Differential expression proteomics of human colon cancer

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Mazzanti, Roberto, Michela Solazzo, Ornella Fantappiè, Sarah Elfering, Pietro Pantaleo, Paolo Bechi, Fabio Cianchi, Adam Ettl, and Cecilia Giulivi. Differential expression proteomics of human colon cancer. Am J Physiol Gastrointest Liver Physiol 290: G1329–G1338, 2006.—The focus of this study was to use differential protein expression to investigate operative pathways in early stages of human colon cancer. Colorectal cancer represents an ideal model system to study the development and progression of human tumors, and the proteomic approach avoids overlooking posttranslational modifications not detected by microarray analyses and the limited correlation between transcript and protein levels. Colon cancer samples, confined to the intestinal wall, were analyzed by expression proteomics and compared with matched samples from normal colon tissue. Samples were processed by two-dimensional gel electrophoresis, and spots differentially expressed and consistent across all patients were identified by matrix-assisted laser desorption ionization-time-of-flight mass spectrometry analyses and by Western blot analyses. After differentially expressed proteins and their metabolic pathways were analyzed, the following main conclusions were achieved for tumor tissue: 1) a shift from β-oxidation, as the main source of energy, to anaerobic glycolysis was observed owing to the alteration of nuclear- versus mitochondrial-encoded proteins and other proteins related to fatty acid and carbohydrate metabolism; 2) lower capacity for Na+/K+ cycling; and 3) operativity of the apoptosis pathway, especially the mitochondrial one. This study of the human colon cancer proteome represents a step toward a better understanding of the metabolomics of colon cancer at early stages confined to the intestinal wall.

protein markers; apoptosis; bioenergetics

Cancer development and progression are multistep processes in which control of cell growth is progressively perturbed resulting from either a loss of tumor suppressor genes or activation of oncogenes. Colorectal cancer represents an ideal model system to study the development and progression of human tumors, because epithelial cells of the colon mucosa often follow a systematic process of cellular proliferation, differentiation, adenoma formation, and, eventually, cancer transformation.

At present, most studies have focused on understanding the genetic changes that occur in tumorigenesis. The global gene expression profiles of normal and cancerous human colonic epithelium had been studied by a relatively new technique known as serial analysis of gene expression, which identifies and quantifies cellular mRNA transcript levels (83). The drawback of concentrating at the transcript level has been demonstrated by independent studies (2, 24, 38) that have shown that correlations between mRNA and protein expression cannot predict protein expression levels from quantitative mRNA data; thus comparative analyses of normal and diseased states at the protein level is most informative.

The first data describing the alteration of protein expression in transformed colon mucosa were published in the beginning of the 1980s (41, 74). In 1983, Anderson et al. (1) described differential distribution of acidic proteins in normal colon mucosa, primary colon adenocarcinoma, and colon metastases to the liver. With the use of the two-dimensional (2-D) gel electrophoresis (GE) approach, several differences in protein expression in the cytosol of colon cancer samples, at various differentiation levels, were observed (50). Unfortunately, none of these proteins were identified. Keesee et al. (44) detected the tumor-specific expression of six nuclear matrix proteins that were also present in a panel of colon tumor cell lines. This study reinforced the concept of differential protein expression by cancerous tissue, and it raised the problem of accuracy at extrapolating results from cancer cell lines to in vivo scenarios (83, 56). Specific expression of several nuclear proteins was observed by Szmyczyk et al. (70) in human colorectal cancer tissue compared with normal matched mucosa. One of these proteins was a 36-kDa polypeptide that was absent in the normal colonic epithelium but present in 83% of the studied carcinomas. This protein might have served as a potential marker for colon malignancy, but, unfortunately, it was not identified, thus limiting its use and understanding of its function.

Recently, a novel approach for purification of epithelial cells from normal and tumor colorectal mucosa has been proposed, and it represents an important step to avoid a cellular contribution other than that of epithelial cells (57) but also may restrict or underestimate the importance of the interactions between epithelial and other tissue cells to the development of a tumor.

The data on specific cancer-related proteins or cancer-related protein expression downregulation are still controversial. Recently, a 2-D electrophoresis database for human colon crypts, colorectal cancer cell lines (i.e., LIM 1215 and HT29), and colonic polyps of multiple intestinal neoplasia and p53-null mice has been set up by the Ludwig Institute for Cancer Research (43, 67, 69). Stulik et al. (69) identified proteins that exhibit differential expression by comparing normal and malignant colon tissues. Most of cancer samples were in stages II and III, spreading from stage I to IV (69). In that study, it was concluded that there were no spots unique for either normal or pathological tissue and, furthermore, that there was no clear

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difference in the expression of the vast majority of spots between normal and neoplastic tissue. Only a few spots for each group seemed to exhibit striking alterations in protein abundance: a fatty acid-binding protein (FABP) and SM22α (an actin-binding protein) were significantly downregulated in tumor tissue, whereas four proteins, all members of S100 family for calcium-binding proteins, were more abundant in transformed colon mucosa.

