Am J Physiol Endocrinol Metab
281: E289–E297, 2001.

Differential gene expression in a murine model of cancer cachexia

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Received 13 October 2000; accepted in final form 6 March 2001

Monitto, Constance L., Dan Berkowitz, Kyoung Min Lee, Sokhon Pin, Daqing Li, Michael Breslow, Bert O'Malley, and Martin Schiller. Differential gene expression in a murine model of cancer cachexia. *Am J Physiol Endocrinol Metab* 281: E289–E297, 2001.—Murine adenocarcinoma 16 (MAC16) tumors and cell lines induce cachexia in NMRI nude mice, whereas histologically similar MAC13 tumors do not. After confirming these findings in BALB/c nude mice, we demonstrated that this tissue wasting was not related to decreased food intake or increased total body oxidative metabolism. Previous studies have suggested that MAC16's cachexigenic properties may involve the production of tumor-specific factors. We therefore screened for genes having increased expression in the MAC16 compared with the MAC13 cell line by performing hybridization to a murine cDNA expression array, by generation and comparison of cDNA libraries from each cell line, and by PCR-based subtractive hybridization. Northern blot hybridization was performed to confirm differences in transcript expression. Transcripts encoding insulin-like growth factor binding protein-4, cathepsin B, ferritin light and heavy chain, endogenous long-terminal repeat sequences, and a viral envelope glycoprotein demonstrated increased expression in the MAC16 cell line. The roles of a number of these genes in known metabolic pathways identify them as potential participants in the induction of cachexia.

murine adenocarcinoma 13 and 16 cells; cDNA expression array

CACHEXIA, OR PROGRESSIVE TISSUE WASTING, is manifested by abnormalities in carbohydrate, lipid, and protein metabolism (34). Cachexia can occur in association with sepsis, trauma, and acquired immunodeficiency syndrome (4), as well as many types of cancer. It is one of the most important factors leading to early morbidity and mortality in cancer, accounting for 10–20% of all deaths, and contributing to deaths from other causes such as infection (11, 40). Despite its profound clinical impact, the mechanisms of cancer cachexia are still incompletely understood. Although anorexia may accompany cachexia, it does not appear to account for the skeletal muscle and adipose tissue wasting seen in cancer, since nutritional supplementation does not reverse the progressive weight loss observed (10), as occurs with refeeding of patients with anorexia nervosa (29). Cytokines, including tumor necrosis factor-α, interleukin (IL)-1, interferon-γ, leukemia inhibitory factor, IL-6, and transforming growth factor-β are thought to play a role in cancer cachexia (17, 23, 24, 26, 42, 43), but therapies aimed at suppressing the cytokine response have been only partially effective in preventing manifestations of the disease (8).

Implantable tumor models in mice provide a means to identify and screen potential candidate molecules important in the induction of cachexia. One such model utilizes two histologically similar colon cancer cell lines, murine adenocarcinoma (MAC)13 and MAC16, derived from tumors initially induced by administration of 1,2-dimethylhydrazine (6). Previous studies have demonstrated that NMRI mice implanted with MAC16 tumor cells develop a 20–30% loss in weight over the course of 1 mo after tumor cell implantation, whereas MAC13-implanted mice maintain their weight (19). The cachexigenic properties of MAC16 tumors do not appear to be cytokine mediated (25) but may involve the production and secretion of tumor-specific factors. Lipolytic factors appear to be produced by MAC16 and, to a lesser extent, MAC13 tumors (2). In addition, a 24-kDa glycopeptide isolated from MAC16 tumors and not present in MAC13 tumors has been shown to induce weight loss in vivo and skeletal muscle proteolysis in vitro (16). Moreover, serum from MAC16-implanted mice also increases muscle proteolysis in vivo (33). These findings suggest that differential production and/or secretion of factors produced by MAC16 tumor cells may play a role in the development of cancer cachexia by inducing lipolysis and proteolysis in peripheral tissues.

In this study, we confirmed the induction of cachexia by MAC16, but not MAC13, tumors in BALB/c athymic nude mice. Hypothesizing that this difference might be related to variations in gene expression between the two cell lines, we then utilized three different methods,

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1) hybridization to a commercially available murine cDNA expression array, *2*) generation and comparison of cDNA libraries from each cell line, and *3*) PCR-based subtractive hybridization, to characterize differences in gene expression between MAC13 and MAC16 tumor cells. Partial profiles of gene expression patterns were generated for the two cell lines by these different approaches, and analysis of these expression patterns has led to the identification of several genes that are overexpressed in MAC16 tumor cells and may play a role in the etiology of cancer cachexia in this model.

MATERIALS AND METHODS

Tumor Cell Lines

MAC13 and MAC16 tumor cells were obtained from Dr. M. Tisdale (Pharmaceutical Sciences Institute, Aston University, Birmingham, UK) and were maintained in vitro in RPMI 1640 medium with L-glutamine (GIBCO BRL, Life Technologies, Rockville, MD) containing 12% fetal bovine serum (HyClone, Logan, UT) and penicillin-streptomycin (100 U/ml and 100 μg/ml, respectively) in a humidified atmosphere with 5% CO_2 at 37°C. Cells were passaged once they achieved 80% confluency.

