An Autoantibody-Mediated Immune Response to Calreticulin Isoforms in Pancreatic Cancer

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ABSTRACT

The identification of circulating tumor antigens or their related autoantibodies provides a means for early cancer diagnosis as well as leads for therapy. We have used a proteomics approach to identify proteins that commonly induce a humoral response in pancreatic cancer. Aliquots of solubilized proteins from a pancreatic cancer cell line (Panc-1) were subjected to two-dimensional PAGE, followed by Western blot analysis in which sera of individual patients were tested for primary antibodies. Sera from 36 newly diagnosed patients with pancreatic cancer, 18 patients with chronic pancreatitis, 33 patients with other cancers, and 15 healthy subjects were analyzed. Autoantibodies were detected against either one or two calreticulin isoforms identified by mass spectrometry in sera from 21 of 36 patients with pancreatic cancer. One of 18 chronic pancreatitis patients and 1 of 15 healthy controls demonstrated autoantibodies to calreticulin isoform 1; none demonstrated autoantibodies to isoform 2. None of the sera from patients with colon cancer exhibited reactivity against either of these two proteins. One of 14 sera from lung adenocarcinoma patients demonstrated autoantibodies to calreticulin isoform 1; 2 of 14 demonstrated autoantibodies to isoform 2. Immunohistochemical analysis of calreticulin in pancreatic/ampullary tumor tissue arrays using an isoform nonspecific antibody revealed diffuse and consistent cytoplasmic staining in the neoplastic epithelial cells of the pancreatic and ampullary adenocarcinomas. The detection of autoantibodies to calreticulin isoforms may have utility for the early diagnosis of pancreatic cancer.

INTRODUCTION

There is, at present, much interest in identifying markers for the early detection of pancreatic cancer. We have implemented a proteomics-based approach to identify tumor markers based on their occurrence as tumor antigens that elicit a humoral response during tumorigenesis. The humoral immune response to cancer in humans has been well demonstrated by identification of autoantibodies to a number of different intracellular and surface antigens in patients with various tumor types (1–4).

Pancreatic cancer has the worst prognosis of all cancers, with a 5-year survival rate of <3%, accounting for the fourth largest number of cancer deaths in the United States (5). It occurs with a frequency of around 9 patients/100,000 individuals, making it the 11th most common cancer in the United States. The poor prognosis for pancreatic cancer is due, in part, to lack of early diagnosis. There is currently no effective biomarker-based strategy useful for the early detection of pancreatic cancer or even to differentiate between pancreatic adenocarcinoma and chronic pancreatitis. In pancreatic cancer, autoimmunity has been shown against several proteins, including MUC1 (6), p53 (7), and Rad51 (8) proteins. MUC1 is a transmembrane glycoprotein involved in cell-cell and cell-extracellular matrix interactions, and MUC1 autoantibodies have been observed in sera from patients with a variety of different tumors (9). In pancreatic cancer, the presence of MUC1 IgG autoantibodies has been shown to be associated with a favorable prognosis (6). The presence of p53 autoantibodies has been observed in 18.2% of patients with pancreatic cancer. However, p53 autoantibodies were also found in 5.3% of patients with acute pancreatitis and 12.1% of patients with chronic pancreatitis, thus the humoral response to p53 was not specific to malignancy. The recombination factor Rad51 is highly expressed in pancreatic adenocarcinoma (10), and Rad51 autoantibodies have been observed in 7% of patients with pancreatic cancer.

It is not clear why only a subset of patients with a particular tumor type develop a humoral response to a particular antigen. Immunogenicity may depend on the level of expression, posttranslational modification, or other types of protein processing, the extent of which may be variable among tumors of a similar type. Other factors that may influence the immune response include variability among tumors and individuals in MHC molecules and in antigen presentation. A large number of autoantibodies have been identified in different tumor types, but in most cases, they occur in less than 50% of sera of patients. Therefore, they are not effective individually for the early detection of cancer. Thus, the development of panels of such autoantibodies directed against a variety of tumor antigens may be effective (11).