The focus of this study was to understand the biochemical differences of human colon cancer and normal colon tissue1 from the same individual by expression proteomics, when the cancer is at relatively “early” stages, providing a more homogenous sampling than in previous studies. In addition, a higher loading of the protein sample followed by staining with a fluorescent dye facilitated the detection of low-abundant proteins, focusing on the following pathways: energy metabolism, Na+/K+ cycling, and apoptosis. This study may help our understanding of functional proteomics in colon cancer. This approach may help to identify proteins that could be useful as a new tool in the diagnosis or prognosis of this type of patients and perhaps to develop new therapeutic strategies to better aim therapy on the neoplastic target while minimizing the impact on normal tissues.

MATERIALS AND METHODS

Patients and Tissue Collection

Tissue samples were obtained from patients (4 men and 4 women; median age: 64 yr; age range: 46–81 yr) who had undergone surgical resection for primary sporadic colorectal adenocarcinomas at the Department of General Surgery, University of Florence, Florence, Italy. None of the patients had taken nonsteroidal anti-inflammatory drugs for at least 3 mo before surgery. All patients were thoroughly informed about the study and gave written consent for the investigation in accordance with the ethical guidelines of Local Ethical Committee.

Matched sets of colorectal carcinomas and normal colon mucosa used for both one-dimensional (1-D) and 2-D GE analyses were obtained within 30 min after surgical resection. The tissue samples were checked and sliced by a pathologist. The diagnoses of all samples were obtained from pathology reports.

Samples of small colorectal carcinomas were collected from the distal colon and rectum and were staged according to American Joint Committee on Cancer classification (33). Four tumors were located in the distal colon, and four tumors were located in the rectum. All eight tumors were adenocarcinomas classified as moderately differentiated. One tumor was stage I (T2, N0, M0); five tumors were stage II (T3, N0, M0; n = 5); and two tumors were stage III (T2, N1, M0). Cancer tissue (from the edge of the tumor) and adjacent normal mucosa (at least 10 cm from the tumor) were excised from each surgical specimen. Samples were washed with cold PBS and were homogenized in a 3:5 volume of lysis buffer (8 M urea, 2 M thiourea, 4% CHAPS, 40 mM Tris, 2% β-mercaptoethanol or 0.1 M DTT, and 1 mM PMSF) using a motorized Teflon/glass homogenizer (Potter-Elvejhem; total clearance: 0.05 mm). The resulting lysate was centrifuged using a refrigerated, high-speed centrifuge (Sorvall) at 12,000 g for 15 min at 4°C. Proteins were resuspended in sample solution (8 M urea, 2 M thiourea, 0.1 M DTT, 2% carrier ampholytes, 2% Triton X-100, and 2% CHAPS), homogenized, and subsequently stored at −80°C for further processing.

Chemicals and Biochemicals

The Multiphor II, DryStrip kit, Immobillon DryStrips with linear pH gradient 3–10, 18 cm, and Pharmalytes pH 8–10.5 and pH 3–10 were from Amersham Pharmacia Biotech (Uppsala, Sweden); the 6,000-V power supply was purchased from Hoefer. 2-D gels were cast and run in a Bio-Rad Multi Cell. Western blot analysis was done using a Bio-Rad Trans-blot apparatus (Richmond, CA). Acrylamide, urea, CHAPS, DTT, and tricine were from USB (Amersham Pharmacia Biotech); 1,4-bis-(acryloyl)pyrrolidine (PDA), SDS, N,N,N,N-tetra-methyl-ethylenediamine, ammonium persulfate, and nitrocellulose membranes were from Bio-Rad; and polyvinylidene difluoride (PVDF) and Nonidet P-40 were purchased from Boehringer (Mannheim, Germany). TRIZMA base, agarose, tricarboxylic acid, glycine, formaldehyde, silver nitrate, methanol, acetic acid, citric acid, iodoacetamide, and sodium deoxycholate (DOC) were from Sigma (St. Louis, MO). Bicinchoninic acid (BCA) assay reagents were from Pierce (Rockford, IL). 1-D electrophoretic gels were cast and run in a Mini-Protein II System. Western blot analyses were performed using Mini Trans-Blot Transfer Cell; all remaining equipment was obtained from Bio-Rad. Deionized water (18 MΩ) prepared with a Barnstead system was used for all buffers.