Mice

All animal procedures were approved by the Johns Hopkins University Institutional Animal Care and Use Committee. Six-week-old male athymic nude mice (BALB/c *nu/nu*) were obtained from the National Cancer Institute (Frederick, MD). Animals had access to standard laboratory rodent chow and water ad libitum throughout the course of the studies.

In Vivo Experiments

Tumor implantation. MAC13 and MAC16 tumor cells grown in culture to ~80% confluency were treated with trypsin, washed with medium, and resuspended at a concentration of 5×10^7 cells/ml in phosphate-buffered saline. A total of 100 μl of this cell suspension was injected subcutaneously into the thigh of each mouse.

Food intake, body weight, and metabolism. Food intake, body weight, and oxygen consumption ($\dot{V}O_2$) were measured every 3–4 days after tumor implantation. Total body $\dot{V}O_2$ was measured using indirect calorimetry with a four-chamber Oxymax system (Columbus Instruments, Columbus, OH), as previously described (3); the first 30 min of data per day were discarded to allow the mice to become acclimatized to their surroundings. $\dot{V}O_2$ per animal per day was calculated as the average $\dot{V}O_2$ over the time period measured. Upon completion of all metabolic measurements on *postimplantation day 25*, mice were euthanized with pentobarbital sodium intraperitoneally, and tumor tissue was excised and stored at −80°C.

In Vitro Techniques

RA isolation and Northern blot analysis. Total RNA was isolated from cell culture and tumor tissue by the RNA-STAT 60 reagent method (Tel-test "B," Friendswood, TX). MAC13 and MAC16 poly(A^+) mRNA were prepared from total RNA with the use of the Promega PolyATract mRNA Isolation System IV kit (Promega, Madison, WI). Northern blot hybridization was performed as previously described (32). Briefly, total RNA samples (10 μg) were separated by electrophoresis on 1% agarose gels containing formaldehyde and then transferred onto nylon membranes by capillary action (Nytran, Schleicher and Schuell, Keene, NH). Blots were prehybridized for 1 h at 65°C in an SDS-PIPES hybridization buffer (32) and hybridized overnight with radiolabeled cDNA probes at 65°C. The membranes were washed three times at 65°C in 0.1× standard sodium citrate (SSC; 20× SSC is composed of 3 M sodium chloride and 0.3 M sodium citrate, pH 7.0) and 0.1% SDS and then exposed to X-ray film with intensifying screens at −80°C. Signals were normalized by probing of the identical blot with probes to β-actin or S26 ribosomal protein cDNA. Signals were quantified using a phosphorimager and image analysis software (Imagequant, Molecular Dynamics, Sunnyvale, CA).

cDNA probes were generated from cDNA fragments by random priming with [α-^{32}P]dCTP, as described by Stratagene (PrimeIT-II kit, Stratagene, La Jolla, CA). cDNA fragments encoding regions of cathepsin B (accession no. Y18463, nt 38–382), insulin-like growth factor binding protein-4 (IGFBP-4; accession no. X81582, nt 86–1028), and calgizzarin (accession no. AI317551, nt 103–503) were generated by polymerase chain reaction (PCR) from a MAC16 cDNA library. Clones encoding cDNAs for mouse ferritin light chain, ferritin heavy chain, mitochondrial uncoupling protein 2, peroxisomal acyl-CoA, and endo B cytokeratin (I.M.A.G.E. Consortium Clone ID nos. 1958025, 1970583, 19733292, 1973572, and 1973584, respectively) were purchased from American Tissue Culture Collection (ATCC; Manassas, VA). β-Actin probe was purchased from Ambion (Austin, TX), and S26 probe was synthesized as previously described (37). Probes for sequences identified by subtractive hybridization were generated from cDNA fragments excised from individual pCR 2.1 constructs (see *PCR-Based Subtractive Hybridization*). Recombinant bacteria were then grown overnight in Luria Broth containing 100 μg/ml ampicillin. For preparation of all probes, plasmid DNAs were isolated by mini- or midiprep (QIAprep Spin Miniprep Kit and Plasmid Midi Kit, Qiagen, Valencia, CA), and fragments of interest were extracted from plasmids after restriction enzyme digestion (*Eco*RI and *Xho*I for ATCC clones and *Eco*RI for pCR 2.1 clones) and separation of DNA fragments on low-melting agarose gels. DNAs were purified from gel slices by gel extraction (QIAquick gel extraction kit, Qiagen).

Atlas cDNA Array Analysis

In accordance with the protocol provided by the manufacturer (Atlas cDNA Expression Array, Clontech Laboratories, Palo Alto, CA), radiolabeled cDNAs were synthesized from poly(A^+) mRNA isolated from MAC13 and MAC16 tumor cells by use of a pool of 588 gene-specific primers. Individual hybridizations of the two different cDNA populations to similar Atlas cDNA arrays were performed, and hybridization intensities of the cDNAs on the arrays were compared. After hybridization, each membrane was washed and exposed to film. After films in which the intensities of the housekeeping genes were similar were obtained, films were scanned and overlaid to allow for identification of comparably expressed and differentially expressed genes.

