Upregulation of thrombospondin-1 and angiogenesis in an aggressive human pancreatic cancer cell line selected for high metastasis

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Abstract
Pancreatic cancer remains a leading cause of death despite its relatively low incidence. As in many other solid tumors, angiogenesis is critical to the growth and metastasis of this cancer. Through serial in vivo passages in mice, we have developed a highly aggressive variant of human pancreatic cancer cell line XPA-1 which shows more rapid primary tumor growth, faster time to metastasis, and more rapid lethality than the parental cell line. The high-metastatic variant developed a much denser tumor vasculature early during growth within the pancreas. Interestingly, examination of the in vitro growth of this aggressive variant yielded no significant difference from the parental cell line. Real-time PCR evaluation of genes involved in angiogenesis revealed a 24-fold increase in Thrombospondin-1 expression in cells derived from the high-metastatic variant when compared with the parental cell line. These findings provide direct evidence that elevated capability for angiogenesis, mediated by specific changes in gene expression, can lead to a large increase in cancer aggressiveness and resulting metastasis. These findings have important implications for the treatment of metastatic disease. [Mol Cancer Ther 2009;8(7):1779–86]

Introduction
Despite its relatively low incidence in the population, pancreatic adenocarcinoma remains the fourth leading cause of cancer-related death in the United States (1), with overall 5-year survival as low as 5% (2). Recent advances in surgical and critical care have had little impact on the epidemiology of this disease, largely because of its often late stage at presentation (3) and its aggressive biology. The precise mechanisms involved in local tumor progression and metastasis in this disease remain poorly understood. Local invasion and distant metastasis occur within complex tumor-host micro-environments and involve signal exchange between cancer cells and host stroma. The tumor microenvironment promotes proliferation and survival of cancer cells in addition to stimulating cancer cell migration and invasiveness (4). Pancreatic adenocarcinoma, in particular, is characterized by a dense stromal reaction, which has been postulated to support tumor invasion (5).

The oxygen and nutrients that are central to cell survival are supplied to tissues by functioning vasculature. New blood vessel formation (angiogenesis) is critical to continued local and systemic progression of rapidly growing tumors. The formation of new blood vessels is initiated by signaling from cancer and stromal cells, including the release of stimulatory factors, which begin a series of events ultimately leading to endothelial cell degradation of extracellular matrix, migration, and proliferation (6, 7). A number of factors involved in angiogenesis are often perturbed in pancreatic cancer, including upregulation of proangiogenic cytokines (8). Pancreatic cancer has been shown to metastasize via hematogenous spread (9), and clinical studies in patients with pancreatic cancer have linked increased tumor angiogenesis with poorer prognosis (10).

The realization of the critical nature of angiogenesis in tumor growth and metastasis has led to the development of pharmacologic agents that block various steps in the process of tumor neovascularization (11). Growing experience with these drugs has underscored the complex nature of tumor angiogenesis, which involves a dynamic interplay between positive and negative secreted regulators in addition to local tissue modulators (6). Indeed, both pro- and anti-angiogenic mediators may play a role in tumor vasculature maturation and function (12).

We describe here a highly aggressive human pancreatic cancer cell line generated through repeated in vivo passage to select highly metastatic cells, which shows both increased tumor vascularularity and upregulation of Thrombospondin-1 (TSP-1), a molecule whose role in angiogenesis remains incompletely understood (13, 14).

Materials and Methods
Cell Culture
Cells were maintained in RPMI 1640 media supplemented with 10% fetal bovine serum (FBS) from Gibco-BRL, Life
Hign-Metastatic Variant of Pancreatic Cancer

The human pancreatic cancer cell line XPA-1 was a gift from Dr. Anirban Maitra at Johns Hopkins University. All media were supplemented with penicillin/streptomycin (Gibco-BRL), L-glutamine (Gibco-BRL), MEM nonessential amino acids (Gibco-BRL), sodium bicarbonate (Cellgro, Herndon VA), and sodium pyruvate (Gibco-BRL). All cell lines were cultured at 37°C with 5% CO₂.

