Characterization of a Human Homologue of Proteolysis-Inducing Factor and Its Role in Cancer Cachexia

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ABSTRACT

Cachexia is an important cause of secondary morbidity and mortality in patients with cancer. Previous studies have suggested that cancer-associated cachexia may be due in part to tumor-specific production and secretion of a glycosylated peptide, proteolysis-inducing factor, originally identified in a murine cancer cachexia model. We report here the cloning of a human cDNA that generates a peptide having high-sequence homology to this proteolysis-inducing factor. Constitutive expression of human proteolysis-inducing factor is low or absent in most normal human tissues but appears to be elevated in some human tumors. Stable forced expression of human proteolysis-inducing factor in multiple murine and human cell lines results in a secreted protein, but no glycosylation of the protein is detected. In addition, tumor xenografts engineered to overexpress human proteolysis-inducing factor protein do not induce cachexia in vivo. These findings raise important questions as to potential cross-species differences in protein sequence and processing of murine proteolysis-inducing factor and human proteolysis-inducing factor, as well as the nature of the relationship between human proteolysis-inducing factor and the development of cancer cachexia.

INTRODUCTION

Cachexia is an important cause of secondary morbidity and mortality in patients with cancer. Despite its profound clinical impact, the mechanisms of cancer cachexia are incompletely understood. Anorexia (3), cytokines (4–8), and transcription factors (9, 10) have all been implicated in, but cannot fully account for, the induction of cachexia.

Implantable tumor models in mice have provided a means to study cancer cachexia. One such model utilizes two histologically similar but not isogenic murine adenocarcinoma (MAC) cell lines, MAC13 and MAC16 (11). When injected into mice, MAC16 tumor cells produce profound cachexia while MAC13 tumor cells do not (12). Using a monoclonal antibody generated from splenocytes of mice with MAC16 tumors and attenuated weight loss, a 24-kDa heat-stable glycopeptide, proteolysis-inducing factor, was isolated from MAC16 tumor cells (13). This peptide was absent from MAC13 tumors (14) but was subsequently identified in cell lysates of other cachexia-causing tumors (15, 16), as well as in the urine of cachectic cancer patients (17). Injection of murine proteolysis-inducing factor into non-tumor-bearing mice induces weight loss in vivo (18), and activates the ATP-ubiquitin–dependent proteolytic pathway (19), suggesting that production and secretion of murine proteolysis-inducing factor by tumors plays a role in the development of cancer cachexia by inducing proteolysis in peripheral tissues.

We report the cloning of a human cDNA, human proteolysis-inducing factor, that generates a protein having high-sequence homology to the proteolysis-inducing factor peptide and likely represents a human homologue to the murine proteolysis-inducing factor. The gene and its protein product display a number of similarities to murine proteolysis-inducing factor, including lack of expression in most normal tissues, sporadic overexpression by select tumors, and secretion by cells that produce it. However, unlike murine proteolysis-inducing factor, human proteolysis-inducing factor does not appear to be glycosylated when expressed in murine or human cell lines and does not induce cachexia when overexpressed by tumor xenografts, throwing into question the identity of murine proteolysis-inducing factor and the role of both murine and human proteolysis-inducing factor in cancer cachexia.

MATERIALS AND METHODS

cDNAs. MCF7 RNA was isolated and obtained from Dr. Nancy Davidson. After DNaseI digestion, cDNA was synthesized from cell line RNA (2 μg) following the manufacturer’s protocol (Thermoscript RT-PCR system; Life Technologies, Inc., Rockville, MD). A commercial panel of cDNAs from normal tissues (heart, brain, lung, liver, skeletal muscle, kidney, pancreas, and placenta) derived from tissue polyA+ RNA was purchased from Clontech (Palo Alto, CA). A commercial panel of cDNAs from normal breast and breast tumors was purchased from Origene (Rapid-Scan Gene Expression Panels; Origene Technologies, Inc., Rockville, MD).

PCR. PCR reaction mixtures (25 μL) consisted of 400 nmol/L of each primer, 200 μmol/L each of dATP, dCTP, dGTP, and dTTP, and 1.25 units of Platinum Taq plus 1× PCR buffer or 1.25 units of Herculase polymerase plus Herculase.
buffer (Stratagene, La Jolla, CA). Primers were purchased from DNAgency (Malvern, PA) and Life Technologies, Inc.