Electronic Subtraction

Directional λZAP2 cDNA libraries were generated from the MAC13 and MAC16 cell lines, and randomly selected clones were isolated and sequenced. The phage cDNA libraries were prepared as previously described (Lambda ZAP II Vector/Gigapack Cloning Kit, Stratagene). Briefly, poly(A^+) RNA (6 μg) was hybridized to the primer 5′-GAGAGA-GAGAGAGAGAGAGAAACTAGTCTGAGT(18)-3′. The RNA was reverse transcribed to generate the first strand of cDNA, and then second-strand cDNA was synthesized. The cDNAs

were blunt ended and ligated to a phosphorylated *Eco*RI adapter. Next, the *Xho*I site within the oligo-dT primer was cut, the modified cDNAs were fractionated on a gel filtration column, and the larger-size cDNAs were pooled. Phage arms were ligated to the cDNAs, the resulting constructs were packaged using GigaPack III Gold, and library titers were determined. The average insert size was 1.7 kb, with cDNA sizes ranging from 0.4 to 12 kb. After mass excision of the MAC13 and MAC16 cDNA libraries, individual, randomly selected clones were arrayed at Lawrence-Livermore National Laboratory, and partial sequences were obtained from the 5′ ends of randomly selected clones by means of the primer −40RP (GIBCO BRL, Life Technologies) and automated fluorescent-dye sequencing. The cDNAs were identified using basic local alignment search tool nucleotide (BLASTN; Ref. 1). Frequency of expression of each cDNA sequenced was quantitated, and cDNAs showing higher levels of expression in the MAC16 compared with the MAC13 library were identified.

PCR-Based Subtractive Hybridization

Suppression-subtractive hybridization, a PCR-based cDNA subtraction method, was used to selectively amplify differentially expressed cDNA fragments while simultaneously suppressing nontarget DNA amplification (7). In accordance with the protocol provided by the manufacturer (Clontech PCR-Select cDNA Subtraction Kit, Clontech Laboratories), mRNAs derived from MAC13 and MAC16 cells were converted into double-stranded cDNAs. Tester (MAC16) and driver (MAC13) cDNAs were hybridized, and the hybrid sequences were removed. The remaining molecules were then subjected to two rounds of PCR to amplify and enrich differentially expressed sequences. These cDNAs were inserted into the pCR 2.1 vector (Original TA Cloning Kit, Invitrogen, Carlsbad, CA) and transferred into *Escherichia coli* NM-522, and plasmids were prepared from eight randomly selected clones and analyzed by Northern blotting and DNA sequencing.

RESULTS

Weight Loss, Food Intake, and Metabolic Data

Previous work by others has demonstrated a 20% weight loss in male NRMI mice after MAC16 tumor cell implantation (6). We performed similar experiments to test this model in BALB/c athymic nude mice. As in NMRI mice, mice implanted with either MAC13 or MAC16 cells developed tumors, and significant weight loss was observed only in those mice implanted with MAC16 tumor cells (Fig. 1*A*). After implantation, animals bearing MAC16 tumors lost ~20% of body weight between *days 14* and *25*, whereas MAC13-implanted mice either maintained or gained weight, despite bearing a similar-size tumor mass. Body weight of MAC16-implanted mice was significantly less than that of MAC13-implanted mice by *postimplantation day 18* ($P < 0.05$).

To determine whether decreases in caloric intake or increases in total body oxidative metabolism could account for this weight loss, we measured food intake and $\dot{V}O_2$. Food intake did not significantly decrease until the mice had begun to lose weight by *day 18* ($P < 0.05$) (Fig. 1*B*). In addition, total body $\dot{V}O_2$ ($ml \cdot kg^{-0.7} \cdot h^{-1}$) did not increase in MAC16-implanted mice during this same time period; in fact, it decreased after the onset of weight loss (Fig. 1*C*). These findings suggest that neither a change in caloric intake nor total body oxidative metabolism was the immediate cause of the observed tissue wasting.

