Research Paper

Stromal Responses to Carcinomas of the Pancreas

Juxtatumoral Gene Expression Conforms to the Infiltrating Pattern and Not the Biologic Subtype

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Received 11/16/04; Accepted 12/23/04

This manuscript has been published online, prior to printing, for Cancer Biology & Therapy Volume 4, Issue 2. Definitive page numbers have not been assigned. The current citation for this manuscript is: Cancer Biol Ther 2005; 4(3):

http://www.landesbioscience.com/journals/cbt/abstract.php?id=1501

On the issue is complete and page numbers have been assigned, the citation will change accordingly.

KEY WORDS
desmoplasia, macrophage, pancreatic cancer, metastasis, IPMN, invasion

ACKNOWLEDGEMENTS

Supported by NIH grants CA62924 and CA106610, the Joseph C. Monastra Fund for Pancreatic Cancer Research, the Jeff Zgonina Fund for Pancreatic Cancer Research, the Michael Rolfe Pancreatic Cancer Foundation, and by The Sol Goldman Pancreatic Cancer Research Center.

ABSTRACT

If there is a “science” of tumor-stromal interactions, there must be a set of biologic rules that are organ-site dependent. One way to explore this hypothesis would be to compare the patterns of gene expression of two biologically distinct neoplasms that arise within the same organ site. Using nonradioactive in situ hybridization, we evaluated the gene expression patterns of three genes previously shown to be robust markers of the juxtatumoral stroma within eight infiltrating ductal adenocarcinomas of the pancreas (ApoC1, ApoD, and MMP11), and compared these patterns to those associated with seven infiltrating colloid and tubular carcinomas arising in association with intraductal papillary mucinous neoplasms (IPMNs), a histologically distinct form of primary carcinoma of the pancreas, two surgically resected samples of chronic pancreatitis and two surgically resected pancreatic cancer liver metastases. Robust juxtatumoral stromal expression was noted for all three markers within all eight conventional infiltrating ductal adenocarcinoma tissues, but not in samples of chronic pancreatitis. Among the carcinomas arising within an IPMN, expression for all three markers was also noted for five of seven infiltrating carcinomas analyzed. However, when labeling for these three markers was analyzed with respect to infiltrative growth pattern, positive labeling was only seen in areas of tubular (ductal-type) growth and not in areas of colloid carcinoma. This observation was further supported by two infiltrating carcinomas arising in an IPMN that showed both tubular and colloid growth patterns within the same neoplasm indicating the host stromal response observed may relate to infiltrative growth pattern rather than the biology of the primary tumor type. Moreover, these robust patterns within conventional infiltrating ductal adenocarcinomas were not retained within matched metastases to the liver, indicating the importance of the tumor microenvironment in the host stromal response. Juxtatumoral stroma was found to be composed of a least two cell types, tumor-infiltrating macrophages and fibroblasts, highlighting the complexity of tumor-stromal interactions within an infiltrating carcinoma. Since the juxtatumoral gene expression response is the strongest indication of direct communication between stroma and cancer cells, we provide evidence of a stereotypical response to infiltrative growth that might predominate in tumor-stromal interactions independent of cancer type, a finding with clinical implications for therapeutic modalities that target this response in human tumors.

INTRODUCTION

A host stromal response to an infiltrating carcinoma, or desmoplasia, is a common feature of neoplasia. This process is characterized by a complex interaction between the host and the invading neoplasm, and is composed of fibroblasts, various inflammatory cells, proliferating vascular structures, as well as normal parenchymal cells undergoing atrophy at the invasive edge of the neoplasm. These components comprise the tumor microenvironment, a highly dynamic process with presumed effects on tumor growth, drug sensitivity and chromatin structure among others.1,2