5'- and 3'-Rapid Amplification of cDNA Ends. Adapted-ligated double-stranded cDNAs derived from breast and pancreatic cancer xenograft RNA were purchased for use in 5'- and 3'-rapid amplification of cDNA ends (RACE) reactions (Marathon-Ready cDNA; Clontech). Overlapping gene-specific primers were synthesized [3'-RACE: human proteolysis-inducing factor-f (sense), 5'-GATCCAGAGGCCGCTCTGCCCCCA-3'; 5'-RACE: human proteolysis-inducing factor-r (antisense), 5'-TGGGTCTTCACCTGCATTTTCCTTT-3'], and 25 μL of 68°C touchdown PCR reactions were performed using these primers and the adapter primer API (5'-CATCCTAATACTGACCTATAGGGGC-3'). After amplification, the PCR product was diluted 1:50; and nested PCR was performed with internal primers [3'-RACE: human proteolysis-inducing factor-r internal (sense), 5'-GGAACCTTGCCATGAAGC-3'; 5'-RACE: human proteolysis-inducing factor-r internal (antisense), 5'-TTGAGCTGCTGATGCTTCAT-3'], and the nested adapter primer AP2 (5'-ACTCACAATAGGCTCAGGCGG-3').

TA Cloning. Freshly synthesized PCR products were ligated into the pCR®2.1 cloning vector following the manufacturer's instructions (Original TA Cloning Kit; Invitrogen Corp., Carlsbad, CA) and transferred into Escherichia coli INV-3a. Transformed bacteria were plated on luria broth ampicillin plates coated with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside, and colonies with inserts were selected by blue/white screening. Recombinant bacteria were then grown overnight in luria broth containing 100 μg/mL ampicillin. Plasmid DNA was isolated by mini or midi prep (QiAprep Spin Miniprep kit and Plasmid Midi kit; Qiagen, Valencia, CA), and fragments of interest were extracted from plasmids after restriction enzyme digestion (EcoRI for pCR®2.1 clones, BamHI and EcoRI, for inserts with restriction sites in the primers) and separation of DNA fragments on 1.2% agarose gels.

RNA Extraction and Northern Blot Analysis. Total RNA was isolated from cell culture and tumor tissue by the TRIzol reagent method (Life Technologies, Inc.). A commercial RNA was isolated from cell culture and tumor tissue by the TRIzol reagent method (Life Technologies, Inc.). A commercial RNA was isolated from cell culture and tumor tissue by the TRIzol reagent method (Life Technologies, Inc.).

Real-Time PCR. Primers and probes were designed to specifically amplify our cDNA [forward (sense) 5'-GCCAT-CAAGCATTAGGCAG-3'; reverse (antisense) 5'-CTTAGTT-TTCCGAGTCCC-3'; Taqman probe 6FAMS-5'-CAGGCAC-CACCAGCAGAGCAG-3'TAMRA]. These primers span two exons to allow gel-based confirmation that amplification signals were only generated from RNA-derived cDNA not genomic DNA. Fluorogenic PCRs were carried out in a reaction volume of 25 μL. Each human proteolysis-inducing factor PCR reaction mixture consisted of 600 nmol/L of each primer, 200 nmol/L probe, 0.5 units of Platinum Taq polymerase, 200 μmol/L each of dATP, dCTP, dGTP, and dTTP, and 1 X PCR buffer. The internal reference gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was amplified using an optimized commercially available PCR mixture containing primers, probe, DNA polymerase, nucleotides, and PCR buffer (Pre-Developed Taqman Assay Reagents, Endogenous Control Human GAPDH; Applied Biosystems, Foster City, CA). The thermal profile for the PCR was 95°C for 15 seconds, 60°C for 30 seconds, and 72°C for 1 minute. Amplifications were carried out in 96-well reaction plate format in a PE Applied Biosystems 7700 Sequence detector. Data obtained after 50 cycles of amplification were analyzed.