The identification of panels of tumor antigens that elicit an immune response may have utility in early cancer diagnosis, in establishing prognosis, and in immunotherapy against the disease. Several approaches are currently available for the identification of tumor antigens. In contrast to identification of tumor antigens based on analysis of recombinant proteins, the proteomic-based approach for the identification of tumor antigens that we have used allows for the identification of autoantibodies to proteins as they occurred in their natural states, in lysates prepared from tumors and tumor cell lines. This technology may uncover antigenicity associated with aberrant post-translational modification of tumor cell proteins. The goal of this study was to implement a proteomic approach for the identification of tumor antigens that elicit a humoral response in pancreatic cancer cell line, Panc-1. To this end, we have used two-dimensional PAGE to simultaneously separate individual cellular proteins from the Panc-1 cell line. The separated proteins were transferred onto polyvinylidene difluoride membranes. Sera from cancer patients were screened individually for antibodies that reacted against the separated proteins by Western blot analysis. Proteins specifically reacting with sera from cancer patients were identified by mass spectrometry. We have identified two calreticulin isoforms as proteins that commonly elicit an antibody response in pancreatic cancer.

MATERIALS AND METHODS

Sera and Cell Lines. Serum and tumor tissue were obtained at the time of diagnosis following informed consent. The experimental protocol was approved by The University of Michigan Institutional Review Board. Sera were

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obtained from 36 patients with pancreatic cancer (all of advanced stage). Sera from 18 patients with chronic pancreatitis, from 15 healthy individuals, and from 33 patients with other cancers (14 with lung cancer and 19 with colon cancer) were used as controls. All subjects that donated sera for this study were between 57 and 74 years of age. The human cancer cell lines used in this study were all individually cultured in DMEM supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 units/ml streptomycin (Invitrogen, Carlsbad, CA).

Two-Dimensional PAGE and Western Blot Analysis. After excision, the tumor tissue was immediately frozen at −80°C, after which an aliquot was lysed in solubilization buffer (8 M urea (Bio-Rad), 2% NP40, 2% carrier ampholytes (pH 4–8; Gallard/Schlessinger, Carle Place, NY), 2% β-mercaptoethanol, and 10 mM phenylmethylsulfonyl fluoride) and stored at −80°C until use. Cultured Panc-1 pancreatic adenocarcinoma cells were harvested in 300 μl of solubilization buffer by using a cell scraper and stored at −80°C until use. Proteins derived from the extracts of either cultured cells or solid tumors were separated into two dimensions as described previously (12). In brief, solubilized proteins were applied onto isoelectric focusing gels. Isoelectric focusing was performed using pH 4–8 carrier ampholytes at 700 V for 16 h, followed by 1000 V for an additional 2 h. The first-dimension gel was loaded onto the second-dimension gel, after equilibration in 125 mM Tris (pH 6.8), 10% glycerol, 2% SDS, 1% DTT, and bromphenol blue. For the second-dimension separation, a gradient of 11–14% acrylamide (Crescent Chemical, Hauppauge, NY) was used. Proteins were transferred to an Immobilon-P polyvinylidene difluoride membrane (Millipore, Bedford, MA) or visualized by silver staining of the gels.

Western Blotting. After transfer, membranes were incubated with a blocking buffer consisting of 10 mM Tris–HCl (pH 7.5), 50 mM NaCl, 1.8% nonfat dry milk, and 0.01% Tween 20 for 2 h. The membranes were incubated for 1 h at room temperature with serum obtained from either patients or healthy individuals as a source of primary antibody at a 1:100 dilution. After three washes with washing buffer (Tris-buffered saline containing 0.01% Tween 20), the membranes were incubated with horseradish peroxidase-conjugated sheep antihuman (Amersham Biosciences, Piscataway, NJ) IgG antibodies at a dilution of 1:1000 for 1 h at room temperature. Immunodetection was accomplished by enhanced chemiluminescence (Amersham Biosciences) followed by autoradiography on Hyperfilm MP (Amersham Biosciences).

Calreticulin Detection by Western Blotting. A rabbit anticalreticulin polyclonal antibody (Affinity Bioreagents, Golden, CO) was used at a 1:1000 dilution for Western blotting and was processed as for incubations with patient sera, with a horseradish peroxidase-conjugated antirabbit IgG (Amersham Biosciences) as the secondary antibody.

