Specific Peptide Ligand for Grb7 Signal Transduction Protein and Pancreatic Cancer Metastasis
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Background: Pancreatic cancer is one of the most aggressive malignancies, with high rates of invasion and metastasis and with generally poor prognosis. We previously found that metastasis was strongly associated with the expression of growth factor receptor–bound protein 7 (Grb7), which contains a Src homology 2 (SH2) domain. In this study, we evaluated Grb7 protein as a molecular target of therapy for metastatic pancreatic cancer. Methods: Grb7 protein expression was measured by immunohistochemistry in 36 human pancreatic cancer specimens and adjacent normal pancreatic tissue. We synthesized a nonphosphorylated peptide inhibitor that binds specifically to the SH2 domain of Grb7. Intracellular signaling was assessed by immunoprecipitation and immunoblot assays in cultured human pancreatic cancer cells. Cell migration was measured with a modified Boyden chamber method. Peritoneal metastasis of the pancreatic cancer cells was measured with a mouse model. All statistical tests were two-sided. Results: We found that 22 (61%) of 36 pancreatic cancer specimens had higher levels of Grb7 protein than their corresponding normal pancreatic tissue specimens. Grb7 expression was statistically significantly different between specimens from patients without lymph node metastasis (stage N0; two of the 10 patients) and patients with lymph node metastasis (stages N1 + N2; 20 of the 26 patients) (P = .006). The Grb7 peptide inhibitor selectively blocked the interaction between Grb7 and focal adhesion kinase and blocked the phosphorylation of Grb7 protein. In vivo Grb7 peptide inhibitor statistically significantly attenuated cell migration (for control peptide, 87.5 cells migrated, 95% confidence interval [CI] = 82.6 to 92.4 cells; for Grb7 peptide, 5.7 cells migrated, 95% CI = 2.3 to 9.0 cells; P < .001) and peritoneal metastasis of the pancreatic cancer cells in a mouse model, as assessed by the number of nodules (control = 72.6 nodules, 95% CI = 55.8 to 89.4 nodules; and for Grb7 peptide = 3.2 nodules, 95% CI = 1.6 to 4.8 nodules; P < .001, t test) and their weight (control = 4.13 g, 95% CI = 3.40 to 4.86 g; Grb7 peptide = 0.19 g, 95% CI = 0.06 to 0.32 g; P < .001, t test). Conclusions: The Grb7 peptide inhibitor appears to be a promising molecularly targeted therapeutic agent against metastatic pancreatic cancer. [J Natl Cancer Inst 2006;98:491–8]

Pancreatic cancer, which is among the most aggressive of all human malignancies, has a 5-year survival rate of less than 1% (1,2). It is the fourth leading cause of cancer death in the United States and the fifth leading cause of cancer death in Japan, and the cause-specific death rate of the pancreatic cancer is gradually increasing worldwide (1). Invasion and metastasis of tumor cells are major risk factors that decrease the clinical prognosis of patients with pancreatic cancer. The mechanism for invasion and metastasis involves the activation of members of the protein tyrosine kinase family, followed by the transmission of intracellular signals (3). The subsequent tyrosyl phosphorylation of intracellular substrates leads to their interaction with Src homology 2 (SH2) domains of downstream signaling molecules (4). Because of the specific role of the SH2 domains in signal transduction pathways, SH2-mediated protein–protein interactions are attractive therapeutic targets for cancer treatment (5).

We have previously identified Grb7, an SH2-containing adaptor protein that is frequently overexpressed in invasive and metastatic human cancer tissues (6). The predicted protein structure of Grb7 shares structural homology with Mig-10, a cell migration molecule from Caenorhabditis elegans, that is required for embryonic cell migration to the neural cleft (7,8). It is noteworthy that the human Grb7 gene is located at chromosomal position 17q12-q22 within an amplicon of the erbB2 gene, which is frequently amplified in various cancer cells (9,10). The Grb7 protein can bind to various protein tyrosine kinases, including members of the erbB family (10–12) and focal adhesion kinase (FAK) (13), whose activities play critical roles in cell migration (14,15). Therefore, competitive targeting of interactions involving the SH2 domain of Grb7 protein might affect the migration of human cancer cells (16–18). Although specific phosphotyrosine-containing peptides with a minimal recognition motif can compete in vitro with phosphorylated protein ligands for SH2 domain binding, these peptides are less able to compete in vivo because the various phosphatases that are present in tissues inactivate these peptides by removing the phosphate groups. This problem prompted us to develop inhibitors that do not require phosphate groups to target SH2 domains, such as the nonphosphorylated cyclic peptides with selective affinity to SH2 domains that we previously developed by use of phage-display technology (19). One of these peptides, termed G7-18NATE, binds specifically to the SH2 domain of Grb7 protein and inhibits the binding of Grb7 protein to various protein tyrosine kinases (20).