Synthesis of Mammalian Expression Vectors Expressing Human Proteolysis-Inducing Factor. Synthetic oligonucleotides (sense 5'-GTGGAATTCGCGCATATACTGACCACTAGTTTCGAGTCTCCTGCCC-3'; antisense 5'-ACTGAATTCGCTCCTTACCCCGCCTGATCCTGGTTATTATTTCC-3') were used to amplify the complete coding sequence of human proteolysis-inducing factor cDNA (+/− stop codon) by reverse transcription-PCR using pancreatic RACE-ready cDNA as template. The PCR products were ligated into the pCRII®2.1 TA-cloning vector, and clones having appropriately sized inserts after restriction digestion were sequenced (AmpliCycle Sequencing kit; Applied Biosystems, Branchburg, NJ). Human proteolysis-inducing factor cDNAs were excised from the pCRII®2.1 cloning vector by restriction digestion, and two mammalian expression cassettes were constructed in which human proteolysis-inducing factor was inserted into the plasmid pcDNA 3.1/Myc-His (+) (Invitrogen) downstream of the cytomegalovirus promoter using bacteriophage T4 DNA ligase. In the first, the complete cDNA coding sequence of human proteolysis-inducing factor, including the stop codon, was inserted. In the second, human proteolysis-inducing factor was cloned in frame with a COOH-terminal tag encoding the myc epitope and a polyhistidine metal-binding peptide.

Western Blot Analysis. A synthetic peptide having the sequence AGEDPGLARQAPKPRKQRS (amino acids 44–62, the most hydrophilic and antigenic portion of the protein) was commercially synthesized, conjugated to a carrier protein, and injected into two rabbits to raise polyclonal antibody following a standard 63-day protocol (Alpha Diagnostics International, Inc., San Antonio, TX). Antiserum raised to a synthetic peptide encoding the murine proteolysis-inducing factor amino acid sequence (anti-CF antisera) was purchased from Alpha Diagnostics. Monoclonal antibody to the glycosylated portion of the murine proteolysis-inducing factor was kindly provided by Dr. Michael Tisdale (Pharmaceutical Sciences Institute, Aston University, Birmingham, United Kingdom). Secondary antibodies (donkey anti-rabbit or sheep anti-mouse immunoglobulin linked to horseradish peroxidase) were purchased from Amersham Pharmacia Biotech (Piscataway, NJ).

Tumor tissue and cells grown in culture were lysed with radioimmunoprecipitation (RIPA) buffer (21). Concentrated conditioned media were obtained by growing cells in a reduced volume of media (1.5 mL/confluent T25 flasks) for 24 hours. Media were aspirated and cleared of cells by passage through a 0.22-μm filter. Protein samples (20 μg of cell lysate or 20 μL of conditioned media) were separated by 4 to 20% SDS-PAGE and transferred to a nitrocellulose membrane. After non-specific sites were blocked by incubation in 5% (wt/vol) nonfat dry milk in 0.1% Tween PBS, the blots were incubated with affinity-purified antibody to human proteolysis-inducing factor (1:1000 cell lysates, 1:500 conditioned media), anti-CF anti-
serum (1:1000) or anti-murine proteolysis-inducing factor antibody (1:1000) for 1 to 2 hours at room temperature. The blots were then washed and incubated with secondary antibody (1:1000) for 1 hour at room temperature. After washing with Tween PBS, bound antibody was visualized by fluorography using an enhanced chemiluminescence kit (Amersham Pharmacia Biotech).

**Cell Lines.** MAC13 and MAC16 tumor cells were obtained from Dr. Michael Tisdale and were maintained in vitro in RPMI 1640 with L-glutamine (Life Technologies, Inc.). HEK293 cells were maintained in DMEM (low glucose), BT-20 cells in MEM, and MCF7 breast tumor cells in MEM supplemented with 0.01 mg/mL insulin. All media were supplemented with 10% fetal bovine serum (Gemini Bio-Products, Woodland, CA), and penicillin-streptomycin (100 units/mL and 100 μg/mL, respectively), and cells were maintained in a humidified atmosphere of 5% CO₂ in air at 37°C.

**Generation of Stably and Transiently Transfected Cell Lines.** pcDNA 3.1/Myc-His(+/human proteolysis-inducing factor(±) plasmids (2 μg) were transfected into MAC13, MAC16, HEK293, and MCF7 cells using Lipofectamine Plus as described by the manufacturer (Life Technologies, Inc.). BT-20 cells were transfected using Lipofectamine 2000 (Life Technologies, Inc.). Selection for transfected cells was based on G418 resistance conferred by the plasmid. After growth in media containing G418 toxin (0.5 to 0.6 mg/mL) for several weeks, surviving colonies were screened for expression of pcDNA3.1/Myc-His(+) plasmid RNA (cell lines transfected with empty vector), or human proteolysis-inducing factor RNA or protein by Northern blot or Western blot analysis.