In-Gel Enzyme Digestion and Mass Spectrometry. For protein identification by mass spectrometry, two-dimensional gels were stained by a modified silver-staining method, and excised proteins were destained for 5 min in 15 mM potassium ferricyanide and 50 mM sodium thiosulfate as described previously (13). After three washes with water, the gel pieces were dehydrated in 100% acetonitrile for 5 min and then dried. Digestion was performed by the addition of 100 ng of trypsin (Promega, Madison, WI) in 200 nm ammonium bicarbonate. After enzymatic digestion overnight at 37°C, the peptides were extracted twice with 50 μl of 60% acetonitrile/1% trifluoroacetic acid. After removal of acetonitrile by centrifugation in a vacuum centrifuge, the peptides were concentrated by using pipette tips C18 (Millipore) and identified by nanoflow capillary liquid chromatography coupled with electrospray quadrupole-time-of-flight tandem mass spectrometry in the quadrupole-time of flight micro (MicroMass, Manchester, United Kingdom). The acquired spectra were processed and searched against a nonredundant SwissProt protein sequence database using proteinLynx Global Server.6

RNA Isolation. Samples of normal pancreas were taken from organ donors provided by the Michigan Transplantation Society (five) or from areas outside regions of pathology in surgically resected pancreata (two). All of the pancreatic cancers were of advanced stage. All samples were processed in a similar manner. Frozen samples were embedded in OCT-freezing media (Miles Scientific, Naperville, IL) and cryotome sectioned (5 μm), and routine H&E stains were evaluated by a surgical pathologist. Areas of relatively pure tumor (at least 70% tumor cells) or normal tissue were microdissected, and these areas were selected for RNA isolation. All grades of differentiation were exhibited by the tumors.

Isolates of human tumor tissue and human tumor cell lines were homogenized in the presence of TRizol reagent (Invitrogen), and total cellular RNA was purified according to the manufacturer’s procedures. RNA samples were further purified using acid phenol extraction and RNeasy spin columns (Qiagen, Valencia, CA). RNA quality was assessed by 1% agarose gel electrophoresis in the presence of ethidium bromide.

Gene Expression Profiling and Statistical Analysis. This study used commercially available high-density oligonucleotide microarrays (U133A; Affymetrix, Santa Clara, CA). Hybridization, scanning, and image analysis of the arrays were performed according to manufacturer’s protocols and as described previously (14, 15). The U133A array consists of 22,283 probe sets, each representing a transcript. Each probe set typically consists of 11 perfectly complementary 25-base-long probes (PM) as well as 11 mismatch probes (MM) that are identical except for an altered central base. A normal pancreas sample was selected as the standard, and probe pairs for which PM-MM ≤100 on the standard were excluded from additional analysis. The average of the middle 50% of the PM-MM differences was used as the expression measure for each probe set. A quantile normalization procedure was used to adjust for differences in the probe intensity distribution across different chips. In brief, we applied a monotone linear spline to each chip that mapped quantiles 0.01 up to 0.99 (in increments of 0.01) exactly to the corresponding quantiles of the standard. For statistical analysis, we first transformed each normalized probe-set expression value, xi, to log [100 + max(0, x)] , which we found stabilized the within group variances between high- and low-expression probe sets. To compare normal and tumor samples, we performed a one-way ANOVA, modeling the log-transformed values for each probe set as having separate means for each group. We calculated fold changes between groups of samples by first replacing mean expression values < 100 units by 100 to avoid negative values or spuriously large fold changes. Code to perform these computations is freely available.7

Determination of Calreticulin mRNA Levels Using Real-Time PCR. Five pancreatic, four lung, three colon and two ovarian cancer cell lines were used to compare the mRNA expression level of calreticulin. Expression levels were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA expression. Oligonucleotide primers and TaqMan probes were designed using the Light Cycler Probe Design Software (Roche Applied Science). Forward and reverse primers for human calreticulin were 5’-CGCC-ATGTCGTACTCC-3’ and 5’-CATAAAAGGCGTGATCTC-3’, respectively (Applied Biosystems). The nucleotide sequence of the forward and reverse primers for GAPDH were 5’-GAAAGGTGAGGAGTGGATC-3’ and 5’-GAAGATGGGTGATGGATTTCT-3’, respectively (Applied Biosystems).