In the study, we analyzed Grb7 protein expression in human pancreatic cancer tissue specimens to determine whether it was associated with the metastatic spread of pancreatic cancer and...
could be a molecular target for metastatic pancreatic cancer therapy. We attached a penetratin sequence to the Grb7 peptide inhibitor G7-18NATE, resulting in G7-18NATE-P, to facilitate its uptake by cells (21). We also evaluated the in vivo effects of the Grb7 peptide inhibitor on cell migration and peritoneal metastasis of the pancreatic cancer cells.

**MATERIALS AND METHODS**

**Tissue Specimens**

We studied 36 specimens (23 from men and 13 from women; mean age = 65.2 years) of primary pancreas adenocarcinomas for Grb7 expression, as described previously (7). Written informed consent from these patients and institutional review board approval were obtained. Tumor pancreatic cancer tissues were fixed with periodate–lysine–paraformaldehyde at 4 °C, embedded in OCT compound, and stored at −80 °C. Sections that were 4 μm thick were cut and then stained with hematoxylin–eosin for histopathologic analysis.

**Immunohistochemistry**

We used immunohistochemistry to evaluate the expression of Grb7 protein in sections of human pancreatic cancer tissue (7,22). The anti-Grb7 polyclonal antibody (product C-20; Santa Cruz Biochemistry, Santa Cruz, CA) was used at a 1:50 dilution in phosphate-buffered saline (PBS) containing 1% bovine serum albumin (Sigma Chemical, St. Louis, MO), followed by incubation for 6 hours at room temperature. Antibody binding was then immunodetected with the avidin–biotin–peroxidase complex, as described by the supplier (Nichirei, Tokyo, Japan). Sections were lightly counterstained with hematoxylin to stain the nucleus. Grb7-positive staining of cancer cells was determined by comparison with the Grb7-negative staining of adjacent normal acinar and duct cells, the internal negative control. Immunohistochemical staining was evaluated under a light microscope by three independent pathologists (K. Taguchi, M. Mori, and S. Ariti). Cancer tissue specimens were scored as positive if more than 20% of the tumor cells stained positive for Grb7; specimens were scored as negative if less than 20% tumor cells were positive for Grb7.

**Immunoblot Analysis**

Pancreatic tissues or cells were prepared for immunoblot analysis as described (7,22). Fixed tissues or cells were lysed in ice-cold Triton buffer (50 mM Tris-HCl at pH 7.5, containing 1% Triton X-100, 2 mM EGTA, 10 mM EDTA, 100 mM NaF, 1 mM Na3P2O7, 2 mM NaVO4, 1 mM phenylmethylsulfonyl fluoride, aprotinin at 25 μg/mL, pepstatin A at 3.5 mg/mL, and leupeptin at 25 mg/mL), followed by centrifugation at 14,000g for 15 minutes at 4 °C. Next, 50 μg of the supernatant per lane was subjected to electrophoresis through sodium dodecyl sulfate–polyacrylamide gels and transferred onto Immobilon-P membranes (Millipore Corp., Bedford, MA). Blots were incubated for 6 hours at 4 °C with specific antibody, and bands were detected via the ECL system (Amersham, Arlington Heights, IL). For immunoprecipitation analysis, the lysate supernatant containing 500 μg of protein was incubated with 10 μg of antibody and precipitated with protein A–agarose for 6 hours at 4 °C. We used anti-Grb7 polyclonal antibody (product sc-606; Santa Cruz Biotechnology, Santa Cruz, CA), anti-Grb2 monoclonal antibody (product sc-8034; Santa Cruz Biotechnology), anti-Grb10 polyclonal antibody (product sc-13955; Santa Cruz Biotechnology), anti-phosphotyrosine monoclonal antibody conjugated to horseradish peroxidase (product PY20H; Transduction Laboratories. Lexington, KY), and anti-FAK monoclonal antibody (clone 77; Transduction Laboratories) and anti–epidermal growth factor receptor (EGFR) monoclonal antibody (clone 77; Oncogene Research Products, Cambridge, MA). All antibodies were used at a 1:200 dilution, except for PY20H, which was used at a 1:1000 dilution. As secondary antibodies, horseradish peroxidase-conjugated anti-mouse immunoglobulin G (IgG) antibody and anti-rabbit IgG antibody were used at 1:10,000 dilutions (products NA931 and NA934; Amersham).