Production of a Stable Red Fluorescent Cell Line (XPA1-RFP)

The pDSRed-2 vector from Clontech Laboratories, Inc. (Palo Alto, CA) was used for stable expression of RFP in the human pancreatic cancer cell line XPA-1. The HindIII/NotI fragment of pDsRed2 (Clontech Laboratories, Inc.) was inserted into the HindIII/NotI site of the pLNCX2 plasmid (Clontech Laboratories, Inc.) containing the neomycin resistance gene to produce the pLNCX2-DsRed2 plasmid. The pLNCX2-DsRed2 plasmids were transfected into PT67 packaging cells using the LipofectAMINE system (Gibco-BRL). Vector production was accomplished via growth in PT67 packaging cells cultured in the presence of 200 to 1,000 μg/mL Geneticin (G418) from Invitrogen Corporation (Carlsbad, CA) for 7 days. Twenty percent confluent XPA-1 cells were incubated with retroviral supernatants of the packaging cells until high levels of DsRed2 expression were observed under fluorescence microscopy. The cells were then harvested and subcultured in selective medium containing G418. The G418 was increased in a stepwise fashion from 200 μg/mL to 2,000 μg/mL. Clones with high DsRed2 expression were isolated and grown for 10 passages in the absence of G418 to select for stable in vitro expression of DsRed2.

Animal Care

Athymic nu/nu nude mice and transgenic nu/nu mice with the nestin-driven green fluorescent protein (GFP) transgene (which express GFP in nestin-positive tissues; ref. 15), both between 4 and 6 weeks of age, were maintained in a barrier facility on high efficiency particulate air (HEPA)-filtered racks. The animals were fed with autoclaved laboratory rodent diet (Teckland LM-485; Western Research Products, Orange, CA). All invasive procedures and imaging were done with the animals anesthetized by intramuscular injection of 0.02 mL of a solution of 50% ketamine, 38% xylazine, and 12% acepromazine maleate. All surgical procedures were done under sterile conditions. All animal studies were conducted in accordance with the principles and procedures outlined in the National Institutes of Health (NIH) Guide for the Care and Use of Animals under Assurance number A3873-01.

Orthotopic Implantation of Pancreatic Tumors

Human pancreatic cancer xenografts from the pancreatic cancer cell line XPA1-RFP were initially established in nude mice by SOI as described above. These tumors were allowed to grow until the animals developed widespread metastases and malignant ascites. At the time of sacrifice, the ascitic fluid was harvested and placed on ice. Four- to 6-week-old female nu/nu mice were anesthetized and a small transverse incision was made in the left lateral flank to the skin and peritoneum. The tail of the pancreas was exposed, and a 1-mm³ piece of tumor tissue was sutured into the pancreatic tail using 8-0 nylon surgical sutures (US Surgical). After six serial passages in this fashion, the resulting primary tumor and malignant ascites were harvested. The primary and malignant ascites were gently dissociated and plated for in vitro culture. After a single in vitro passage for expansion, the passage-6 ascites and passage-6 primary cells were stored in liquid nitrogen for later analysis.

Animal Imaging

Mice were imaged using the Olympus OV100 Small Animal Imaging System (Olympus Corp., Tokyo, Japan), containing an MT-20 light source (Olympus Biosystems Planegg, Germany) and DP70 CCD camera (Olympus Corp.) on the basis of the expression of fluorescent proteins, a procedure developed in our laboratory (17, 18). All images were analyzed using Image-J (NIH, Bethesda, MD) and were processed for contrast and brightness with the use of Photoshop element-4 (Adobe Systems Inc., San Jose, CA).

Blood Vessel Measurement

Tumors harvested from nestin-GFP mice, which express GFP nude in nascent blood vessels, as established by our laboratory (15), were weighed and measured for volume and gently compressed between two glass slides as previously described (19). Sequential images at high magnification using the GFP narrow bandpass filter were obtained with the OV100 Small Animal Imaging System. All GFP-expressing blood vessels were measured using Image-J software and calculated as blood vessel density (blood vessel length/tumor volume = mm/mm³). Tumor volume was calculated as length × height × width × 0.52.

Histology

Primary and metastatic tumor samples were removed en bloc with surrounding tissue at the time of sacrifice, fixed in Bouin’s Solution, and embedded in paraffin prior to sectioning and staining with hematoxylin and eosin (H&E) for standard light microscopy. All slides were imaged using...
Table 1. Gene expression in the high-metastatic variant