The first-strand cDNA was synthesized with SuperScript First-Strand Synthesis System for reverse transcription-PCR according to the manufacturer’s instructions (Invitrogen). Quantitative PCR reaction was carried out in 96-well optical reaction plates using cDNA derived from 50 ng of total RNA for each sample in a volume of 25 μl. PCR was performed on the ABI Prism 7700 Sequence Detector (Applied Biosystems). The cycling conditions were 10 min at 95°C followed by 55 cycles at 95°C for 30 s, 60°C for 45 s, and 72°C for 45 s.

To control for the variation in the amount of starting RNA among samples, we performed amplification of GAPDH mRNA as an internal reference against which other RNA values were normalized. Additionally, the real-time PCR

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6 Internet address: www.micromass.co.uk.

products were purified by QIAQuick Gel Extraction kit (Qiagen) and subjected to DNA sequencing to verify the identity of the real-time PCR products.

Pancreas/Ampullary Tumor Tissue Array and Immunohistochemistry. A tissue array containing triplicates of 4 normal pancreas, 12 nonpancreas normal tissues, 47 pancreatic adenocarcinomas, 31 ampullary adenocarcinomas, and 2 large cell anaplastic carcinomas was constructed as described previously (9). The cases were randomly selected from the University of Michigan Pathology archives. Immunohistochemistry for calreticulin was performed using the same rabbit polyclonal antibody (30 min incubation at room temperature) at 1:200 using citrate buffer (pH 6.0) and microwave antigen retrieval (10 min) and the Dako automated instrument (Dako Cytomation, Carpinteria, CA). Primary antibody was detected using the Dako Envision kit.

RESULTS

Pancreatic Tumor Proteins Recognized Specifically by Sera from Newly Diagnosed Patients with Pancreatic Cancer. Panc-1 pancreatic tumor cell line proteins were separated by two-dimensional PAGE and transferred onto Immobilon-P polyvinylidene difluoride membranes. Sera obtained from 36 newly diagnosed patients with pancreatic cancer, from 18 patients with chronic pancreatitis, from 33 patients with other types of cancers, and from 15 healthy donors were screened individually for the presence of antibodies to Panc-1 pancreatic tumor cell line proteins (Table 1). Each membrane was treated

Fig. 1. A silver-stained image of the Panc-1 pancreatic tumor cell line (A) compared with a Western blot of the Panc-1 cell line with normal serum (B) and serum from a patient with pancreatic cancer (C).

Fig. 2. Western blot analysis of calreticulin with a polyclonal anticalreticulin antibody (A) and sera from a pancreatic cancer patient (B) and from a healthy individual (C).
with one serum sample as the primary antibody and with sheep anti-human IgG as the secondary antibody. In general, most pancreatic patient sera reacted against multiple spots (Fig. 1; Fig. 2). Some of the reactive protein spots were observed in the control sera and thus were considered to represent nonspecific reactivity. The reactive proteins most commonly observed with pancreatic cancer patient sera but not with noncancer controls included two proteins (spots 1 and 2) with an estimated molecular mass of 55–60 kDa and an isoelectric point of 4.4. These two proteins frequently showed concordant reactivity with the same sera suggesting, given their close proximity in two-dimensional gels, that they represented isoforms of the same protein. The protein from spot 1 showed reactivity with sera from 17 of 36 patients with pancreatic cancer (47.2%), with sera from 1 of 18 patients with chronic pancreatitis (5.6%) and with sera from 1 of 15 healthy donors (6.7%). The protein from spot 2 showed reactivity in 16 of 36 patients with pancreatic cancer (44.4%), in 0 of 18 patients with chronic pancreatitis and in 0 of 15 healthy donors (Table 1). The number of pancreatic cancer patients’ sera that showed reactivity with one or both spots was 21 of 36 (58.3%). Reactivity directed against the protein in spot 1 was found in sera from 1 of 14 patients with lung cancer; reactivity directed against the protein in spot 2 was found in 2 of 14 lung cancer patients. None of the sera from 19 colon cancer patients exhibited reactivity against the protein in either spot (1 or 2).