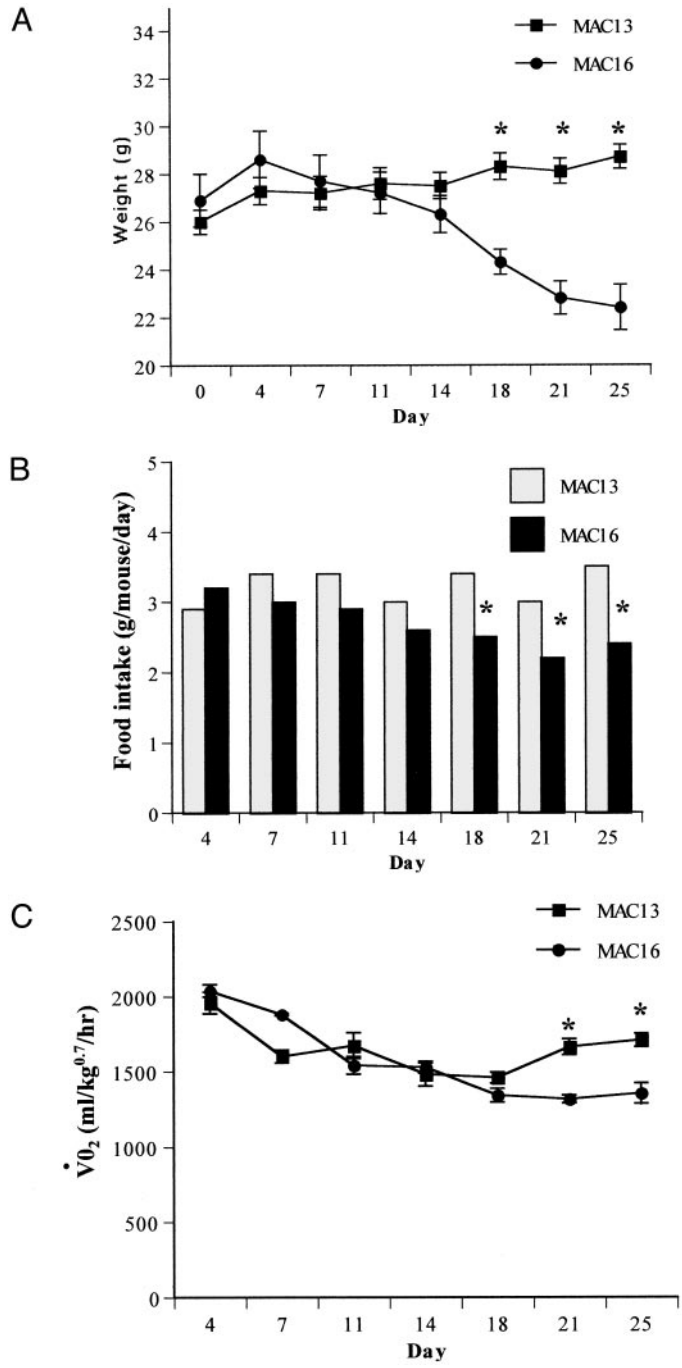

Fig. 1. Mice implanted with murine adenocarcinoma (MAC)16 tumors develop cachexia, whereas those implanted with MAC13 tumors do not. *A*: weight of MAC13 and MAC16 tumor-bearing mice ($n = 7$ per group). *B*: food intake of mice whose body weights are represented in *A* ($n = 7$ per group). *C*: total body oxygen consumption of MAC13- and MAC16-implanted mice ($n = 4$ per group). *$P < 0.05$.

Identification of Upregulated Gene Transcripts

In light of previous studies suggesting that MAC16 tumors may induce cachexia by means of increased

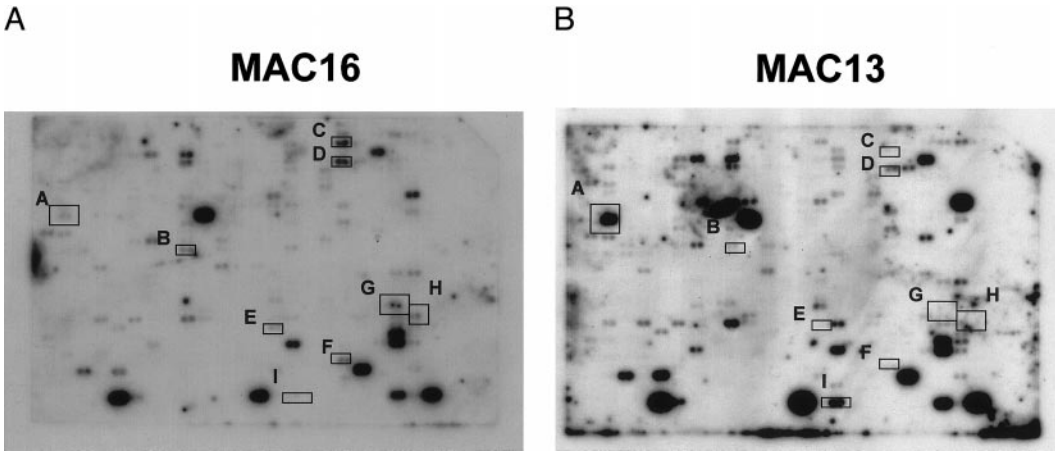

Fig. 2. Atlas cDNA hybridization arrays for MAC13 and MAC16 mRNA. Radiolabeled cDNA was synthesized from poly(A^+) mRNA (1 μg) isolated from MAC13 and MAC16 tumor cells by use of a pool of 588 gene-specific primers. Individual hybridizations of the 2 different RNA populations were performed and membranes were washed and exposed to film. After films were obtained in which the intensity of the housekeeping genes was similar, they were scanned and overlaid to allow for identification of comparably and differentially expressed genes. Genes that appeared significantly different upon comparison of MAC13 and MAC16 probed arrays are denoted by a □ and identified by grid location in Table 1.

synthesis of lipolytic and proteolytic tumor gene products (2, 16, 36), we used three different approaches: hybridization to a cDNA expression array, comparison of cDNA libraries from each cell line, and PCR-based subtractive hybridization, to identify genes with elevated expression in MAC16 vs. MAC13 tumor cells. Differential expression of select genes identified by these preliminary screens was confirmed by Northern blot analysis. Because previous experiments had demonstrated the presence of putative cachexia-inducing factors in tumor tissue, serum, and possibly urine, we focused our attention on secretory products and genes with known metabolic impact in our secondary Northern blot screening.

