Peptidomics-based Approach Reveals the Secretion of the 29-Residue COOH-Terminal Fragment of the Putative Tumor Suppressor Protein DMBT1 from Pancreatic Adenocarcinoma Cell Lines*

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Abstract

Deleted in malignant brain tumors 1 is a putative tumor suppressor protein in brain, lung, esophageal, gastric, and colorectal cancer. Here we report the mass spectrometric identification of a 3335 Da peptide, which was found in serum-free conditioned medium from 5 of 15 pancreatic adenocarcinoma cell lines but not from 35 carcinoma cell lines and 2 nonmalignant pancreatic duct cell lines. The peptide was the 29 COOH-terminal amino acids from deleted in malignant brain tumors 1. It is suggested that the peptide is generated inside the cells by limited proteolysis and extracellularly secreted. Our peptidomics-based approach will help screen candidate marker peptides for a particular type of cancer.

Introduction

Peptides that are secreted from a particular type of cancer cells and stable in body fluids may serve as a tumor marker. This notion is supported by the establishment of pro-gastrin-releasing peptide (31–98; proGRP) as a useful tumor marker specific to small cell lung carcinoma lines (7). The discovery process for new peptide markers may be expedited by recent advances in MS3. SELDI MS is a type of affinity MS that shares a basic idea with the MALDI MS (3). SELDI permits the differential display of proteins on various crude biological samples (4); however, CM has attracted little attention because low concentrations of cell-derived proteins provide an obstacle to the discovery of target proteins, still less peptides. To overcome this issue, we established a single-step sample preparation method for enriching peptides in SFCM amenable to SELDI MS (5, 6).

Despite low protein concentrations (10–50 μg/mL), SFCM from cultured cells has several advantages in screening secretory polypeptides. First, even established cell lines are shown to secrete tumor markers currently used in clinical practice, as exemplified by the production of the peptide GRP in small cell lung carcinoma lines (7). Second, SFCM contains an appreciable amount of serum components that would otherwise mask the detection of cell-derived peptides present in much lower abundance. Third, the relative homogeneity of SFCM favors the biochemical purification of a target. Fourth, it is supposed to be rich in stable peptides that can survive a culture condition of 37°C for a given time period.

To screen for potential marker peptides of pancreatic adenocarcinoma, we performed SELDI MS on SFCM from various carcinoma cell lines as well as nonmalignant pancreatic duct cell lines. We found a peak with a mass of 3335 Da in 5 of 15 pancreatic adenocarcinoma cell lines. Two types of tandem mass spectrometric techniques identified the peptide as the COOH-terminal fragment of DMBT1, the putative tumor suppressor protein in malignant brain tumors and some digestive tract cancers (8). Our data suggest that this peptide is cleaved from the parent DMBT1 protein by limited proteolysis and secreted to CM.

Materials and Methods

Cell Lines. The sources of cell lines were as follows: pancreatic carcinoma (PANC-1, FA-6, GGP-1, Capan-1, BxPC-3, AsPC-1 SUIT-4, PSN-1, YPK-1, YPK-2, PK-1, PK-8, PK-45P, PK-59, JHP-1, KP-1N, KP-2, and H-48NN), colorectal carcinoma (HT-29, COLO 201, LS 180, and LoVo), gastric carcinoma (MKN-1, MKN-7, MKN-28, and MKN-74), and mammary carcinoma (MDA-MB-231, MDA-MB-361, SK-BR-3, and T-47D) were described previously (5, 6). Colorectal carcinoma (DLD-1, WiDr, HCT-15, and SW480), gastric carcinoma (Kato III, NUGC-4, H-111, SH-10, and HGC-27), cholangiocarcinoma (HuCCT1 and TFK-1) were from the Cell Resource Center for Biomedical Research (Tohoku University, Miyagi, Japan). Prostate carcinoma (LNCap, FGC, and Du 145), mammary carcinoma (MDA-MB-468 and MCF7), choriocarcinoma (JEG-3), embryonal carcinoma (Tera-1), and normal diploid embryonic lung fibroblast (MRC-5) were purchased from American Type Culture Collection (Manassas, VA). Gastric carcinoma (HSC-41 and HSC-43) were described in Ref. 9. Prostate carcinoma (PC3) was from the Japanese Cancer Research Bank. GGP-1 was provided by Dr. Haruo Iguchi (National Kyushu Cancer Center, Japan). All of the cancer cell lines but PANC-1, GGP-1, FA-6, Du 145, HCT-43, Kato III, NuGC-4, MKN-1, T-47D, JEG-3, HGC-27, and Tera-1 were established from adenocarcinomas. HPD4 and H6C7 cell lines were cultured as described previously (6). The synthetic furin inhibitor dec-RVKR-cmk was purchased from Alexis Co Biochemicals (San Diego, CA). Brefeldin A was from Wako Chemicals (Osaka, Japan).