Western Blot Analyses

Tumors and normal mucosa samples were homogenized in ice-cold buffer [0.9% NaCl, 20 mM Tris-HCl (pH 7.6), 0.1% Triton X-100, 1 mM PMSF, and 0.01% [euepeptin] and centrifuged at 4°C for 10 min at 10,000 g. The protein content was determined by using a BCA protein assay kit (Pierce) with BSA as the standard. Samples were mixed 1:1 with Laemmli buffer under reducing conditions and boiled. SDS-PAGE was performed using 12% and 5% acrylamide for the separating gel and stacking gel, respectively. Proteins were transferred onto nitrocellulose membranes (Pierce). Blots were blocked with 5% BSA in PBS (Pierce) and incubated overnight at 4°C with the primary antibody [anti-B cell-associated protein 31 (BAP31) goat polyclonal antibody from Santa Cruz Biotechnology, Santa Cruz, CA]. Blots were further incubated with the appropriate secondary antibodies from Santa Cruz Biotechnology for 1 h at room temperature, incubated with SuperSignal West Pico Chemiluminescent Substrate (Pierce) for 5 min, and then exposed to CL Xposure Film (Pierce). Western blots for human caspase-9, apoptotic peptide-activating factor (ApaF)-1, the β-subunit of F0F1-ATPase, and cytochrome c oxidase subunit II were performed using anti-caspase-9 from Cell Signaling Technologies (no. 9502), anti-ApAF1 from Cell Signaling Technologies (no. 4452), anti-β-subunit of F0F1-ATPase (A-21351 from Molecular Probes), and anti-cytochrome c oxidase subunit II (A-6404 from Molecular Probes) following the manufacturer’s protocols. Blots were obtained by transferring the 2-D GE of the samples and probing sequentially (previous stripping) for the proteins. Secondary antibodies were from Santa Cruz Biotechnology. The images were taken with a Kodak 2000MM imager.

2-D GE

The first-dimensional IEF was performed on precast 18-cm IPG strips (Amersham Pharmacia Biotech) at 20°C with a maximum current setting of 50 μA/strip using an Amersham Pharmacia IPGphor IEF unit. Commercial strips with linear immobilized pH 3–10 gradient were used for isoelectric focusing. These strips were wetted with rehydration buffer containing 2 M thiourea, 7 M urea, 4% CHAPS, 0.5% Triton X-100, 100 mM DTT, and 0.4% Pharmalytes (pH 3–10) overnight in a leveled acrylic strip holder. The amount of protein loaded was 0.8–1 mg protein for preparative gels. After rehydration,
the IEF run was carried out using the manufacturer’s conditions for linear strips using a power supply MPS 3500 XL. Before the 2-D SDS-PAGE was carried out, strips were subjected to a two-step equilibration. The first was with an equilibration buffer consisting of 6 M urea, 30% glycerol, 2% SDS, 50 mM Tris-HCl (pH 6.8), and 1% (wt/vol) DTT. The second step was with a buffer consisting of 6 M urea, 30% glycerol, 2% SDS, 50 mM Tris-HCl (pH 8.8), and 2.5% (wt/vol) iodoacetamide. After the IPG strips were transferred onto the 2-D SDS-PAGE gel, a molecular mass marker strip was included, and the strips were sealed in place with 0.75% agarose. SDS-PAGE was performed on 1.0-mm-thick 10% polyacrylamide gels at a constant voltage at 13°C for 5 h using a Hoefer SE600.

Gel Staining

Silver staining of the gels was performed as follows: gels were fixed in 50% methanol-5% acetic acid in water for 30 min followed by washing in 50% methanol in water for 10 min. Gels were then washed again with water for 60 min and sensitized with 0.02% sodium thiosulfate for 2 min. After the gels were rinsed twice with water for 1 min each, they were incubated in chilled 0.1% silver nitrate for 40 min at 4°C. After the silver nitrate was discarded and gels were rinsed with two changes of distilled water for 1 min each, they were developed in 0.04% formalin (35% formaldehyde in water) in 2% sodium carbonate. When the desired intensity was attained, the developer was discarded, and the gel was incubated with either 1.46% EDTA disodium dihydrate or 1% acetic acid for 10 min to stop the development. The staining procedure was completed by three rinses with water for 5 min each. Stained gels were scanned using a Kodak Imager. Sypro RubyRed staining was performed by following the manufacturer’s conditions, and images were taken with a Fluorochrom imager.

Statistical Analysis and Image Analysis of 2-D Gels

Silver-stained or Sypro-stained protein spots were excised manually with a sterile scalpel blade and transferred to a sterile microcentrifuge tube. Each excised spot was destained, washed, reduced with DTT, alkylated with iodoacetamide, and digested in gel with trypsin. The peptide extraction was performed with ammonium bicarbonate and acetonitrile.

