in our laboratory indicate that transformation of 3T3 cells with ras or myc can increase GAPDH mRNA levels. It is therefore interesting to note that a 2- to 4-fold increase in myc mRNA expression has been reported in HPAF cells compared to normal human pancreas (43).

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