**Tumor Implantation.** All procedures were reviewed and approved by The Johns Hopkins University Institutional Animal Care and Use Committee. In vivo studies were performed on 6 to 8-week-old female BALB/c nude mice acclimated to their surroundings and allowed free access to food and water for a minimum of 1 to 2 weeks before experimentation. MCF7 tumor cells, transfected MCF7 tumor cells overexpressing human proteolysis-inducing factor, and transfected tumor cells containing an empty pcDNA3.1/Myc-His(+) expression vector were grown in culture. After induction of general anesthesia (400 mg/kg i.p. avertin), 5 × 10⁶ tumor cells in 100 μL of PBS were mixed with 100 μL of freshly thawed Matrigel (Becton Dickinson, Bedford, MA; ref. 22) and injected into the left thigh of athymic nude mice (n = three to six mice per group). In addition, because growth of MCF7 tumors is estrogen dependent (23), a 60-day time release 17β-estradiol pellet (0.5 mg; Innovative Research of America, Sarasota, FL) was also implanted s.c. in the right suprascapular region of each mouse at the time of tumor cell injection. Food intake, body weight, and tumor size were subsequently monitored every 3 to 7 days. Upon completion of data collection, mice were euthanized with pentobarbital (100 mg/kg i.p.), and tumor tissue was excised, weighed, and stored at −80°C. Tumor protein was subsequently solubilized by homogenization in radioimmunoprecipitation buffer at 4°C and analyzed for expression of human proteolysis-inducing factor protein as described above. Differences in body weight, food intake, and tumor size over time between mice implanted with MCF7 cells containing pcDNA3.1/Myc-His(+) human proteolysis-inducing factor(+) or pcDNA3.1/Myc-His(+/human proteolysis-inducing factor(−) vectors are reported as mean ± SD and evaluated by Student’s t test, ANOVA, and nonparametric statistics where appropriate. Significance is defined as P < 0.05.

**RESULTS**

**Cloning of a Human Peptide Sequence Homologous to Proteolysis-Inducing Factor.** Initial searches of publicly accessible nonredundant and expressed sequence tag nucleotide databases (24) with the 20 amino acid mouse peptide sequence (TBLASTN/nr and dbest) revealed no mRNA or cDNA sequences identical to the murine proteolysis-inducing factor peptide. However, screening of protein databases (BLASTP) identified a human peptide with >90% homology to murine proteolysis-inducing factor This peptide, identified as survival-promoting peptide (SWISS PROT accession number P81605), had been purified from medium conditioned by Y79 human retinoblastoma cells, as well as a mouse hippocampal cell line (H33.1) exposed to H₂O₂. Its peptide sequence [X-Asp-Pro-Glu-Ala-Ala-Ser-Ala-Pro-Gly-Ser-Gly-Ash-Pro-(CyS)-His-Glu-Ala-Ser-Ala-Gln-X-Glu-Ash-Ala-Gly-(Glu)-Asp-Pro] is 10 amino acids longer than murine proteolysis-inducing factor, and their common sequence differs only at the first (undefined versus Tyr) and fifteenth (CyS versus Ser) amino acid positions (25).

Given the high degree of sequence homology, as well as the presence of this survival-promoting peptide in media conditioned by oxidatively stressed tumor cells, it seemed likely that the two peptides were human and mouse homologues. Therefore, the 30 amino acid human peptide sequence was used to search the unfinished human genome sequences (TBLASTX/htgs). Using this approach, we identified two neighboring sequences in Homo sapiens chromosome 12 clone RP11-681J20 [accession number AC025686 (bases 153,735–153,771 and 155,185–155,236)] that were consistent in structure with two exons separated by one intron.4 When adjointed, these sequences generated the entire known human peptide sequence (amino acids 2–30).

On the basis of the genomic sequence data, forward and reverse primers were designed, which amplified a 1-kb PCR product from human genomic DNA. Subsequently, a 90-bp PCR product from cDNA was confirmed in human breast cancer tissue. This PCR product was ligated into the TA-cloning vector, and plasmids containing the PCR product were isolated. Sequence data obtained from these plasmids were identical with the reported human genomic sequence.