We have previously reported that the host stromal response to an infiltrating cancer is, in part, comprised of two distinct and reproducible regions termed the panstromal compartment and the juxtatumoral compartment.3,4 Whereas the panstromal compartment encompasses all stromal cells within the desmoplastic response to an infiltrating tumor, the juxtatumoral compartment corresponds to those stromal cells immediately adjacent to the neoplastic cells and represents the putative site of tumor-host interactions.5 Our previous studies have indicated three genes in particular, apolipoprotein C-1 (ApoC1), apolipoprotein D (ApoD) and MMP11, are robust and specific markers of the juxtatumoral stroma within infiltrating ductal adenocarcinomas of the pancreas. This specialized
The juxtatumoral region was confirmed in breast cancer, another tumor type with a prominent stromal component, although differences in the juxtatumoral stroma gene expression between pancreatic and breast carcinomas indicated potentially important differences in the desmoplastic response. These observations in turn raised questions regarding the desmoplastic response to human neoplasms, i.e., do histologically different neoplasms arising from the same organ produce similar or different host stromal responses? Is the gene expression response of the host stroma to primary neoplasms similar to or different from the stromal response to metastatic tumors? To explore the variability of the host stromal response to infiltrating cancers, we compared the patterns of juxtatumoral gene expression between two histologically distinct forms of primary carcinoma of the pancreas-conventional infiltrating ductal adenocarcinomas and intraductal papillary mucinous neoplasms (IPMNs) having associated infiltrating colloid or tubular adenocarcinomas-and compared these patterns to samples of chronic pancreatitis and metastatic pancreatic carcinoma (Fig. 1). In doing so, we sought to define the differences and similarities of stromal gene expression patterns in relation to the organ site.

**MATERIALS AND METHODS**

Tissue specimens. Formalin-fixed and paraffin-embedded tissues from were collected from the Surgical Pathology files of The Johns Hopkins Hospital. Tissues used included 17 pancreaticoduodenectomy (Whipple resection) specimens representing eight patients with a conventional infiltrating pancreatic duct adenocarcinoma, seven patients with an infiltrating carcinoma arising in an intraductal papillary mucinous neoplasm (IPMN), and two patients with chronic pancreatitis. For two of the eight patients with an infiltrating pancreatic duct adenocarcinoma, a matched metastatic pancreatic carcinoma to liver was also available. Hematoxylin and eosin-stained sections of each paraffin-embedded tissue were examined to confirm the presence of infiltrating adenocarcinoma or chronic pancreatitis within the section.

Preparation of riboprobes. Riboprobes specific to the juxtatumoral stromal markers apolipoprotein C-1 (ApoC1), apolipoprotein D (ApoD) and MMP11 were generated following the methods reported previously.

Briefly, DNA templates were generated by PCR with incorporation of a T7 promoter into the antisense or sense primer. Following phenol:chloroform purification of amplified DNA, 200 ng of the DNA templates were used to generate either antisense or sense riboprobes by in vitro transcription with digoxigenin labeling reagents and T7 polymerase according to the
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**Non-radioactive in situ hybridization of Paraffin-embedded tissues.** Five-micron thick sections were cut from the paraffin blocks, deparaffinized in xylene, and hydrated in graded concentrations of ethanol for 5 minutes each. Sections were incubated with 1% hydrogen peroxide, followed by digestion in 10 µg/ml of Proteinase K at 37°C for 30 minutes. Sections were hybridized overnight at 15–25°C below the Tm calculated for each individual riboprobe with a 200 ng/ml dilution of either antisense or sense riboprobes in mRNA hybridization buffer (DAKO, Carpinteria, CA). The following day, sections were washed in 2X SSC (0.3 M sodium chloride and 0.03 M sodium citrate) and incubated with a 1/35 dilution of RNase A cocktail (Ambion, Austin, TX) in 2X SSC for 37°C. Next, sections were stringently washed in 2X SSC/50% formamide twice, followed by one wash at 0.08X SSC at 5–8°C below the calculated Tm. For signal amplification, a horseradish peroxidase (HRP)-conjugated rabbit anti-digoxigenin antibody (DAKO) was used to catalyze the deposition of biotinyl-tyramide, followed by secondary streptavidin complex (GenPoint Kit, DAKO). The final signal was developed with DAB chromagen (GenPoint Kit, DAKO) and the tissue counterstained in hematoxylin for 15 seconds.