Preparation of SFCM. Cancer cell lines were grown in RPMI 1640 (Sigma) supplemented with 10% FCS, penicillin, and streptomycin in a 100-mm dish unless otherwise stated. After reaching confluency, SFCM was prepared as described previously (5). After a 24-h incubation, CM was centrifuged, and supernatant was stored at -20°C.

Sample Preparation and SELDI MS. CM (750 μl) was diluted with an isovolume of 0.17 N acetic acid and applied on a SCX membrane (Millipore) as described previously (6). For reproducibility, each sample was added to two separate spots on a SELDI C16 hydrophobic interaction chip (Ciphergen Biosystems, Fremont, CA), and samples from at least more than two different passages were examined. Mass spectra acquisition and peptide differential display were performed as described previously (5). Bovine insulin was used as an external standard, with mass accuracy of a PBS II SELDI mass spectrometer (Ciphergen) being better than 1000 ppm.

Partial Purification of the 3335-DA Peptide for MS/MS. Capan-1 SFCM was processed through SCX membranes. Disulfide bridges in the SCX eluate...
were reduced in 50 mM NH4HCO3 and 50 mM DTT at 56°C for 30 min. Cysteine residues were subsequently modified by iodoacetamide at a final concentration of 200 mM at 37°C.

Cysteine residues were subsequently modified by iodoacetamide at a final concentration of 200 mM at 37°C. The sample was separated by gel filtration with a Superdex Peptide PC 3.2/30 column (Pharmacia) on a Smart HPLC system (Pharmacia). Target fractions were located by SELDI MS.

**MS/MS for Peptide Identification.** The peptide-rich fraction was divided in half and lyophilized. One aliquot was reconstituted in 5 μl of 0.5% trifluoroacetic acid/50% acetonitrile and added to a SELDI C16 chip. The chip was mounted with 2.5-dihydroxybenzoic acid and analyzed on a mass spectrometer that combines a SELDI ion source with a selection quadrupole (Q), a collision cell (q), and a time-of-flight (TOF) fragment ion analyzer (SELDI QqTOF; Refs. 4, 10). Each peptide of interest underwent collision-induced dissociation to acquire tandem mass spectra. The other aliquot was reconstituted in 20 μl of 25 mM NH4HCO3 containing 2.5 ng of sequencing grade lysyl-C endopeptidase (Roche) and incubated overnight at 37°C. The digest was loaded to an automated μLC ESI MS/MS system, consisting of a HPLC Magic 2002 (Michrom BioResources, Auburn, CA) connected on-line to a LCQ Deca iontrap tandem mass spectrometer (Thermoquest, San Jose, CA). The HPLC system was first equilibrated with 0.1% formic acid-5% ACN, and then a linear gradient of 5–60% ACN was applied >20 min for analysis.

Tandem mass spectra were searched against theoretical tandem mass spectra of peptide sequences in the Nr and Swissprot databases by the Ms-Tag program 4 to establish from normal pancreatic ducts, were used as a control for the nonmalignant pancreatic duct cell lines, many of which (38 of 50) are derived from adenocarcinoma. Five carcinoma (PANC-1, FA6, and QGP-1), 6 mammary carcinoma, 8 colo-

**Antibodies.** Monoclonal antibody (anti-DMBT1h12), raised against the first NH2 terminus of DMBT1(26–40), was provided by Dr. Jan Mollenhauer (11). The antiserum to detect the target COOH-terminal peptide, designated L129, was prepared against a synthetic peptide (LQTPPRREEEPR) corresponding to DMBT1(2415–2426).