On the basis of this sequence, primers were constructed to generate larger fragments of the cDNA by RACE. Because of our confirmation of the 90-bp proteolysis-inducing factor sequence in breast cancer cDNA and because proteolysis-inducing factor had previously been identified in the urine of cachectic pancreatic cancer patients, we initially screened cDNAs from both breast and pancreatic cancer xenografts. 5′-RACE generated a ~200-bp sequence, whereas 3′-RACE generated a

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400-bp fragment. Overlapping portions of the two sequences (~90 bp) were aligned, and a final 446-bp sequence was identified (Fig. 1A). Sequence analysis of this cDNA revealed two potential start codons and a stop codon generating a 106–109 amino acid peptide, which includes the survival-promoting peptide/proteolysis-inducing factor sequence (Fig. 1B).

The entire 22-kb clone H. sapiens chromosome 12 clone RP11-681J20 was also evaluated using the GENSCAN 1.0 program (26). This program predicted a gene including a promoter region (bases 136,138–136,177), an initial exon containing a start codon (bases 152,589–152,646), a second exon consistent with the 5′-end of our known peptide (bases 153,733–153,771), a third exon that encoded the 3′-end of our known peptide, as well as additional sequence (bases 155,185–155,286), a possible fourth exon (bases 155,630–155,719), a termination exon, which included multiple stop codons (bases 156,136–156,246), and a polyadenylation signal (bases 158,101–158,106). On the basis of these predicted exons, primers were designed, and PCR products of appropriate length were amplified from breast cancer-derived cDNA, confirming the presence of five transcribed exons and a putative transcript of ~500 kb in length.

**Gene Expression.** Northern blot analysis with a full-length human proteolysis-inducing factor probe was carried out on a commercially purchased multiple tissue Northern blot, as well as Northern blots containing RNA extracted from MAC13, MAC16, and MCF7 breast cancer cells. We were unable to demonstrate measurable expression of human proteolysis-inducing factor mRNA in any of those tissues or cell lines.

Given the absence of human proteolysis-inducing factor expression in normal tissue by Northern blot analysis, real-time PCR was performed to improve our detection sensitivity. Primers and probes were designed to specifically amplify the human proteolysis-inducing factor cDNA and the internal reference gene GAPDH in fluorogenic real-time PCR reactions. Screening commercially prepared human normal tissue cDNAs, we found that human proteolysis-inducing factor expression was very low to absent in normal tissues. No amplification of human proteolysis-inducing factor was observed in skeletal muscle and kidney cDNA, and human proteolysis-inducing factor amplification thresholds were <1000-fold those observed with GAPDH in heart, brain, placenta, lung, liver, and pancreas. We also screened a commercially prepared cDNA panel consisting of 12 nonpaired normal breast and breast tumor samples. In the normal breast samples, we generally observed minimal to absent proteolysis-inducing factor amplification. However, human proteolysis-inducing factor expression was significantly elevated in three breast tumors, with transcript levels 25 to 200% that of GAPDH, a finding consistent with abundant expression of human proteolysis-inducing factor in certain tumors (Fig. 2).

To characterize protein expression, we screened mouse tumor cell lines (MAC13 and MAC16), breast tumor cell lines (MCF7 and BT-20), and the HEK293 cell line with anti-proteolysis-inducing factor antibody. We did not observe the presence of any protein signal consistent in size with the complete human proteolysis-inducing factor protein sequence (~14 kDa), a potential glycosylated human proteolysis-inducing factor protein (presumably 20 kDa larger), nor the 24-kDa murine proteolysis-inducing factor glycopeptide fragment reported previously (Fig. 3A).

Antibody binding to a 14-kDa protein was observed when probing cell lysates from MAC16, HEK293, MCF7, and BT-20 cells stably transfected with pcDNA/human proteolysis-inducing factor mammalian expression cassettes, whereas HEK293 cells stably transfected with a myc-His–tagged human proteolysis-inducing factor construct expressed a 24-kDa protein. Interestingly, stably transfected MAC13 cells (clones 3, 8, and 27) expressed human proteolysis-inducing factor RNA, but protein expression was absent on Western blot analysis. Identical sized bands were also observed when probing cell
lysates from transfected MAC16, HEK293, MCF7, and BT-20 cells with anti-CF antiserum (Fig. 3A). No larger, glycosylated constructs were observed in any cell line stably transfected with either expression vector, and absence of antibody binding was observed when lysates from native and transfected MAC16, BT-20, and MCF7 cells were hybridized with the monoclonal antimurine proteolysis-inducing factor antibody (unpublished data). Secretion of human proteolysis-inducing factor in vitro was confirmed by probing Western blots of conditioned media from transfected HEK293, MAC16, BT-20, and MCF7 cells with anti-human proteolysis-inducing factor antibody (Fig. 3B).