**Histologic evaluation of tissue sections.** In situ hybridization labeling of mRNA expression in the paraffin-embedded tissues was evaluated by three of the authors at a multiocular microscope (Ricci F, Hruban RH, Iacobuzio-Donahue C). For each case, the labeling pattern obtained following in situ hybridization was evaluated for the presence or absence of gene expression individually within each sample of infiltrating carcinomas or chronic pancreatitis. In those cases determined to be positive, gene expression was scored as occurring within the entire stromal region of the tumor or the stroma immediately adjacent to tumor epithelium (juxtatumoral stroma).

**Immunohistochemistry.** Unstained five-micron sections were cut from each paraffin block and deparaffinized by routine techniques before placing in 200 ml Target Retrieval Solution, ph 6.0 (Dako, Envision Plus Detection Kit, Carpinteria, CA) for 20 minutes at 100°C. After cooling for 20 minutes, slides were quenched with 3% H2O2 for 5 minutes, before incubating with the appropriate dilution of each primary antibody (1:4000 dilution of mouse monoclonal anti-human CD68, clone KP1; 1:800 dilution of mouse anti-human alpha actin, clone ASM1; 1:100 dilution of mouse monoclonal anti-human desmin, clone D33; Dako) for 30 minutes using the Dako Autostainer. Labeling was detected with the Dako Envision system following the manufacturer’s protocol. Labeling was detected by adding biotinylated secondary antibodies, avidin-biotin complex, and 3, 3’-diaminobenzidine. All sections were counterstained with hematoxylin. Immunolabeling patterns were evaluated by two of the authors (Ricci F, Iacobuzio-Donahue C).

**RESULTS**

Juxtatumoral stroma expression in infiltrating ductal adenocarcinoma of the pancreas. The mRNA expression patterns of three genes, ApoC-1, ApoD, and MMP11, were used to evaluate the host stromal response to neoplasms arising in the pancreas. These three genes were previously identified as markers of the juxtatumoral stroma in infiltrating ductal carcinomas of the pancreas. Consistent with our prior findings, ApoC1, ApoD, and MMP11 were strongly expressed throughout the juxtatumoral stroma of all primary infiltrating ductal adenocarcinomas analyzed (Fig. 2A–C). ApoD expression was also detected within small intra-pancreatic nerves, as we have previously described.

A robust stromal reaction is also a feature of chronic pancreatitis, a nonneoplastic condition of the pancreas in which atrophy of the pancreatic parenchyma is associated with fibrosis and inflammation (Fig. 1C). Therefore, to determine if ApoC1, ApoD and MMP11 are specific markers of desmoplastic stroma or generally expressed within stromal reactions in the pancreas, we also determined the expression of these three markers among
Intraductal papillary mucinous neoplasms (IPMNs) are one such distinct neoplasm that arises within the main pancreatic duct system, in contrast to conventional ductal adenocarcinomas that are believed to arise from a genetic progression involving the duct epithelium lining smaller ducts of the pancreas (known as pancreatic intraepithelial neoplasia, PanIN).2-13 Invasive carcinomas arising in an IPMN are separately categorized into colloid (mucinous noncystic carcinomas) or tubular (resembling usual ductal adenocarcinomas) types.1-3,14,15 Infiltrating carcinomas and IPMNs are composed of at least two cell types, tumor-infiltrating cells, indicating the juxtatumoral stromal compartment that corresponds to the alpha-actin positive fibroblasts adjacent to the neoplastic cells, indicating the juxtatumoral stromal compartment that we examined is comprised of at least two cell types, tumor-infiltrating macrophages and proliferating fibroblasts (Fig. 3).