Identification of the Reactive Proteins as Isoforms of Calreticulin. The proteins of interest were extracted from the gels after two-dimensional PAGE and silver staining. The proteins were digested with trypsin, and the resulting peptides were analyzed by electrospray quadrupole-time of flight tandem mass spectrometry. The acquired spectra were processed and searched against a nonredundant SwissProt protein sequence database using the ProteinLynx global server. The two proteins were identified (Fig. 3) as being isoforms of calreticulin (SwissProt accession no. P27797). Identity with calreticulin was confirmed with two-dimensional Western blotting using Panc-1 whole-cell extracts and an anticalreticulin rabbit polyclonal antibody.
Role of Glycosylation in Calreticulin Antigenicity. We sought to determine whether calreticulin glycosylation contributed to immunogenicity. Solubilized proteins from the Panc-1 pancreatic tumor cell line were subjected to N-deglycosylation by a combination of endoglycosidase F, endo-α-N-acetylgalactosaminidase, and α-2,3,6,8,9-neuraminidase. The resulting products were separated by SDS electrophoresis and analyzed by Western blotting. Although the deglycosylated positive control revealed a demonstrable mobility shift by SDS-PAGE, the deglycosylating enzyme treatment did not result in any mobility shifts of calreticulin. Thus, endoglycosidase F-sensitive glycosylation does not appear to play a role in the observed immunogenicity of the calreticulin isoforms (data not shown).

mRNA Expression of Calreticulin. To examine whether the immunogenicity of calreticulin in pancreatic cancer could be due to elevated transcriptional mechanisms, the expression of calreticulin mRNA was examined in different cell lines and tumor tissues. To examine calreticulin expression in all cell lines, including five pancreatic tumor cell lines, four lung tumor cell lines, three colon tumor cell lines, and two ovarian tumor cell lines, we performed real time-PCR using the expression level of GAPDH as an internal control. After normalization, the calreticulin:GAPDH ratio was calculated from each cell line (Fig. 4). In general, we found that the level of mRNA expression in the pancreatic tumor cell lines was significantly higher than the other cell lines examined, suggesting that overexpression of calreticulin may be a possible contributing factor in its immunogenicity. Therefore, we examined calreticulin expression in eight pancreatic adenocarcinomas, in four samples of chronic pancreatitis, and in seven samples of normal pancreas by microarray analysis (Fig. 5). The expression of calreticulin mRNA was approximately 2-fold higher in pancreatic tumors as compared with normal pancreas (P = 0.006). It is important to note, however, that the pancreatic adenocarcinomas were microdissected and are derived from ductal epithelium. Because the normal pancreas is primarily acinar, it may be that the difference in gene expression noted in the pancreas tumors is entirely related to the differences in the epithelium analyzed rather than any differences that arose in the tumors.

Analysis of Calreticulin Expression by Two-Dimensional PAGE. We hypothesized that there might be changes in the levels of calreticulin total protein or isoforms that could lead to antigenicity in pancreatic cancer. Using two-dimensional PAGE, we examined the expression of calreticulin isoforms 1 and 2 in a variety of tissues and tumor types. All calreticulin isoforms were present in different cell lines, including 6 pancreatic tumor cell lines, 4 lung tumor cell lines, 9 colon tumor cell lines, and 33 ovarian tumor cell lines, at similar expression levels. A similar pattern of expression was also observed in 6 pancreatic tumors, 38 lung tumors, 7 colon tumors, and 25 ovarian tumors (Fig. 6). All isoforms were also observed in a variety of normal tissues and in gastric, esophageal, and brain tumors (data not shown). These results suggest that all calreticulin isoforms, including isoforms 1 and 2, were ubiquitously expressed and that the
level of protein expression was unlikely to contribute to the antigenicity of calreticulin.