**In vivo Tumor Studies.** To determine the production and effects of human proteolysis-inducing factor in vivo, mice were implanted with native MCF7 tumor cells or cells expressing pcDNA3.1/Myc-His(+)human proteolysis-inducing factor(+) plasmids. Estrogen-supplemented mice implanted with native MCF7 cells grew tumors but did not develop anorexia or wasting (Fig. 4A). MCF7/human proteolysis-inducing factor(+) tumors clearly produced human proteolysis-inducing factor in vivo (Fig. 4B), yet we observed no significant differences in feeding [average daily food intake: 5.0 ± 0.6 g per MCF7/human proteolysis-inducing factor(−) mouse versus 4.7 ± 0.7 g per MCF7/human proteolysis-inducing factor(+) mouse] or weight change between groups of mice containing human proteolysis-inducing factor(+) and human proteolysis-inducing factor(−) tumors over the course of the study (Fig. 4C). Furthermore, instead of losing weight after tumor implantation, all mice had a corrected weight (total body weight − tumor weight) in excess of their preimplantation weight at sacrifice [17.4 ± 0.9 versus 20.1 ± 0.4 g, MCF7/human proteolysis-inducing factor(−); 17.4 ± 0.9 versus 19.7 ± 1.3 g, MCF7/human proteolysis-inducing factor(+), P < 0.05]. Nine mice [six human proteolysis-inducing factor(−) and three human proteolysis-inducing factor(+)] were sacrificed 55 days after implantation (implanted estrogen pellets were only active for 60 days, and tumor growth is marginal beyond that time; unpublished data). An additional three mice [all human proteolysis-inducing factor(+)] were sacrificed 43 days after implantation as a consequence of excessive tumor growth as assessed by weekly visual inspection and/or measurement. All human proteolysis-inducing factor(+) mice grew tumors larger than those MAC16 tumors reported to induce cachexia (27), suggesting that weight maintenance was not a result of inadequate tumor growth over the course of the study. In addition, MCF7/human proteolysis-inducing factor(+) tumors were significantly larger than MCF7/human proteolysis-inducing factor(−) tumors (0.61 ± 0.28 versus 0.30 ± 0.12 g; P < 0.05), supporting a previously hypothesized role for human proteolysis-inducing factor peptide fragments in promoting cell survival (25).

**DISCUSSION**

We report here the DNA and protein sequences of a potential human homologue for a proteolysis-inducing factor ini-
tially isolated from cachexia-causing MAC16 tumors, identified by rapid amplification of cDNA ends and in silico analysis of the human genome. After complete cloning of the human proteolysis-inducing factor cDNA, we found that the sequence had previously been patented in 1998 by Incyte Genomics [patent 5834192: human cachexia-associated protein (HCAP)]. Subsequently, the sequence reported was also identified as a serial analysis of gene expression transcript in a breast tumor library (28) and by screening a subtracted cDNA library of primary melanoma and benign melanocytic nevus tissues (dermcidin; Ref. 29).

In keeping with previous studies documenting proteolysis-inducing factor peptide production by select tumors, human proteolysis-inducing factor (HCAP/dermcidin) transcripts appear to be absent or at most minimally present in all normal tissues, with the exception of select regions of the brain and skin (28, 29). Transcripts are present, however, in a subset of breast tumors (28) and were also recently been identified in prostate cancer tumors and tumor cell lines (30). Moreover, consistent with previous reports of the presence of proteolysis-inducing factor in the urine of cachectic cancer patients, human proteolysis-inducing factor is indeed a secretory protein. The full-length human proteolysis-inducing factor protein contains a hydrophobic 5′-peptide motif (amino acids 1–14), consistent with a signal peptide.5 Western blot analysis of conditioned media from cell lines expressing human proteolysis-inducing factor demonstrated in vitro secretion, and a truncated peptide consisting of 46 terminal amino acids of dermcidin has also been identified in human sweat (29).