<table>
<thead>
<tr>
<th>Type of Gene</th>
<th>Fold Difference (P6/P0)</th>
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<tbody>
<tr>
<td></td>
<td>0.1–0.5</td>
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<tr>
<td>Angiogenic factors</td>
<td>VEGFC</td>
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<tr>
<td>Adhesion molecules</td>
<td>ANGPT1</td>
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<tr>
<td>Prostates, inhibitors, and other molecules</td>
<td>ANGPT4</td>
</tr>
<tr>
<td>Transcription factors and others</td>
<td>CXCL10</td>
</tr>
<tr>
<td>Cytokines and chemokines</td>
<td>CXCL10</td>
</tr>
<tr>
<td>Other factors involved in angiogenesis</td>
<td>CXCL10</td>
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<tr>
<td>Other growth factors and receptors</td>
<td>CXCL10</td>
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<td>Adhesion molecules</td>
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NOTE: Multiple genes involved in angiogenesis, including growth factors and receptors, adhesion molecules, proteases, matrix proteins, and transcription factors, were evaluated for expression using real-time PCR via the RT2 Profiler PCR Array System. The table lists the genes of interest evaluated and their fold increase or decrease in the high-metastatic variant as compared with the parental cell line. In contrast to many of the angiogenesis-related genes evaluated, TSP-1 showed a 24-fold increase in expression in the high-metastatic variant. Complete abbreviations of genes can be found at http://www.sabiosciences.com/.
an inverted Nikon DE-300 microscope and Spot camera RD. All images were analyzed using Image-J and were processed for contrast and brightness with the use of Photoshop element-4.

**In vitro Proliferation**

All cell lines evaluated were thawed from frozen stocks and assayed within two passages after thaw. The aggressive (generated from either primary tumor or malignant ascites) and parental cell lines (XPA1-RFP) were plated in 96-well plates in triplicate at 5,000 cells per well. Four days later, proliferation was quantified using an XTT assay from Bio-tium Inc. (Hayward, CA). The XTT developing reagent was added to the wells and allowed to incubate for 4 hours at 37°C. Plates were then read using a standard plate reader at OD 490 nm. OD readings between the three cell lines were evaluated by ANOVA using NCSS Statistical Software (Kaysville, UT) for statistically significant differences in cell proliferation.

**RNA Isolation from Cultured Cells**

Total RNA was isolated from the high-metastatic and parental cell lines using the RNeasy kit from Qiagen (Valencia, CA). Briefly, cells were grown in RPMI as described. At 75% confluence, the cells were scraped from the dish, washed once using ice-cold phosphate buffered saline (PBS) from Gibco-BRL, and counted. The cells were then pelleted and homogenized. An equal volume of 70% ethanol was added to the homogenized lysate, and the mixture was applied to an RNeasy midi-spin column. The RNA was washed several times while on the column according to the manufacturer’s instructions and eluted using RNase-free water. Total RNA concentration and purity in the eluted samples obtained was quantitated using a

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**Figure 1.** Malignant ascites were isolated from tumor-bearing animals and serially passaged into the pancreas of 4- to 6-week-old female nu/nu mice. The aggressive high-metastatic variant grew more rapidly in vivo and was more rapidly lethal than the parental cell line XPA1-RFP. Measurement of tumor surface area (measured in mm²) via whole-body imaging showed much more rapid primary growth of tumors generated from the high-metastatic variant cell line. **A**, representative animals from each group imaged using the Olympus OV-100 show the earlier time to widespread metastasis in the aggressive variant. **B**, survival data from each group show more rapid mortality in animals bearing tumors from the aggressive variant. **C**, the solid line indicates growth of tumors in animals implanted with the aggressive variant, whereas the dotted line represents the parental line.
NanoDrop ND-100 UV spectrophotometer from NanoDrop Technologies, LLC (Wilmington, DE).

**cDNA Preparation**

Following RNA preparation, the samples were treated with DNase to ensure elimination of genomic DNA, and the extracted RNA was converted to cDNA using the RT² First Strand Kit from SuperArray Bioscience Corporation (Frederick, MD). Briefly, 25 to 50 ng RNA was combined with 2 μL genomic DNA-elimination buffer and brought up to a final volume of 10 μL using RNase-free H2O. This mixture was incubated at 42°C for 5 minutes, then chilled on ice. Ten microliters of RT Cocktail was then added to this mixture and incubated at 42°C for exactly 15 minutes followed by 5 minutes at 95°C. Ninety-one microliters ddH2O was added to each 20 μL cDNA synthesis reaction, and the diluted cDNA mixture was stored at −20°C until used for gene expression profiling.

**Gene Expression Profiling**

The RT² Profiler PCR Array System (SuperArray, Bioscience Corp.) was used to evaluate the aggressive and parental cell lines for differential gene expression. The genes evaluated in this PCR array include multiple genes involved in...
in angiogenesis and vascular homeostasis (Table 1). Real-time PCR detection was carried out per the manufacturer's instructions. The experimental cocktail was prepared by adding 550 μL of the SuperArray RT2 qPCR master mix and 448 μL ddH2O to 102 μL of the diluted cDNA mixture. For real-time PCR detection, 10 μL of this cocktail was added to each well of the 384-well PCR array. The array was then cycled on a real-time thermal cycler through the following program: 1 cycle of 10 minutes at 95°C followed by 40 cycles of 15 seconds at 95°C and of 1 minute at 60°C. SYBR Green fluorescence was detected from each well during the annealing step of each cycle. Values were exported to a template Excel file for analysis. Analyses of the raw data were done through the Superarray Data Analysis Web Portal (SuperArray Bioscience Corp.).