Atlas cDNA array. Gene expression array analysis was performed to screen for differences in expression of 588 known mouse cDNAs (for full list of genes and array grid locations see http://www.clontech.com/atlas/genelists/index.html). We identified nine genes that showed different spot intensities upon comparison of MAC13 and MAC16 probed arrays (Fig. 2 and Table 1). Two of these genes, RNA polymerase I termination factor and murine ornithine decarboxylase, had increased intensity in the MAC13 cell line, whereas the remaining genes were higher in the MAC16 cell line. Although attention has been focused in other cancer cachexia models on the role of IL-6 and other cytokines as potential contributors to cachexia, expression of IL-6 was not increased in MAC16 compared with MAC13 cells by either array or Northern blot analysis (data not shown). Northern blot analysis of tumor-derived RNA and quantitation of signals using a phosphorimager revealed more than a twofold increase in expression of IGFBP-4, and more than a fourfold increase in expression of cathepsin B in MAC16 compared with MAC13 total RNA (Fig. 3).

Electronic subtraction. cDNA libraries were generated for the MAC13 and MAC16 cell lines. Randomly selected clones were arrayed, and partial sequences were obtained from the 5′ ends of 231 MAC16 and 649 MAC13 clones (for full list see Entrez Browser nucleotide database, with Schiller and MAC13 or MAC16 as queries). These clones included 489 sequences present in the nonredundant database, 129 sequences present in the expressed sequence tag (EST) database, and 29 unique sequences. Frequency of clone expression was

Table 1. *Genes with differential expression between MAC13 and MAC16 cell lines as determined by cDNA hybridization array*

Box	Grid Location*	Accession No.	Gene	Cell Line Having Increased Expression
A	A2j	X83974	RNA polymerase I termination factor	MAC13
B	B1n	U40930	Oxidative stress-induced protein mRNA	MAC16
C	C2b	J04696	Glutathione S-transferase-μ	MAC16
D	C2d	D30687	Glutathione S-transferase-π	MAC16
E	E6h	M34510	CD14 antigen	MAC16
F	F2l	X81582	Insulin-like growth factor binding protein-4	MAC16
G	F5f	X63615	Ca^{2+}/calmodulin-dependent protein kinase II	MAC16
H	F6g	M14222	Cathepsin B	MAC16
I	G14	M10624	Murine ornithine decarboxylase	MAC13

*See Clontech website for array locations (www.clontech.com).

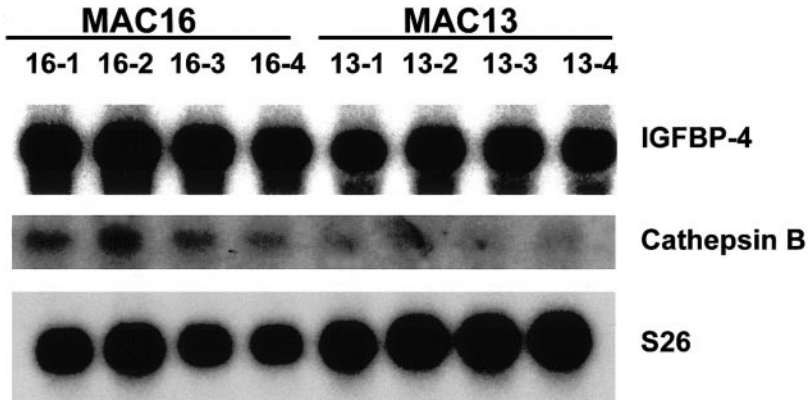

Fig. 3. Northern blot analysis of select genes with increased expression in MAC16 tumors identified by Atlas cDNA array. Each lane contains tumor RNA (10 μg). All signals were normalized to S26. Nos. 13-1, -2, -3, -4 and 16-1, -2, -3, -4 refer to individual MAC13 and MAC16 tumors, respectively. Insulin-like growth factor binding protein (IGFBP)-4 (2.6 kb) and cathepsin B (2.2 kb) messages were present at elevated levels in MAC16, compared with MAC13, tumors ($P < 0.05$).

tabulated, and a normalized ratio was calculated on the basis of the relative contribution of MAC16 and MAC13 clones to the total number of clones sequenced (1:2.8).