Survival-promoting peptide has been shown to be induced in the setting of oxidative stress (25), and expression analysis suggests that MAC16 cells are subject to more oxidative stress than MAC13 cells. Transcripts encoding oxidative-stress induced mRNA, ferritin, and long terminal repeats, all consistent with cellular oxidative stress, were previously shown to be up-regulated in MAC16 as compared with MAC13 cells (31). Our tumor implantation studies are also consistent with a survival promoting component of human proteolysis-inducing factor given the significantly greater in vivo tumor growth of the human proteolysis-inducing factor (+) than human proteolysis-inducing factor (−) MCF7 clones, although further study is needed to substantiate this preliminary finding. Indeed, Porter et al. (28) have reported an association between dermcidin expression in breast tumors and a more aggressive clinical course. Similarly, MAC16 tumors, unlike MAC13 tumors, are generally refractory to standard cytotoxic agents (32).

To further study the expression of human proteolysis-inducing factor protein, we transfected human proteolysis-inducing factor into a number of murine and human cell lines. MAC13 cells, which are histologically similar to MAC16 cells but do not produce murine proteolysis-inducing factor, expressed human proteolysis-inducing factor mRNA, but they appear to either not synthesize or rapidly degrade the resultant protein. Stably transfected MAC16 cells, which do express murine proteolysis-inducing factor, were able to synthesize human proteolysis-inducing factor as an unglycosylated protein. Because it is possible that MAC16 cells might not be able to process a human protein, we subsequently transfected a number of human cell lines, including breast cancer and kidney cell

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5 Internet address: http://www.cbs.dtu.dk/services/SignalP.
lines, with a plasmid containing the gene construct. In all instances, as with MAC16 cells, only unprocessed protein was produced as confirmed by Western blot analysis using antibodies to three (two peptide and one carbohydrate) epitopes, two of which bind to murine proteolysis-inducing factor sequences, and one that binds to the full-length human proteolysis-inducing factor peptide. This finding has also been confirmed by other researchers (28). It is conceivable that our failure to identify glycosylated forms of human proteolysis-inducing factor is due to our use of different protein isolation techniques than those originally described (17), resulting in either loss of processed human proteolysis-inducing factor glycoprotein or a change in its antigenicity. However, glycosylated HCAP was reported to be immunoprecipitated in a lysis buffer very similar to the one used in this article (30), making this possible explanation less likely.

Wang et al. (30) recently reported an association between HCAP expression and weight loss in a murine prostatic cancer xenograft model, but this study failed to provide a negative control, i.e., isogenic tumors lacking HCAP/human proteolysis-inducing factor transcripts that do not induce cachexia. To document complete processing in vivo and to determine whether human proteolysis-inducing factor overexpression is sufficient to convert a noncachexia causing tumor into a cachexia causing one, we stably transfected noncachexia-causing MCF7 tumor cells with human proteolysis-inducing factor or an empty control plasmid followed by implantation of these cells into nude mice. However, in contrast to studies of the cachexia-inducing ability of HCAP, implantation and growth of tumors with forced expression of human proteolysis-inducing factor and abundant protein production resulted in stable or increased whole animal weights.

There are a number of possible explanations as to the discordance between our findings regarding human proteolysis-inducing factor and murine proteolysis-inducing factor. First, the actions of human proteolysis-inducing factor might have been altered by our model system in which mice receive supplemental estrogen to promote tumor growth. We believe this is unlikely, however, given the occurrence of cachexia clinically in patients with breast cancer (33), who presumably have appropriate estrogen levels, as well as the identification of proteolysis-inducing factor in cachectic patients with breast cancer (17). Recent studies have also demonstrated that MCF7 tumor cells genetically engineered to overexpress a secreted form of interleukin 1 α induce cachexia in an estrogen-supplemented nude mouse model (34). In addition, in separate studies, we have also found that male mice implanted with cachexia-inducing MAC16 tumor cells, which were engineered to express human proteolysis-inducing factor, did not develop more or more rapid weight loss than mice implanted with native MAC16 cells (unpublished data).

Another possibility is that our xenografts do not secrete human proteolysis-inducing factor. However, whereas we cannot measure serum levels of human proteolysis-inducing factor in vivo (serum levels of proteolysis-inducing factor/murine proteolysis-inducing factor have never been successfully measured in humans or mice), we do know that the human proteolysis-inducing factor is secreted by all cell lines tested in vitro, and dermcidin is also secreted in vivo (29).