**Immunohistochemical Analysis of Calreticulin.** Calreticulin expression in pancreatic and ampullary tumors was assessed by immunohistochemistry (Fig. 7, A and B), using a rabbit polyclonal anticalreticulin antibody and the pancreatic/ampullary tumor tissue array. Diffuse and consistent cytoplasmic immunoreactivity for calreticulin was observed in the majority of the pancreatic and ampullary adenocarcinomas. There were no significant staining differences with regard to tumor differentiation. Normal pancreatic ductal epithelium exhibited minimal reactivity, whereas normal pancreatic islets exhibited intense immunoreactivity, and normal exocrine pancreas exhibited moderate reactivity (Fig. 7C).

**DISCUSSION**

We have implemented a proteomics-based approach to identify proteins that elicit a humoral response in pancreatic cancer patients. This approach allows screening by Western blot analysis of patient sera for antibodies that react against separated tumor cell proteins. This study was focused on a search for autoantibodies to pancreatic tumor proteins present in the Panc-1 cancer cell line. We have shown that a humoral response directed against calreticulin isoform 1 or 2, or both, occurred in 58.3% of pancreatic cancer patients. One of 18 chronic pancreatitis patients (5.6%) and 1 of 15 healthy controls (6.6%) demonstrated autoantibodies to calreticulin isoform 1; none demonstrated autoantibodies to isoform 2. None of the sera from patients with colon cancer exhibited reactivity against these proteins. One of 14 (7.1%) sera from lung adenocarcinoma patients demonstrated autoantibodies to calreticulin isoform 1. Two of 14 (14.3%) demonstrated autoantibodies to isoform 2.

Calreticulin is an abundant, high-capacity Ca\(^{2+}\)-binding protein found in the endoplasmic reticulum (ER) lumen of most cells of human origin. It has been shown to play a role in the regulation of a variety of cellular functions within the ER lumen (chaperone functions and \(\text{Ca}^{2+}\) storage and signaling) and calreticulin-dependent modulation of cell adhesion and gene expression at extra-ER sites (16). In particular, calreticulin interacts with \(\text{N}\)-linked oligosaccharides on nascent proteins in the ER lumen, with \(\text{Ca}^{2+}\) binding essential for this function.

It has been demonstrated that calreticulin elicits a humoral response in a variety of autoimmune diseases (17). Peptides transported into the lumen of the ER associate with calreticulin, as well as with protein residues...
unable to demonstrate aberrant related to the level of protein expression. Furthermore, we were expressed in pancreatic tumor cell lines at either the mRNA or protein similar type. We have demonstrated that calreticulin is not overexpressed in the Panc-1 tumor cell line, it did not elicit immunoreactivity. This suggests that a specific mechanism of calreticulin processing may exist during carcinogenesis that may differ between tumor types.

A prerequisite for an immune response against a cellular protein is its presentation as an antigen. It is not clear why only a subset of patients with a specific tumor type develop a humoral response to a particular antigen. Immunogenicity may depend on the level of expression, posttranslational modification, or other types of processing of a protein, the extent of which may be variable among tumors of a similar type. We have demonstrated that calreticulin is not overexpressed in pancreatic tumor cell lines at either the mRNA or protein level, compared with lung, colon, or ovarian tumor cell lines in our study. Thus, the immunoreactivity of calreticulin is unlikely to be related to the level of protein expression. Furthermore, we were unable to demonstrate aberrant N-linked glycosylation of calreticulin in the pancreatic tumor cell lines (data not shown). It is possible that the antigenicity to the calreticulin isoforms may be arising from the aberrant expression of an unrelated protein in pancreatic cancer that generates an epitope that cross-reacts with calreticulin.

Although the calreticulin autoantibodies were largely restricted to patients with pancreatic cancer among the subject groups we investigated, additional studies are needed to determine the specificity of the calreticulin antibodies to pancreatic cancer. For example, although increased levels of calreticulin antibodies were found in pancreatic cancer, compared with chronic pancreatitis and other control groups, the relationship between tumor burden, tumor staging, and antibody levels needs additional clarification. Assessment of the utility of calreticulin autoantibodies as diagnostically markers in pancreatic cancer also needs to be addressed in additional studies. It is clear, however, that the proteomic approach that we have implemented, which allows for the screening of native proteins as they are expressed in tumor cells, has the potential to identify novel proteins that may have clinical utility in cancer.

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