Although electronic subtraction provides digital data, it is most useful in identifying abundant mRNAs (39). Hence, given the limited size of our total database (880 clones) and our inability to differentiate with any degree of certainty between the probable frequency of sequences expressed between zero and one time in each library, we focused our attention on more frequently expressed transcripts and excluded all single transcripts (MAC13: 405 sequences; MAC16: 146 sequences) from further study. The electronic subtraction data suggested that 16 transcripts that occurred at least two times in the MAC16 library might have increased expression in MAC16 compared with MAC13 cells (Table 2). Six of these transcripts, including a known secretory protein (ferritin), as well as a protein capable of influencing cellular metabolism (mitochondrial uncoupling protein 2), were selected for secondary screening by Northern blot analysis. Using this approach we confirmed a four- to fivefold increase in expression of both ferritin heavy-chain and ferritin light-chain mRNA in MAC16 cell culture and tumor tissue compared with MAC13 cell culture and tumor tissue (Fig. 4). In contrast to our electronic subtraction data, we observed comparable expression levels of endo B cytokeratin and mitochondrial uncoupling protein 2, and increased expression of the calcium binding protein calgizzarin (35) was observed in MAC13 cell culture-derived RNA. We were unable to detect expres-

Table 2. *Sequences demonstrating differential expression between MAC16 and MAC13 cells, as determined by electronic subtraction of cDNA libraries*

Accession No.	Description	Observed Frequency in MAC13 Library	Observed (Normalized) Frequency in MAC16 Library
AI272495	Cytoplasmic γ-actin	6	1(2.8)
AI272439	A-X actin	4	0
AI317552	A disintegrin and metalloproteinase domain 9	0	2(5.6)
AI317589	ATP synthase-ϵ chain, mitochondrial precursor	0	2(5.6)
AI317551	Calgizzarin	0	2(5.6)
AI316072	Carbonic anhydrase II	0	2(5.6)
AI316385	Chaperonin 10	0	2(5.6)
AI316312	Elongation factor 1-α	4	0
AI316327	Elongation factor 2	4	0
AI316613	Endo B cytokeratin	0	2(5.6)
AI272586	Ferritin heavy chain	1	3(8.4)
AI316335	Ferritin light chain	4	4(11.2)
AI316279	Guanine nucleotide-binding protein-β-like protein	5	0
AI265349	Mouse IgE-binding factor	52	4(11.2)
AI317585	Mouse IgG related glycoprotein-70	0	2(5.6)
AI272494	Ly-6 alloantigen	1	2(5.6)
AI272538	Laminin receptor	13	0
AI272637	Cytosolic malate dehydrogenase	0	2(5.6)
AI272610	Mitochondrial uncoupling protein 2	0	2(5.6)
AI272607	Murine leukemia virus long-terminal repeat	1	2(5.6)
AI272632	Nucleoplasmin 3	0	2(5.6)
AI316609	Peroxisomal acyl-CoA oxidase	0	2(5.6)
AI316068	Protein phosphatase PP2A	0	2(5.6)
AI272467	Retroviral transposon sequence	7	0
AI316406	Human DNA sequence for RP5-1104E15	0	2(5.6)
AI272449	Type IIB intracisternal A particle	4	0
AI316283	Ubiquitin	8	0

Only sequences occurring ≥2 times are presented in this table.

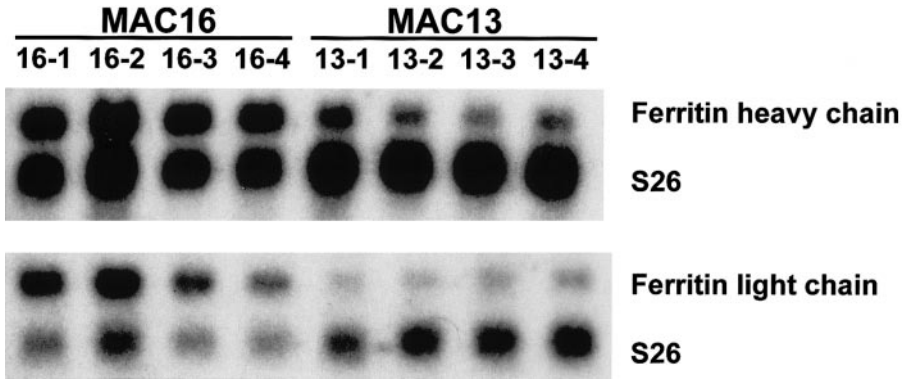

Fig. 4. Northern blot analysis of ferritin light-chain and ferritin heavy-chain expression. Each lane contains tumor RNA (10 μg). All signals were normalized to S26. Ferritin light chain (0.8 kb) and heavy-chain (1 kb) messages are present at >4-fold higher levels in MAC16 cells ($P < 0.05$).

sion of peroxisomal acyl-CoA oxidase message by Northern blot analysis for either cell line with our probe. With examination of the 29 novel sequences, each was found to occur only once in the combined library database, suggesting that this technique was not helpful in identifying differential expression of novel clones.