**Real-Time Quantitative Genomic Polymerase Chain Reaction Analysis**

Pancreatic tissue preparation, microdissection of pure tumor cell populations from fixed tissue, and nucleic acid extraction from paraformaldehyde-fixed tissues for polymerases chain reaction (PCR) analysis were carried out as described in our previous report (6). Amplification of Grb7 and erbB2 sequences was carried out by real-time PCR with the ABI PRISM 7700 Sequence Detection System (Applied Biosystems, Foster City, CA), as described by Walch et al. (23). The following primers and probes were prepared to amplify genomic erbB2 sequences (sense, 5′CA GAAGGTCTACATGGTGCTTC-3′; antisense, 5′TTCCGAGC GGCCAAGTC-3′; probe, 5′-TGGAGAGTGTGGCGCTGATC ACA-3′), Grb7 sequences (sense, 5′-CAAGGTCTCAGCTCTACTCA TGCTG-3′; antisense, 5′-ACGGTGACTCGTCATACGCTG-3′; probe, 5′-AGGATCGGGGCACCATGCAGTACGTG-3′), and globin sequences (sense, 5′-ACCCTTAAGCTGTCTGTTGG-3′; antisense, 5′-GGAGTTGACAGATCCCCAAA-3′; probe, 5′-CTA CCCCTGACCCAGGTTCTTTGAGTC-3′). Copy numbers of Grb7, erbB2, and globin genes per cell were calculated by linear regression analysis from an external standard curve generated from pooled genomic DNA from human blood cells. Cut points for altered Grb7 and erbB2 copy numbers were defined by the mean value of the Grb7/globin and erbB2/globin expression ratios determined in normal pancreatic tissues plus four standard deviations. Cut points were 5.17-fold for Grb7 and 5.05-fold for erbB2.

**Synthesis of Small Interfering RNA and Peptides**

The targeted sequence for human Grb7 small interfering RNA (siRNA) (5′-AAGCAGUCAAACGUGUACGUG-3′) was designed as described (24). The siRNA was synthesized and labeled with Alexa Fluor 488 by QIAGEN Inc. (Hilden, Germany). The negative control siRNA, labeled with Alexa Fluor 488 (5′-AA UUCUCGAACGGUGUACGUG-3′; product 1022563), was purchased from QIAGEN. Thioether-cyclized peptides were synthesized by Bio-Synthesis Inc. (Lewisville, TX), as previously described (20). Briefly, the peptides were synthesized on a Rink Amide resin (product 01-64-0013; Novabiochem) by standard solid-phase synthesis with fluorenylmethoxycarbonyl (Fmoc) chemistry (25). N-a-Fmoc-S-p-methoxytrityl-l-cysteine [Fmoc-lys(Mmt)-OH] (04-12-1061; Novabiochem) was used in this synthesis. After attaching the amino acids and bromoacetylating the amino-terminal peptide, the protected cysteine side chain was deprotected with 1% trifluoroacetic acid (TFA) in CH2Cl2, and then the peptide was cyclized in 1% N,N-diisopropylethylamine.
in dimethylformamide for 24 hours. The cyclic peptide was cleaved from the support under standard conditions (5% 2-[(2-hydroxy-1,1-bis(hydroxymethyl)ethyl)amino]ethanesulfonic acid–95% TFA). The peptides were purified by reverse-phase high-pressure liquid chromatography on a Phenomenex Jupiter C18 column (solution A = 0.5% TFA in water; solution B = 0.5% TFA in acetonitrile) gradient = solution A to solution B over 40 minutes). To improve the uptake of the Grb7 peptide inhibitor G7-18NATE by cells for in vivo studies, we covalently attached a penetratin peptide (21) to G7-18NATE to produce G7-18NATE-P (WFEGYDNFP-CQIKWFQNRMKWK). As a control, we used the penetratin peptide without an attached peptide (RQIKIWFQNRMKWK).