Results and Discussion

Serial in vivo passage of malignant ascites from human pancreatic cancer-bearing nu/nu mice was used to generate a highly aggressive passage-6 generation cell line with much more rapid primary tumor growth and faster time to distant metastasis. The tumors generated from the parental cell line XPA1-RFP grew slowly (Fig. 1A) with eventual metastasis to multiple extra-pancreatic sites. Mice bearing these tumors showed widespread metastasis 9 to 12 weeks after implantation (Fig. 1B) and died within 12 to 14 weeks (Fig. 1C). Repeated in vivo passage of malignant ascites from XPA1-RFP tumor-bearing mice into the pancreas of recipient nude mice yielded a much more aggressive cell line. Tumors generated from the aggressive variant grew rapidly (Fig. 1A) and showed widespread metastasis within 3 to 5 weeks (Fig. 1B). Time to death in this group was from 3 to 8 weeks (Fig. 1C).

Orthotopic parental and aggressive-variant tumors metastasized to distant sites in a similar distribution and yielded histologically similar lesions. Mice bearing tumors generated from the parental cell line XPA1-RFP developed lesions in the liver, spleen, abdominal lymph nodes, mesentry, and lung by 9 to 12 weeks after implantation. Similarly, the aggressive variant metastasized to liver, spleen, abdominal lymph nodes, mesentry, and lung. Whereas the metastatic lesions in the animals bearing tumors from the aggressive variant were found in the same distribution as their parental line, they developed much more rapidly than tumors generated from the parental cell line, within 3 to 5 weeks of tumor implantation. Microscopic evaluation of the metastatic and primary tumors from animals after implantation of either the parental or aggressive-variant cell lines revealed lesions that were histologically indistinguishable (Fig. 2).

In addition to faster growth in vivo, tumors from the aggressive cell line had greater blood vessel density at 8 days after implantation. Cells generated from the aggressive variant were assayed for their early in vivo growth and angiogenesis within the pancreas of transgenic nude mice with
Within the pancreas of living animals and metastasized earlier to distant sites, the cell line generated from this variant did not grow more rapidly in culture. This suggested that the aggressive nature of this variant was related to something other than increased cell proliferation, and the adaptations responsible for its aggressive in vivo behavior may be related to the pancreatic microenvironment present within the living animal. Indeed, evaluation of early orthotopic tumors generated from the aggressive-variant and parental cell lines revealed larger tumors at 8 days postimplantation with greater blood vessel density in the aggressive tumors. Interestingly, real-time PCR evaluation of cells from this aggressive variant showed a 24-fold increase in TSP-1 RNA expression when compared with the parental, less aggressive, cell line.

TSP-1 is a trimeric 450-kDa glycoprotein present in platelets and the extracellular matrix. This protein participates in platelet aggregation as well as in the tissue response to injury (20, 21). The role of TSP-1 in angiogenesis has been somewhat controversial, with both stimulatory and inhibitory functions reported (13, 14, 22, 23). Qian and colleagues found that bovine aortic endothelial (BAE) cell tube formation was enhanced by low levels of TSP-1 but inhibited at higher levels (14). TSP-1 mediated this effect on tube formation through the upregulation of matrix metalloproteinase-9 (MMP-9) in the BEA cells. Nicosia and colleagues showed that matrix-bound TSP-1 was able to promote growth of microvessels and fibroblast-like cells in culture in a dose-dependent fashion (22). TSP-1 has also been shown to be involved in platelet-stimulated smooth muscle proliferation (24), which may play a role in vascular remodeling. Our results suggest an important role for TSP-1 in elevated angiogenesis and metastasis. These findings have important implications for the treatment of metastatic disease.

Future experiments will evaluate protein expression of TSP-1 in the aggressive-variant cell line as compared with both the parental cell line and normal pancreatic tissue, as well as gene and protein expression of TSP-1 in tumors growing within the mouse pancreas. In addition, the time-dependent induction of TSP-1 during tumor development and metastasis will be evaluated, as will the effect of in vitro and in vivo downregulation of the TSP-1 gene.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

References


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