PCR-select subtraction. To screen more extensively for potential novel and/or rare transcripts differentially expressed in the MAC cell lines, PCR-select subtraction was performed. Eight of the resulting cDNAs were partially sequenced, and expression levels were compared by Northern blot analysis. By comparison of these partial sequences to the GenBank database, five clones were initially found to demonstrate homology to previously identified genes, whereas two (*clones 6* and *18*) were homologous to sequences reported in the EST database (Table 3). One additional novel clone, *clone 10*, was subsequently used to further probe a MAC16 cDNA library, and a larger clone was identified and sequenced and was found to have high homology to the 3′ end of a retroviral envelope protein sequence (data not shown). Northern blot analysis revealed no difference in expression of *clone 7* (calcium-binding protein ALG-2) in MAC16 vs. MAC13 cells, less than a twofold increase in expression of *clone 1* (human putative enterocyte differentiation-promoting factor) in MAC16 vs. MAC13 tumor-derived RNA, and two- to threefold increases in expression of *clones 5* (goblet cell mucus-secreting protein GOB-4), and *6* (AI482147) in MAC16 cells. *Clones 10* (retroviral envelope glycoprotein), *12* (mouse endogenous retrovirus 3′ long-terminal repeat), and *18* (AA395982) were highly expressed by MAC16 cells but demonstrated barely detectable expression in MAC13 cells, and this finding was confirmed in tumor-derived RNA for *clones 10* and *18* (Fig. 5). These data indicate that PCR select-subtractive hybridization is useful in identifying previously unreported novel and known differentially expressed genes in this cancer cachexia model.

DISCUSSION

Implantable tumors in animals provide a model to further elucidate the cellular mechanisms involved in cancer cachexia. In the present study, we focused on two murine adenocarcinoma cell lines with readily observable differences in their ability to induce cachexia in mouse models. Despite generation of these cell lines by similar treatment and their similar histological appearance, MAC13 and MAC16 tumors are quite distinct in their ability to induce weight loss. As previously demonstrated in NMRI mice, we confirmed that MAC16 tumor cell implantation and growth induced substantial weight loss in BALB/c nude mice. On the basis of our metabolic and food intake data, this wasting does not appear to be related to alterations in caloric intake, as may occur with tumor necrosis factor-induced anorexia (21) or to differences in total body $\dot{V}O_2$. In addition, weight loss induced by MAC16 tumors was neither strain specific nor dependent on host T-cell-mediated immunity.

Table 3. *cDNAs having increased expression in MAC16 cells, as identified by PCR-select hybridization*

Clone No.	Homologous Sequence (Accession No.)	Transcript Size, kb	Northern Blot Analysis (Tissue Analyzed)
1	Human putative enterocyte differentiation promoting factor (U622136)	1.4	MAC16 > MAC13 (cell culture and tumor)
5	GOB-4 (AB016592)	0.7	MAC16 > MAC13 (cell culture)
6	EST-Soares mouse NbMH *M. musculus* cDNA clone (AI482147)	1.5	MAC16 > MAC13 (cell culture)
7	*M. musculus* calcium-binding protein ALG-2 (U49112)	1.0	MAC16 = MAC13 (cell culture)
10	Envelope glycoprotein (AI615643)	3.3	MAC16 ≫ MAC13 (tumor)
11	*M. musculus* pendulin (U34229)	2.4	MAC16 > MAC13 (cell culture)
12	Mouse endogenous retrovirus 3′ LTR with inserted LTR-IS element (K02892)	5.0	MAC16 ≫ MAC13 (cell culture)
18	EST-Soares mouse lymph node (AA395982)	2.5	MAC16 ≫ MAC13 (cell culture and tumor)

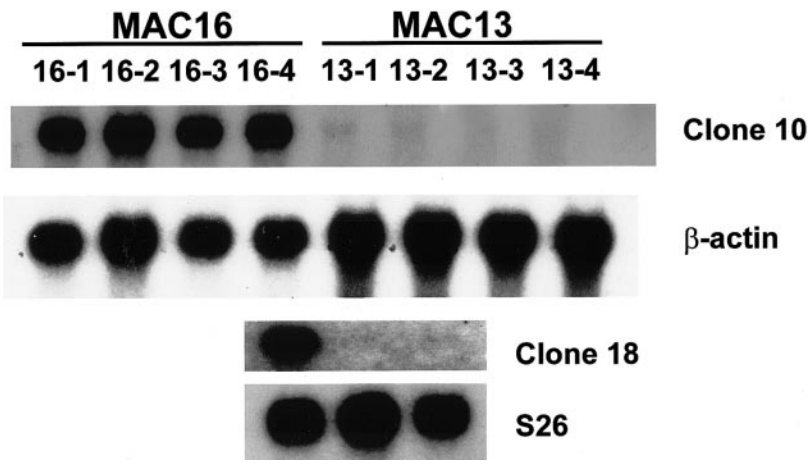

Fig. 5. Northern blot analysis of *clones 10* (envelope glycoprotein) and *18* (EST AA395982), identified by PCR-select subtraction. Each lane contains tumor RNA (10 μg). All signals were normalized to β-actin or S26. Transcripts for *clones 10* and *18* were detectable in MAC16, but not MAC13, tumors.