Cell Culture, Transfection, Proliferation, and Migration Assays

Human pancreatic cancer cell lines, Panc-1, MiaPaca2, PK8, and KLM1, were cultured in RPMI 1640 medium (Sigma) supplemented with 10% heat-inactivated fetal bovine serum (Sigma). Multichannel confocal laser–scanning microscopy was used for analysis of the living cell cultures. Panc-1 cells were transfected with siRNA oligonucleotides by use of Lipofectamine 2000 Reagent (GIBCO BRL, Life Technologies, Inc., Gaithersburg, MD) essentially as described elsewhere (7), except that we used 60% confluent cells.

Assays of cell proliferation and migration were performed as described previously (7,22). For proliferation assays, 1 × 10^5 cells were cultured on 10-cm plastic dishes, and the number of cells was counted every other day for 8 days. For migration assays, we used a modified Boyden chamber and 24-well plates containing polyethylene terephthalate filter inserts (8-µm pores) that were coated with fibronectin (Collaborative Biomedical Products, Bedford, MA). First, 1 × 10^4 cells were suspended in serum-free medium containing 0.5% bovine serum albumin and placed in the upper chamber, and then the lower chamber was filled with the same medium. After a 16-hour incubation, cells were fixed with 5% glutaraldehyde in PBS and stained with 0.5% trypan blue in 2% Na2CO3. Cells on the upper side of the filter were carefully removed with a cotton swab, and cells invading the lower side of the filter were counted by microscopic examination. The mean migration rate derived from three independent experiments was determined for each clone.

Mouse Model of Pancreatic Cancer Peritoneal Metastasis

To produce experimental tumor metastases (26), we suspended 5 × 10^6 Panc-1, MiaPaca2, or KLM1 cells in 200 µL of RPMI 1640 medium and injected them intraperitoneally into BALB/c nu/nu mice at 5 weeks of age (mouse CAnN.Cg-Foxn1nu/CrlCrlj; Charles River Japan, Inc., Yokohama, Japan). Mice were randomly assigned (five mice per group) to treatment with the Grb7 peptide inhibitor (10 mmol/kg) or the control peptide (10 mmol/kg); the peptides were injected intraperitoneally at the time of cell implantation and every third day for 2 weeks. On day 14, all mice were killed by cervical dislocation under anesthesia, macroscopic nodules on the peritoneal surface were counted and excised, and each nodule was weighed. Data presented are the means of three independent experiments.

To investigate whether the Grb7 peptide inhibitor had adverse side effects, we injected BALB/c nu/nu mice with the Grb7 peptide inhibitor at 1, 10, or 100 mmol/kg intraperitoneally every third day for 4 weeks and determined the body weight of the mice and any histologic changes of the major tissues, including liver, kidney, spleen, and lung. The growth of subcutaneously injected pancreatic tumor was also assessed as described previously (27). All in vivo procedures were approved by the animal care committee of Tokyo Medical and Dental University.

Statistical Analysis

Statistical significance was assessed by a chi-square test or Fisher’s exact test with a single degree of freedom. Two-sided Student’s t tests (StatView software, Abacus Concepts, Inc., Berkeley, CA) were used to analyze differences between continuous values of two independent groups. P values of less than .05 were considered to have statistical significance. All statistical tests were two-sided.

RESULTS

Grb7 Expression in Clinical Samples of Pancreatic Cancer

To determine whether Grb7 protein has clinical significance in human pancreatic cancer, we assessed the expression of Grb7 protein in human pancreatic cancer tissue specimens obtained from surgical resection by immunohistochemical analysis. We found that 22 (61%) of the 36 pancreatic cancer specimens examined had higher expression of Grb7 protein than the adjacent normal tissue, which had little if any expression in all 36 specimens (Fig. 1, A). We confirmed overexpression of Grb7 protein in some pancreatic cancers by Western blot analysis of tumor tissue lysates and also detected phosphorylation of Grb7 protein on tyrosine residues in tumors overexpressing Grb7 protein (Fig. 1, B).