**IP-SELDI MS.** Culture supernatant (500 μl) was incubated overnight at 4°C with L129 antiserum (1:100) and then with 20 μl of slurry of Protein G Sepharose (Pharmacia) for 30 min. After five washings with saline, the beads were immersed in 10 μl of 0.2% trifluoroacetic acid to release bound material. Four μl of the supernatant were analyzed by SELDI MS. To detect intracellular peptides, cells were rinsed with saline and lysed in IP buffer [0.5% TX-100, 5 mM EDTA, 1 mM PMSF, 20 μM dec-RVKR-cmk, and Tris-HCl (pH 7.5)]. Cleared supernatant was likewise immunoprecipitated.

**Immunoblotting.** Immunoblotting was performed as described previously (11). Anti-DMBT1h12 and L129 antiserum were used at a dilution of 1:2000 and 1:4000, respectively.

**Results**

**SELDI MS Profiling of SFCM to Screen for Differentially Expressed Peptides in Pancreatic Adenocarcinoma Cell Lines.** CM was analyzed to profile peptides from 50 carcinoma cell lines, including 15 pancreatic adenocarcinoma, 3 pancreatic nonadenocarcinoma (PANC-1, FA6, and QGP-1), 6 mammary carcinoma, 8 colorectal adenocarcinoma, 11 gastric carcinoma, 3 prostate carcinoma, 2 cholangiocarcinoma, 1 choriocarcinoma, and 1 embryonal carcinoma cell lines, many of which (38 of 50) are derived from adenocarcinoma. The nonmalignant pancreatic duct cell lines H6C7 and HPDE4 (6), established from normal pancreatic ducts, were used as a control for pancreatic adenocarcinoma. In a previous study, we established that normal pancreatic duct cells are reduced in 50 mM NH4HCO3 and 50 mM DTT at 56°C for 30 min. Because no mass shift was observed for the 3335-Da peptide after this chemical modification, we conclude that it has no cysteine residues.

Identification of the COOH-Terminal 29-Amino Acid Fragment of DMBT1 by MS/MS. Before MS/MS identification, cysteine residues included in the processed CM from CAPAN-1 were reduced and carbamidomethylated for the possible presence of disulfide bonds. The peptide-rich fraction separated by gel filtration was analyzed on a SELDI QqTOF mass spectrometer. The monoisotopic peak of the 3335-Da peptide (MH+ 3334.737 Da) was fragmented by collision-induced dissociation to acquire tandem mass spectra (Fig. 2). Database searching with the Ms-Tag program retrieved the last 29-amino acid COOH-terminal fragment of DMBT1 as the top hit, its calculated mass (MH+ 3334.739 Da) being practically identical to the observed mass. Of 29 monoisotopic product ions used in the Ms-Tag search, 22 peptides were matched with accuracy on average better than 13 ppm. The finding was additionally confirmed by μLC ESI MS/MS analysis (12) of the remaining sample fraction after digestion with lysyl C endopeptidase. This resulted in the identification of two peptides with m/z 925 and 2428 as the fragments of DMBT1(2398–2405) and DMBT1(2406–2426), respectively. This was in agreement with the retrieved DMBT1 sequence (DVGSYQEKDVVLGPIQLQTPPRREEEP), which should be cleaved at a single lysine residue to generate 8- and 21-amino acid fragments.

The 3335-Da Peptide Preferentially Occurs in Pancreatic Adenocarcinoma Cell Lines. To immunologically confirm that the MS/MS-identified 3335-Da peptide was the COOH-terminal fragment of DMBT1, we raised antiserum to perform IP-SELDI MS. It was revealed that L129 antiserum but not preimmune serum recovered this peptide from culture supernatant of Capan-1 cells grown in the presence of 10% FCS (Fig. 3A). Under this condition, cell debris was not at all observed after centrifugation (data not shown), suggesting that the peptide is secreted. IP-SELDI MS was used to assess peptide amounts in CM semiquantitatively (Fig. 4A). Capan-1 showed the highest level of expression, followed by SUIT-4, PSN-1, YPK-2, and PK-8. In agreement with the initial SELDI MS screening, the 3335-Da peptide was not detected in other cell lines. Normal embryonic lung fibroblast MRC5 and normal mammary epithelial cells, both of which were difficult to harvest SFCM, did not express the peptide (data not available).
shown). Thus far examined, this peptide occurred preferentially in pancreatic adenocarcinoma cell lines.