Although it is also possible that the two genes, murine proteolysis-inducing factor and human proteolysis-inducing factor/HCAP/dermcidin, do not represent homologues, this is highly unlikely based on examination of the human genome. Although a 20-amino acid sequence has been described for murine proteolysis-inducing factor, only the first 14 amino acids of the human sequence have been reported (17), and human proteolysis-inducing factor demonstrates 100% homology to this sequence. In addition, the first tyrosine of murine proteolysis-inducing factor was not identified by screening the human genome for sequences homologous to survival-promoting peptide because it occurs at a splice site, yet it was identified by computer-assisted genome screening and confirmed by RACE. Subsequent database analyses of a now largely complete human genome have also failed to identify any other human chromosomes having neighboring sequences, which, when adjointed, generate a coding sequence having high homology to murine proteolysis-inducing factor with a serine in place of a cysteine (National Center for Biotechnology Information/Genome/Map Viewer). Moreover, although screening of the now largely complete murine genome has failed to identify any potential murine DNA sequences encoding the complete murine proteolysis-inducing factor peptide sequence (National Center for Biotechnology Information Home/Genomic Biology/Mouse), a rat dermcidin precursor mRNA has recently been identified (NM_173108; National Center for Biotechnology Information Sequence Viewer), which displays 100% amino acid and nucleotide homology to the human sequence described here. This finding makes it highly unlikely that a murine protein would differ significantly from the reported human and rat sequences.

Finally, it is known that cellular glycosylation pathways can differ in cancer cells (35), hence, it is possible that our MCF-human proteolysis-inducing factor xenografts did not induce cachexia because the protein was not able to be processed properly in this system. Unfortunately, the exact enzymes required to generate proteolysis-inducing factor from human proteolysis-inducing factor/HCAP/dermcidin, as well as the necessary abundance of enzymes required to do so and therefore the optimal model system, are unknown. We attempted in this study to get around this limitation by expressing human proteolysis-inducing factor in multiple human cell lines, as well as MAC16 cells, which are known to process the murine peptide, and hence, by definition, should contain sufficient quantities of the necessary enzymes of glycosylation to process an almost identical human peptide. However, we were unsuccessful using this approach.

The absence of any glycosylated forms of human proteolysis-inducing factor as well as the absence of murine proteolysis-inducing factor’s putative N-glycosylation site (amino acids 13–15, Asn-Pro-Ser) led us to question whether human proteolysis-inducing factor could be glycosylated in human tumors. Speculating that our negative results might be because of the (theoretical) absence of glycosylation sites on the human proteolysis-inducing factor peptide caused by the cysteine/serine substitution, we subsequently constructed a plasmid expressing a cysteine/serine mutant protein. However, when that protein was also expressed in vitro, we were again unable to identify any glycosylated forms of the protein (data not shown).

Since it was first reported in 1996, proteolysis-inducing factor

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6 K. Polyak, personal communication.
has been the only (glyco)protein ever shown to be produced by tumors and capable of inducing muscle proteolysis in vitro and in vivo. Reliable blockade of the activity of proteolysis-inducing factor could potentially ameliorate the cachexia commonly seen in cancer and improve the quality of life of millions of patients. Characterization of the protein is, therefore, of significant clinical importance. Accurate identification of the peptide sequence of proteolysis-inducing factor is critical to further work given that the carbohydrate portion of the moiety is of high complexity and has not been fully characterized to date. We report here the cloning of a human gene human proteolysis-inducing factor, which likely represents a human homologue of the murine factor. The gene and its protein product display a number of properties attributed to murine proteolysis-inducing factor, including absence of expression in most normal tissues, sporadic overexpression by select tumors, and secretion by cells that produce it. However, human proteolysis-inducing factor peptide lacks a putative glycosylation site and does not appear to be glycosylated when expressed in murine or human cell lines. In addition, forced expression of human proteolysis-inducing factor does not induce cachexia de novo in a murine xenograft model. These findings raise important questions as to potential cross-species differences in protein sequence and processing of murine proteolysis-inducing factor and human proteolysis-inducing factor, as well as the nature of the relationship between human proteolysis-inducing factor (HCAP/dermcidin) and the development of cancer cachexia.

REFERENCES

Characterization of a Human Homologue of Proteolysis-Inducing Factor and Its Role in Cancer Cachexia


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