The human Grb7 gene is located at chromosomal position 17q12-q22 within an ampiclon of the erbB2 gene, which is frequently amplified in pancreatic cancer cells (28). To determine whether the Grb7 and erbB2 genes were amplified in our tumor specimens, we used quantitative PCR, as described elsewhere (23). We found that both Grb7 genes (median = 12.0 copies per cell, interquartile range = 8.9–23.3 copies per cell, and range = 7.2–36.2 copies per cell) and erbB2 genes (median = 14.8 copies per cell, interquartile range = 9.8–18.3 copies per cell, and range = 7.9–23.3 copies per cell) were amplified in 13 of the 22 tumors that overexpressed Grb7 protein, but we did not detect amplification of Grb7 or erbB2 genes in any of the tumors that did not overexpress Grb7 protein (P<.001; t test). Thus, overexpression of Grb7 protein might be associated with gene amplification of the 17q12-q22 chromosomal region in some pancreatic tumors.

Grb7 overexpression also appeared to be associated with lymph node status (Fig. 1, C). Among patients without lymph node metastases, Grb7 overexpression was observed in pancreatic cancer specimens from only two (20%) of the 10 patients. Among patients with regional lymph node metastases, Grb7 overexpression was observed in pancreatic cancer specimens from 12 (67%) of the 18 patients with N1 stage disease and from all eight (100%) patients with N2 stage disease. Grb7 expression was statistically significantly different between specimens from patients without (stage N0; two of the 10 patients) and specimens from patients with...
Grb7 siRNAs labeled with an anti-Grb7 monoclonal antibody. Similar tissues gave similar results. Immunohistochemistry used an antibody to detect Grb7 protein in pancreatic cancer tissue and adjacent normal tissue are shown. Immunohistochemistry detected an antibody to detect Grb7 protein in pancreatic cancer cells (original magnifications: upper = ×100, lower = ×200). Positive staining = solid arrowheads; negative staining = open arrowheads. Lack of staining for Grb7 protein was lower than that in nontransfected cells and Grb7 siRNA tranfection reduced the migration of pancreatic cancer cells (Fig. 2, B). We found that expression of Grb7 protein in cells transfected with control siRNA (control siRNA = 84.8 cells migrated; Grb7 siRNA = 20.7 cells migrated; difference = 64.2 cells migrated, 95% confidence interval [CI] = 47.3 to 81.1 cells migrated; P<.001; Fig. 2, C). As in our previous results with antisense Grb7 (7), these results indicate that Grb7 overexpression plays a role in cancer cell migration.

**Inhibition of Grb7 Gene Expression with Grb7 siRNA in Human Pancreatic Cancer Cells**

To explore the biological role of Grb7 overexpression in the cancer phenotype, we used knock-down analysis of Grb7 gene expression in human pancreatic cancer cells (24). We transfected Grb7 siRNAs labeled with a fluorescent dye into Panc-1 human pancreatic cells (Fig. 2, A) and assessed the expression of Grb7 protein by immunoblotting with anti-Grb7 polyclonal antibody. We found that expression of Grb7 protein in cells transfected with Grb7 siRNA was lower than that in nontransfected cells and in cells transfected with control siRNA (Fig. 2, B). We then used these transfected cells to investigate whether migration into fibronectin, as measured in a modified Boyden chamber (22), was altered in cancer cells transfected with Grb7 siRNA. Grb7 siRNA tranfection reduced the migration of pancreatic cancer cells, compared with that of nontransfected control cells or cells transfected with a control siRNA (control siRNA = 84.8 cells migrated; Grb7 siRNA = 20.7 cells migrated; difference = 64.2 cells migrated, 95% confidence interval [CI] = 47.3 to 81.1 cells migrated; P<.001; Fig. 2, C). As in our previous

**Effect of Grb7 Peptide Inhibitor on Intracellular Signaling, Proliferation, and Migration of Human Pancreatic Cancer Cells**