**Capan-1 Expresses a Large Amount of DMBT1 Protein.** Immunoblotting was performed on total cell lysate to examine DMBT1 protein expression. With Capan-1 cells, L129 and anti-DMBT1h12 both specifically recognized a 250-kDa protein on a SDS-PAGE gel (Fig. 3B). In addition, the antiserum could immunoprecipitate the 250-kDa protein from Capan-1 cell extract, followed by immunoblotting with anti-DMBT1h12 (data not shown). Because the coding region of the prototype DMBT1 mRNA has 2426 amino acid residues (8), this band would represent the whole DMBT1 protein. As for SUIT-4, PSN-1, YPK-2, and PK-8, which displayed the 3335-Da peptide in CM, no specific bands were detected in immunoblots made on total cell lysate (data not shown).

**The 3335-Da Peptide Is Intracellularly Generated and Extracellularly Secreted.** The amino acid sequence immediately NH₂-terminal to the DMBT1 cleavage site, RSKR, is reminiscent of the consensus recognition site for the proprotein convertase furin (13). It is reported that glioma cells release active furin into culture supernatant, thereby releasing bioactive transforming growth factor β molecules from precursor molecules (14). This conversion is shown to be inhibited by the furin synthetic inhibitor dec-RVKR-cmk (14). To examine if our peptide would be cleaved outside cells, culture supernatants from Capan-1 cells treated with the furin inhibitor for 48 h were analyzed. However, extracellular cleavage was negated because the inhibitor did not affect the peptide amount (Fig. 4B). Next, Capan-1 cell extract was immunoprecipitated with L129 antiserum, in the presence of 5 mM EDTA, 1 mM PMSF, and 20 μM dec-RVKR-cmk to suppress possible in vitro cleavage. The peptide was already present in cell extract (Fig. 4C), suggesting that it is intracellularly cleaved, presumably by furin-like proprotein convertase(s). Consistent with this, increasing concentrations of Brefeldin A, an inhibitor of translocation of secretory proteins from the endoplasmic reticulum to the Golgi apparatus (15), lowered the peptide secretion (Fig. 4D).

**Discussion**

Peptides and small proteins (<1 kDa) are not yet covered by standard proteomics research tools, typically two-dimensional gel electrophoresis in combination with MS (12). The term “peptidomics,” advocated by two independent groups (16, 17), refers to alternative strategies to comprehensively analyze peptides in complex biological mixtures in which they are fractionated by liquid chromatography and then identified by MS/MS. In this study, we adopted a different peptidomics approach by using mass spectrometric techniques to identify secretory cancer marker peptides; SELDI MS was used to screen differentially expressed peptides in SFCM, which were subsequently identified with MS/MS techniques.

The COOH-terminal 29-amino acid fragment of DMBT1, with a molecular mass of 3335 Da, was identified as a peptide apparently specific to pancreatic adenocarcinoma cells. Our IP-SELDI studies indicate that the peptide was intracellularly generated before secretion. Thus, we could reveal a hitherto unknown molecular form of DMBT1 in pancreatic adenocarcinomas. Another noteworthy point is that our sample preparation also allowed the acquisition of mass spectra by the conventional MALDI MS for peptide profiling in CM (data not shown). This also means that we can take advantage of the ability of MALDI collision-induced dissociation to identify individual peptides in mixtures (10). In fact, a number of proteins or peptide fragments known to have a secretory property are being identified.5 Taken together, these findings indicate the validity of our approach to screening secretory marker peptides.

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5 K. Sasaki, manuscript in preparation.
The DMBT1 gene is now considered a candidate tumor suppressor gene for brain, lung, esophageal, gastric, and colorectal cancer (8). Immunohistochemical studies using anti-DMBT1h12 reveal that the skin, colon epithelium, and renal collecting tubule show immuno-reactivity (11, 18). On the basis of these observations, we suspected that the 3335-Da peptide might be produced by some normal tissues and released into circulation. Thus far, the peptide was not detected by IP-SELDI MS in 2 μl of serum from two normal subjects (purchased from Biowhittaker, Walkersville, MD). The development of a sensitive assay for this peptide could be helpful for further evaluation.