Gene expression in variant forms of pancreatic cancer. In addition to conventional duct adenocarcinomas, the pancreas gives rise to a variety of other biologically distinct primary neoplasms. Intraductal papillary mucinous neoplasms (IPMNs) are one such distinct neoplasm that arises within the main pancreatic duct system, in contrast to conventional ductal adenocarcinomas that are believed to arise from a genetic progression involving the duct epithelium lining smaller ducts of the pancreas (known as pancreatic intraepithelial neoplasia, PanIN).2-13 Invasive carcinomas arising in an IPMN are separately categorized into colloid (mucinous noncystic carcinomas) or tubular (resembling usual ductal adenocarcinomas) types.1-3,14,15 Infiltrating carcinomas and IPMNs not only are morphologically distinct from infiltrating ductal adenocarcinomas, but the pattern of molecular genetic alterations present in these two tumor types also differs.16 These differences provide an opportunity to explore the patterns of juxtatumoral stroma expression in two neoplasms from the same organ. Therefore, we determined the juxtatumoral expression of ApoC, ApoD and MMP11 within the host stromal response in seven infiltrating carcinomas arising in association with intraductal papillary mucinous neoplasm (IPMN) of the pancreas. Two of the infiltrating carcinomas were colloid carcinomas, two were tubular carcinomas, and three had a mixed colloid-tubular pattern.

Five of seven infiltrating carcinomas arising in an IPMN showed strong labeling for all three markers (ApoC1, ApoD and MMP11) within the stromal response. In all cases, expression of all three markers was noted in a juxtatumoral distribution similar to that seen for conventional ductal adenocarcinomas. In addition, juxtatumoral expression within these five infiltrating carcinomas was noted only within areas of tubular (ductal-type) morphology, but not within the stroma associated with invasive colloid carcinomas (Figs. 3 and 4). In the remaining two infiltrating carcinomas, both with pure colloid morphology, no expression of ApoC1 or MMP11 were noted whereas focal expression of ApoD was noted in a panstromal distribution.

Immunoprofiles of normal and neoplastic pancreatic stroma. The host stromal response is comprised of a variety of cell types, including fibroblasts and inflammatory cells. Thus, to define the cell type(s) that expressed the markers identified by in situ hybridization, we performed immunohistochemical analysis using anti-CD68 (a macrophage marker), anti-smooth muscle alpha actin (a fibroblast marker) and anti-desmin (a myofibroblast marker). Immunolabeling for CD68 was noted throughout the stroma of all cases of infiltrating carcinoma analyzed (both conventional or arising in an IPMN) as well as within the stroma of chronic pancreatitis, indicating macrophages are a prominent cellular component of stromal reactions to both neoplastic and inflammatory processes within the pancreas (Fig. 3). Immunolabeling for alpha-actin showed a panstromal pattern of expression infiltrating carcinomas and chronic pancreatitis tissues similar to that observed for anti-CD68 antibody among, consistent with the prominent fibroblastic component of stromal tissues. In contrast, all cases of infiltrating carcinoma or chronic pancreatitis were negative for desmin.

The expression patterns of ApoC1, ApoD and MMP11 determined within conventional ductal adenocarcinomas and tubular carcinomas arising in IPMNs were compared to the cell types identified by immunohistochemical labeling. ApoC1 and ApoD expression within juxtatumoral stroma coincided with CD68 expression in those same cases, whereas MMP11 expression corresponded to the alpha-actin positive fibroblasts adjacent to the neoplastic cells, indicating the juxtatumoral stromal compartment that we examined is comprised of at least two cell types, tumor-infiltrating macrophages and proliferating fibroblasts (Fig. 3).

Gene expression of the stromal response in metastases to the liver. The liver is a frequent site of metastasis for a variety of primary carcinomas, including pancreatic cancers. Thus, we also determined the expression of the juxtatumoral stromal markers ApoC1, ApoD and MMP11 within two liver metastases from ductal adenocarcinomas of the pancreas for which the primary carcinoma was also analyzed. In contrast to the primary carcinomas that showed strong positive expression for all three juxtatumoral stroma markers, ApoC1 or ApoD expression was focal and noted in only one of the two liver metastases, while no expression of MMP11 was seen within the stromal response of either lesion (Fig. 5). These patterns of expression did not appear due to a decreased representation by macrophages or fibroblasts within the sections analyzed, as immunolabeling for both CD68 and smooth muscle alpha actin confirmed the presence of these cell types throughout the stromal response. Nor were the differences seen due to mRNA integrity of the tissue sections, as in situ labeling using Hevin riboprobes (an endothelial marker) were positive in all cases (data not shown).