We have previously described the Grb7 peptide inhibitor G7-18NATE (Fig. 3, A), which binds specifically to the SH2 domain of Grb7 protein and inhibits the association of Grb7 protein with various members the protein tyrosine kinase family (10). In this study, we used G7-18NATE-P (i.e., G7-18NATE to which we added a penetratin sequence to facilitate the intracellular uptake of the inhibitor). By use of multichannel confocal laser-scanning microscopy, we found that Panc-1 human pancreatic cancer cells can take up fluorescently labeled G7-18 NATE-P (Fig. 3, B). In our previous studies, we found that Grb7 protein can interact with and phosphorylate FAK in fibronectin-stimulated cells (22). To further assess intracellular Grb7 signaling, Panc-1 cells were stimulated with fibronectin, and the interaction of endogenous Grb7 protein with FAK and the phosphorylation of Grb7 protein on tyrosine residues were examined by immunoprecipitation with anti-FAK and anti-Grb7 antibodies. Both the interaction between Grb7 and FAK and the level of phosphorylated Grb7 were reduced after treatment with the Grb7 peptide inhibitor.
G7-18NATE-P (Fig. 3, C). To investigate the specificity of the Grb7 peptide inhibitor for these activities, we repeated the experiments but assessed Grb2 and Grb10, other endogenous SH2-containing proteins. We found that G7-18NATE-P did not affect the interaction of Grb2 with FAK or with EGFR (Fig. 3, C) and that the peptide inhibitor did not affect tyrosyl phosphorylation of Grb10 (Fig. 3, C). The proliferation of pancreatic cells treated with the Grb7 peptide inhibitor was not statistically significantly different from that of untreated cells (Fig. 3, D).

Fig. 2. RNA interference of Grb7 expression in human pancreatic cancer cells. A) Transfection of fluorescent-labeled siRNAs in the Panc-1 living human pancreas cancer cells. Fluorescently labeled siRNAs are shown as green on multichannel confocal laser-scanning microscopy. B) Expression of Grb family proteins in fibronectin-stimulated Panc-1 cells transfected with Grb7 siRNA or with control siRNA or nontransfected control Panc-1 cells (none). C) Fibronectin migration assay. The number of cells migrating into the fibronectin was determined for Panc-1 cells transfected with Grb7 siRNA or with control siRNA or nontransfected control Panc-1 cells (none) with a modified Boyden chamber method. The mean migration rate, expressed as cell numbers, derived from three independent experiments for duplicates per point (control siRNA = 84.8 cells and Grb7 siRNA = 20.7 cells; difference = 64.2 cells, 95% confidence interval = 47.3 to 81.1 cells; P < .001). All statistical tests were two-sided.

Effect of Grb7 Peptide Inhibitor on Metastasis of Pancreatic Cancer

To determine whether the Grb7 peptide inhibitor inhibited the metastasis of pancreatic cancer cells, we used a murine model of peritoneal metastasis (26). We first injected Panc-1 human pancreatic cancer cells intraperitoneally into BALB/c nu/nu mice and then injected the Grb7 peptide inhibitor or a control peptide intraperitoneally every third day for a period of 2 weeks. Four weeks after the first injection of the Panc-1 cells, mice were killed, the numbers of peritoneal metastatic nodules were counted, and then each nodule was weighed. Fewer peritoneal metastases of the pancreatic cancer cells were found in mice treated with the Grb7 peptide inhibitor than in mice treated with a control peptide (Fig. 5). Pancreatic cancer cells were found in the control mice but not in mice treated with the Grb7 peptide inhibitor (Fig. 5, A). In addition, the number of tumor nodules per mouse (control = 72.6 nodules, 95% CI = 55.8 to 89.4 nodules; Grb7 peptide = 3.2 nodules, 95% CI = 1.6 to 4.8 nodules; P < .001, t test) and the total nodule weight per mouse (control = 4.13 g, 95% CI = 3.40 to 4.86 g; Grb7 peptide = 0.19 g, 95% CI = 0.06 to 0.32 g; P < .001, t test) were statistically significantly lower in mice treated with the Grb7 peptide inhibitor than in mice treated with control peptide (Fig. 5, B). Similar results were found with other human pancreatic cancer cell lines that overexpressed Grb7 (such as MiaPaca2 cells) but not with lines that did not overexpress Grb7 (such as KLM1 cells) (data not shown). However, treatment with the Grb7 peptide inhibitor appeared to have no effect on the growth of subcutaneously injected pancreatic tumor (data not shown).

To investigate whether the Grb7 peptide inhibitor had adverse side effects, we injected BALB/c nu/nu mice with the Grb7 peptide inhibitor intraperitoneally every third day for 4 weeks and determined the body weight of the mice and the histologic changes of the major tissues, including liver, kidney, spleen, and lung. We found no clinically significant adverse effects, even when we injected a 10-fold higher concentration than was used to investigate metastasis.