SAGE gives a clue to the abundance of mRNA transcripts. SAGE is supposed to produce a comprehensive profile of gene expression and can be used to search for tumor markers at the mRNA level (19). The National Cancer Institute SAGE database6 lists 113 libraries (as of Feb. 15, 2002) from various sources, which contains four pancreatic cancer cell lines, two primary cultures of normal pancreatic duct cells, and two surgical specimens of pancreatic cancer. DMBT1 mRNA is given one well-characterized SAGE tag (CTTCTCATCT), significant numbers of which are found in the pancreatic cancer cell lines Capan-1, Capan-2, and HS766T but not found in the two libraries from primary duct cells. These SAGE data support our immunoblotting data, especially DMBT1 overexpression in Capan-1. Of note, two libraries from a single patient with gastric adenocarcinoma contain a significant number of DMBT1 tags. Although our 11 gastric carcinoma cell lines did not express the 3335-Da peptide, additional clinical evaluation should also be made on gastric adenocarcinomas.

The DMBT1 gene has a NH2-terminal signal peptide sequence (8). Because the secretory nature has been suggested by immunohistochemical studies alone (11, 18), we addressed this issue in a straightforward way using Capan-1 cells. However, our attempt to detect the whole DMBT1 protein from 1 μl of Capan-1 CM by IP immunoblotting was unsuccessful, even in the presence of the protease inhibitors EDTA and PMSF to minimize degradation. In contrast, the small 3335-Da peptide was consistently recovered in the same immunoprecipitates after a 4 h-incubation period. In addition, the peptide tended to accumulate in CM up to 72 h, suggesting that it is relatively stable.

Fig. 4. Detection of the 3335-Da peptide by IP-SELDI MS. A, semiquantitative analysis of the peptide in CM from normally growing cells. The right panel shows examples for negative expression. B, Capan-1 cells were exposed to dec-RVKR-cmk for 48 h, which was replenished every 12 h because of its labile property in CM (14). C, recovery of the 3335-Da peptide from Capan-1 cell lysate. D, Brefeldin A experiments. Capan-1 cells exposed to the drug for 20 h decreased the peptide secretion in a dose-dependent manner. The values indicate a signal to noise ratio ± SE from three independent measurements.

fied peptide, RSKR, suggests its cleavage by furin or furin-like processing proteases, which are ubiquitously expressed in all cell types (13). The occurrence of the peptide in cell extracts strongly suggests that the peptide is intracellularly cleaved from the parent DMBT1 protein. To our knowledge, this is the first demonstration that a defined portion of a candidate tumor suppressor protein is secreted by a particular cell type. Additional studies will be needed to identify the responsible enzyme. Because COOH-terminal peptides cleaved by furin or furin-like proteases are known to function as an extracellular mediator of signaling (13), the 3335-Da peptide may have any biological role in pancreatic cancer.

Our study points to a new approach to marker discovery. It should be stressed that other techniques currently applied to marker discovery such as the genome-wide analysis of mRNA expression or the conventional proteome analysis (19, 20) would not lead to the identification of this peptide. Overall, our peptidomics-based approach for the discovery of candidate secretory marker peptides was verified by the identification of the last COOH-terminal 29 amino acids of DMBT1 in pancreatic adenocarcinoma. Although the expression and secretion of this peptide in the CM seemed to be limited to pancreatic adenocarcinoma cell lines thus far, additional studies are needed to demonstrate that the cleavage is specific to these cells. In the upcoming study, we are going to evaluate its occurrence on clinical specimens and sera if it would serve as a tumor marker specific to pancreatic adenocarcinomas. Besides the search for tumor markers, our approach will greatly expedite the discovery of differentially expressed secretory peptides in a particular context.

Acknowledgments

We thank Dr. Yi Min She (Department of Physics & Astronomy, University of Manitoba, Canada) and Dr. Kenji Saitoh (Ciphergen Biosystems) for MS/MS analysis of the peptide, and Dr. Ian Mollenhauer (Deutsches Krebsforschungszentrum, Heidelberg, Germany) for the monoclonal antibody.

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