Given the identical origin of these two cell lines, it is likely that their genetic profiles are not vastly different. We hypothesized that differences in a limited number of genetic events and/or gene expression may reveal differences that underlie important phenotypic characteristics, such as the ability to induce cachexia. To screen and compare the genetic expression profiles of these two cell lines, we utilized three different but complementary approaches. Although each technique was limited and did not allow a comprehensive screening of all genes expressed by the two cell lines, each approach identified additional genes that were subsequently confirmed to be differentially expressed in these cell lines. In this manner, we were able to identify several genes and ESTs having higher levels of expression in MAC16 compared with MAC13 tumor cells.

A number of the genes identified by this screen are known to be upregulated in cancer. IGFBP-4 (20), cathepsin B (31), ferritin (22), endogenous long-terminal repeat sequences (28), and envelope protein-related transcripts (9) have previously been shown to be expressed in colon cancer cell lines. However, this is the first study to report differential expression of these genes between two adenocarcinoma cell lines with differing ability to induce cachexia. Of note, at least four of the genes identified by this screen are secretory proteins (IGFBP-4, ferritin light chain, ferritin heavy chain, and GOB-4). In addition, the normally intracellular protein cathepsin B has been demonstrated to be released from adenocarcinomas (15, 27). This is particularly relevant because previous work demonstrating cachexia-inducing effects of serum from MAC16-implanted mice, as well as isolation of proteolysis- and lipolysis-inducing factors, suggests that secretory products produced by this tumor may be important in cachexia.

Because this study screened for differences in gene expression between only two cell lines, the general role of this differential expression in the overall development of cachexia is unknown. However, the biological attributes of a number of these genes identify them as potential participants in the induction of cachexia. IGFBP-4 binds to IGF, inhibiting its interaction with the IGF receptor (12), and overproduction of IGFBP-4 could induce cachexia by preventing IGF-mediated anabolic effects in muscle and adipose tissue (18). In addition, IGFBP-4 might enhance muscle proteolysis by preventing IGF-mediated degradation of an mRNA transcript encoding the 14-kDa ubiquitin-conjugating enzyme E2, which catalyzes the first, irreversible step in the ubiquitin-dependent proteolytic system (41). Increased expression of the lysosomal protease cathepsin B might contribute to cachexia by inducing proteolysis by nonubiquitin-dependent pathways locally within the tumor. In addition, if released from tumor cells, cathepsin B might induce abnormal proteolysis of extracellular factors and tissues. The increase in both ferritin light- and ferritin heavy-chain mRNA in MAC16 tumor cells suggests that these cells may have an enhanced requirement for iron. By acting as an iron sink, the tumor might promote peripheral tissue degradation to provide a source of additional iron. Finally, increases in viral envelope glycoprotein or an endogenous long-terminal repeat sequence might promote cachexia by inducing the production of cytokines or arachidonic acid metabolites (38) or by promoting the transcriptional activation (5) of other intracellular cachexia-causing genes not identified by our screens.

A number of other sequences identified have homology to transcripts for proteins that have a less-established role in pathways that may be relevant to cachexia. For example, human putative enterocyte differentiation-promoting factor is a ubiquitin-conjugating E2 enzyme variant that lacks enzymatic activity but may be involved in the control of differentiation by altering cell cycle distribution (30). GOB-4 most likely generates a secretory protein involved in mucus-secreting function, in keeping with the gastrointestinal origin of these tumors (13), whereas pendulin (*clone 11*) acts as a blood cell tumor suppressor and is required for normal growth regulation (14). Variation of expression levels of these genes appears more likely to be related to the degree of differentiation of these tumor cell lines and may not be associated with modulation of adipose tissue or protein catabolism. Finally, *clone 18* demonstrates barely detectable expression in MAC13 and high expression in MAC16 cell lines, but a poten-

tial role for this gene awaits further identification of its full-length sequence and characterization of its protein product.

There are a number of limitations to this study. Our approach provides a sampling, but not a comprehensive screening, of differences in gene expression between these two cell lines. Also, in our screen, we focused our attention on genes for secretory peptides with increased expression in MAC16 tumor cells. Hence, we may have failed to identify important factors because they were not identified by our screen or because they were related to decreased expression of genes in MAC16 cells or increased expression of genes in MAC13 cells. Moreover, clinically important effects may be the result of small changes in expression of critically important genes, whereas we focused on genes demonstrating more substantial differences in expression. A further limitation is the restriction of our screen to changes in mRNA expression, which provides little insight into differences induced by posttranslational modification of proteins.

Screening of tumor cell line gene expression profiles between a cachexigenic and a similar, noncachexigenic cell line identified differences in expression of genes important in known metabolic pathways. Further study is required to determine whether these observed changes translate into changes in protein production and/or activity to better characterize their contribution to the cachectic phenotype. Reproduction of wasting in mice by one or more of these protein products will further support their role in cancer-induced cachexia.

The authors thank Dr. Michael Tisdale for provision of MAC13 and MAC16 cells, David Palmer for technical assistance, and Drs. Michael Wang and David Sidransky for advice.

C. L. Monitto is a Foundation for Anesthesia Education and Research/Baxter Pharmaceutical Research Starter Grant Recipient. This work was performed by the Department of Anesthesiology and Critical Care Medicine at the Johns Hopkins Hospital, Baltimore, MD.

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