**DISCUSSION**

In this study, 61% of 36 pancreatic cancers were found to have higher levels of Grb7 protein than corresponding normal pancreatic tissue. The increased cellular levels of Grb7 appeared to be associated with regional lymph node metastatic spread of human pancreatic cancer (P = .006). Because the Grb7 SH2-mediated interactions might be an attractive candidate for the therapeutic targets of cancer invasion and metastasis (17, 22), we synthesized a thioether-cyclized peptide that selectively inhibited the interactions of Grb7 via its SH2 domain with protein tyrosine kinases (20). In vitro, the Grb7 inhibitor blocked binding to FAK and phosphorylation of the endogenous Grb7 protein in human pancreatic cancer cells. In vivo in a mouse model of metastatic pancreatic cancer, the Grb7 peptide inhibitor completely attenuated cell migration (control = 81.8 cells migrated versus Grb7 peptide = 4.3 cells migrated; difference = 77.5 cells migrated, 95% CI = 64.2 to 90.7 cells migrated; P < .001) and peritoneal metastasis, as assessed by the number of tumor nodules (control = 72.6 nodules and Grb7 peptide = 3.2 nodules; difference = 69.4 nodules, 95% CI = 52.7 to 86.1 nodules; P < .001) or by the weight of the tumor nodules (control = 4.13 g and Grb7 peptide = 0.19 g; difference = 3.94 g, 95% CI = 3.34 to 4.54 g; P < .001). Our results suggest that the Grb7 peptide inhibitor might be a promising agent for molecular targeted therapeutics against the metastatic pancreatic cancer.

SH2 domains are relatively small protein modules of approximately 100 amino acids that recognize sequences containing...
Fig. 3. Grb7 peptide inhibitor. A) Schematic structural formula of the modified nonphosphotyrosine cyclic Grb7 peptide inhibitor G7-18NATE (WFEGYDNTFPC). The cyclic peptide was synthesized by a thioether bond method, as described (20). A modified form of G7-18NATE with a penetratin sequence attached (G7-18NATE-P; WFEGYDNTFPC-RQIKIWFQNRRMKWKK) was used for the in vivo studies. B) Intracellular localization of fluorescent-labeled G7-18NATE-P in the living cells of human pancreas cancer. After cells were incubated for 6 hours at 37 °C, the peptide (green) was detected by multichannel confocal laser-scanning microscopy in living Panc-1 cells. C) Immunoblot analysis. Top) Expression (blot Grb7), focal adhesion kinase (FAK) binding (blot FAK/Grb7), and tyrosyl phosphorylation of Grb7 protein (blot Grb7/pY). Panc-1 cells were plated on fibronectin (10 μg/mL) and then incubated with 10 mM control peptide (RQIKIWFQNRRMKWKK), 10 μM G7-18NATE-P, or vehicle for 1 hour at 37 °C. Grb7 was detected with anti-Grb7 protein antibody C-20. To detect the interaction between FAK and Grb7 protein (FAK/Grb7), 500 μg of cell lysate were immunoprecipitated with 10 μg of anti-FAK antibody for 6 hours at 4 °C, and the immunoprecipitate was subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis, transferred onto Immobilon-P membranes (Millipore Corp.), and then immunoblotted with anti-Grb7 antibody. Bottom) Expression of Grb2 (blot Grb2), interaction of FAK with Grb2 (blot FAK/Grb2), interaction of epidermal growth factor receptor (EGFR) with Grb2 (blot EGFR/Grb2), expression of Grb10 (Grb10), and expression of tyrosyl-phosphorylated Grb10 (blot Grb10/pY). Grb2 was detected with anti-Grb2 monoclonal antibody. The interaction of FAK and Grb2 was detected by immunoprecipitation with anti-FAK antibody, followed by immunoblotting with anti-Grb2 antibody. The interaction of EGFR and Grb2 was detected by immunoprecipitation with anti-EGFR antibody, followed by immunoblotting with anti-Grb2 antibody. The expression of Grb10 was detected with anti-Grb10 polyclonal antibody. The expression of tyrosyl-phosphorylated Grb10 was detected by immunoprecipitation with anti-Grb10 antibody, followed by immunoblotting with anti-phosphotyrosine antibody. D) Growth curves. The proliferation of Panc-1 cells treated with control peptide, G7-18NATE-P, and vehicle was determined by counting the number of cells every other day for 8 days (7, 22). Data are expressed as averaged cell numbers, determined from three independent experiments with duplicate samples. Error bars = 95% confidence interval (CI). E) Fibronectin migration assay. We treated 1 × 10⁴ Panc-1 cells with 10 μM G7-18NATE-P, 10 μM control peptide, or vehicle. The number of cells migrating into the fibronectin was determined for each culture, and the mean migration rate was determined from three experiments for duplicate samples. The mean number of cells migrating in cultures treated with G7-18NATE-P (5.7 cells, 95% CI = 2.3 to 9.0 cells) was statistically significantly less than that of cultures treated with a control peptide (87.5 cells, 95% CI = 82.6 to 92.4 cells) (P <.001). Error bars = 95% CIs. All statistical tests were two-sided.