**DISCUSSION**

Numerous independent lines of investigation indicate the tumor microenvironment plays an important role in cancer initiation and progression.17 Thus, the host connective tissue, with all its components (fibroblast, inflammatory and endothelial cells), can be regarded as an integral part of an invasive carcinoma. This desmoplastic reaction is particularly evident in pancreatic ductal adenocarcinomas making this an ideal tumor type for understanding the biology of tumor-associated stroma.
The robust juxtatumoral stromal expression of ApoC1, ApoD, and MMP11 observed in both conventional ductal adenocarcinomas arising from PanIN lesions and tubular (ductal-type) carcinomas arising in association with IPMNs indicate that the host stromal response generated within the pancreas is similar to carcinomas with infiltrative-type growth despite their origin in precursor lesions associated with differing molecular events. In striking contrast to infiltrating ductal or tubular type carcinomas, colloid carcinomas of the pancreas are not associated with ApoC1, ApoD or MMP11 juxtatumoral stromal expression, but instead have an expression pattern more similar to that of the long-standing fibrosis associated with chronic pancreatitis. As chronic pancreatitis is a well-known feature of IPMNs due to the obstruction of the duct system by the thick mucin produced by the neoplasm, the similarity of the stromal expression patterns suggest that the growth properties of colloid carcinomas may be due to the dissection of fibrotic stroma by the abundant extracellular mucin production rather than actual invasion of the surrounding parenchyma. Differences in the expression profiles, genetic alterations, protein expression and clinical outcome of invasive tubular versus colloid carcinomas arising within IPMNs have been reported. Our findings are consistent with these observed differences, and suggest that the presence of a host stromal response to an infiltrating carcinoma of the pancreas may relate to its biologic aggressiveness.

In datasets obtained from expression profiling of resected tumor tissues, highly expressed genes may indicate overexpression by a particular cell type or by increased representation of a cell type(s) as compared to reference samples. Immunohistochemical labeling for the cell types that comprise the stromal response indicate that both patterns account for the juxtatumoral gene expression patterns detected in infiltrating pancreatic cancers by expression profiling methods. Specifically, MMP11 was found to be overexpressed by proliferating fibroblasts within the juxtatumoral stroma compared to the virtually undetectable expression of this enzyme by fibroblasts within chronic pancreatitis tissues, consistent with a variety of studies implicating matrix metalloproteinases as regulators of tumor invasion. In contrast to the overexpression of MMP11 by juxtatumoral stromal fibroblasts, ApoC1 and ApoD expression detected in resected pancreatic cancer tissues is likely due to increased numbers of tumor-infiltrating macrophages present within the juxtatumoral compartment. Apolipoprotein expression by macrophages is consistent with the known roles of these gene products as protein components of the plasma lipid transport system, a finding of interest in light of a proposed role of macrophages in promoting solid tumor formation and invasive growth. Apolipoprotein expression has been identified by expression profiling in a variety of human tumor types. Thus, the findings of robust apolipoprotein gene by expression profiling may reflect the presence of tumor-promoting macrophages within the host stromal response to these primary neoplasms as well.

While numerous investigations have focused on tumor-stromal interactions at the site of primary invasion, few have addressed these interactions in relation to the primary versus secondary sites of growth. Our preliminary data of the host response in matched primary and metastatic disease suggests that the host stromal response generated to an infiltrating carcinoma may relate, in part, to the primary versus secondary organ microenvironment. The concept of a tumor microenvironment has long been hypothesized and has a rich history of literature to match, and we provide additional unbiased data in support of this concept. Of course, our findings do not rule out the possibility that the unique molecular or epigenetic features of a primary neoplasm that affect stromal gene expression are not selected for within the metastatic site. Nonetheless, these divergent patterns highlight the complex relationship of the target organ site to the tumor microenvironment, and may in part relate to the modest results of clinical trails designed to target stromal interactions within human neoplasms.

In summary, we show that the desmoplastic response appears to have a unique patterns of gene expression that relate to the tumor microenvironment (primary or secondary) in addition to the molecular features of the neoplasm. Future studies that identify new targets for clinical imaging, serological diagnosis, or drug delivery systems should take advantage of these features so that clinical efforts may become better tailored to the organ specific patterns of the host response.

References