phosphorylated tyrosine residues, thereby facilitating phosphorylation-dependent, protein–protein interactions that result in signal transduction (4). Small-molecule inhibitors that can disrupt these interactions should be useful in modulating the function of the SH2-containing proteins and may ultimately be used as pharmaceutical agents (5). The difficulty in designing cellularly active SH2 inhibitors has been the requirement of phosphorylated tyrosine for high-affinity binding (29). Peptide phage displays used to identify the ligands that bind to the SH2 domains have advantages over other methods, such as synthesis of phosphopeptides (19). The peptide phage display method can be used to select phosphorylated ligands because bacteriophages lack tyrosine kinase activity, and thus the naturally occurring peptides on the phage contain no phosphorylated tyrosine residues (30). The nonphosphorylated peptide that binds to the Grb7 SH2 domain has the advantage of in vivo stability over other SH2
ligands that possess a highly charged phosphate group (20). When phosphate groups are attached to a macromolecule, the ability of the compound to enter cells is reduced, and the phosphorylated compound is unstable in vivo because of the presence of endogenous phosphatases (18). In vivo stability is important to the development of inhibitors that are cellularly active and clinically effective.

Recent advances in molecular biology have provided a detailed understanding of the molecular events in pancreatic carcinogenesis (31,32) and may now offer new approaches to the treatment of pancreatic cancer. As molecular targets are identified, novel agents that are specific for such targets can be designed that might improve the treatment of pancreatic cancer. Trials have evaluated several biological agents, including humanized monoclonal antibodies that inhibit members of the protein tyrosine kinase family, cetuximab that inhibits EGFR, and trastuzumab that inhibits erbB2 (33,34). Other small molecules that inhibit the EGFR tyrosine kinase, such as ZD1839 and OSI-774, are also being investigated for activity in advanced pancreatic cancer (35,36). Because Grb7 protein binds to many oncogenic protein tyrosine kinases, such as EGFR (11), erbB2 (10), erbB3 (12), and erbB4 (12), combination therapy with the protein tyrosine kinase-targeting agents and the Grb7 peptide inhibitor may be a novel therapeutic intervention.

Several limitations of this study must be noted. First, a small number of pancreatic cancer specimens was studied in our clinical investigation. Much larger clinical studies are required to determine whether Grb7 protein is associated with patient survival or outcome. Second, we could not determine the mechanism of action of the Grb7 peptide inhibitor in vivo. The actual effects of the peptide on actively invading tumors should be analyzed. Finally, the actual cellular and molecular functions of Grb7 have not been defined in detail (16,17). Guan and colleagues (37) have reported that the association of Grb7 with phosphatidylinositol 3-kinase mediates the signal transduction pathways in FAK-induced cell migration. Recently, PREL1, another homologue of Grb7 and Mig-10 proteins, was found to bind to the actin cytoskeleton via Ena/vasodilator-stimulated phosphoproteins, which are important regulators of the actin polymerization machinery (38). Further analyses are required to identify the downstream targets of the Grb7-mediated signaling pathways to cancer metastasis.

In view of the evidence presented on the relationship between Grb7 expression and tumor progression, potent Grb7 antagonists may be promising anticancer agents for treatment of human cancers (16). Because of the antimitotic activity and the low toxicity of the Grb7 peptide inhibitor in mice, the potential of this inhibitor or its analogues should be investigated in additional preclinical experiments as treatments for human cancers that
overexpress Grb7 (17). The Grb7 peptide inhibitor should also be evaluated as a lead compound for the development of new drugs to treat diseases in which Grb7 is overexpressed, and it may also aid in elucidating the function of Grb7 in cancer cells (18). In addition, clinical studies should be designed to evaluate the effects of the peptide inhibitor in humans against pancreatic cancer and other cancers that overexpress Grb7, such as esophageal (6), gastric (9), and breast (10) cancers.

REFERENCES


NOTES

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