Matrix-Assisted Laser Desorption Ionization-Time-of-Flight Mass Spectroscopy Analysis of Tryptic Peptides

Samples were speed vacuumed to dryness after proteolytic digestion and then reconstituted to 15 μL with 95:5 water-acetonitrile and 0.1% trifluoroacetic acid (TFA), vortexed gently, and centrifuged at 2,000 g for 30 s. The desalting/concentration procedure was performed by using C18 ZipTip according to Millipore (Billerica, MA) instructions. A C18 resin was hydrated with 2 × 10-μL aliquots of 50:50 water-acetonitrile and 0.1% TFA and then equilibrated with 2 × 10-μL aliquots of aqueous 0.1% TFA. Peptides were bound to C18 by pipetting up and down in the sample five times with the pipettor set to 10 μL. Desalting was performed by pipetting 5 × 10-μL aliquots of aqueous 0.1% TFA through the resin. Elution was performed by pipetting 1.5 μL of 60:40 water-acetonitrile and 0.1% TFA over the resin three to four times. One microliter of the extracted sample from each tube was dispensed onto a matrix-assisted laser desorption ionization (MALDI) sample plate along with 1 μL of matrix solution consisting of 10-mg/ml α-cyano-4-hydroxycinnamic acid (Sigma) in 0.1% TFA and 50% acetonitrile and spotted on a MALDI target. Mass analyses were performed according to a previously published method (Ref. 21 and the references therein) using a PerSeptive Biosystems Voyager-DE STR MALDI-time of flight (TOF) mass spectroscopy (MS) system (Framingham, MA) located at the Mass Spectrometry Facility (University of Minnesota). Samples were allowed to dry under ambient conditions. For each sample, the average of 256 spectra was acquired in the delayed extraction and reflector mode. The average of four scans (each containing 64 spectra) that passed the accepted criterion of peak intensity was automatically selected and saved. Spectra were automatically calibrated upon acquisition using a two-point calibration with residual porcine trypsin autolytic fragments [842.51 and 2,210.10 (M + W) ions]. Assignment of peaks was done manually; measured peptide masses were excluded if their masses corresponded to trypsin autodigestion products or if they were from identified proteins adjacent to the spot being analyzed.

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The proteins were identified by searching in SWISSPROT and National Center for Biotechnology Information nonredundant databases using MS-Fit (Protein Prospector, University of California, San Francisco, CA) and peptide mapping (Profound, version 4.10.5, Rockefeller University). All mass searches were performed using a mass window between 1,000 and 100,000 Da and included human, rat, and mouse sequences. The search parameters allowed for oxidation of methionine, NH2-terminal acetylation, carboxamidomethylation of cysteine, and phosphorylation of serine, threonine, and tyrosine. The criteria for positive identification of proteins were set as follows: 1) at least four matching peptide masses; 2) 50 ppm or better mass accuracy; and 3) molecular mass and isoelectric point (pl) of identified mature proteins should match estimated values (within a 20% error) obtained from image analysis.

RESULTS AND DISCUSSION

Cancer development and progression are multistep processes. The knowledge of these complex mechanisms may strikingly improve diagnosis, therapy, and prognosis of cancer patients. Colorectal cancer is an interesting model to study the transition from normal epithelium to cancer and cancer progression, because, in most cases, cancer arises from normal epithelium through a step-by-step process. Several lines of evidence suggest that the tumor may share some of the features of the tissue in which it develops, but often some different characteristics appear that lead to disruption of cell growth control and to cancer progression.

The purpose of this study was to understand the biochemical differences of certain metabolic pathways in human colon cancer and normal colon mucosa by identifying proteins differentially expressed that may be useful as a diagnostic or prognostic marker or as a drug target. To this end, colon samples from normal tissue and tumors from each patient were analyzed by 2-D GE. Gel images of control and tumor samples were superimposed for each patient to detect differentially analyzed by 2-D GE. Gel images of control and tumor samples performed with low protein loading is shown. Images were scanned with a flatbed scanner and saved as TIFF files. Warping and geometrical distortion were adjusted using Phoretix software. The files were colored in Adobe Photoshop (red control and green tumor), overlaid, and flatten. Arrows indicate some of the differentially expressed proteins in 2-D GE. Thick arrows indicate proteins solely found in normal tissue, whereas thin arrows point at proteins found solely in tumor tissues. Other experimental details are explained in MATERIALS AND METHODS.

Energy Metabolism in Colon Tissue

ATP is mainly provided by β-oxidation of short-chain fatty acids, for they are the preferred metabolic fuel in normal colonocytes (4, 30, 61). These fatty acids (e.g., acetate, propionate, and butyrate) are generated at high levels in the colon by bacterial fermentation of dietary fiber and unabsorbed carbohydrates (4, 30, 61). If ATP is mainly provided by β-oxidation followed by oxidative phosphorylation in normal colonocytes, then it is expected that proteins associated with this pathway would be differentially expressed. Confirming this view, NADH-ubiquinone oxidoreductase B1b (a component of complex I of the mitochondrial electron transport chain) and the β-subunit of F0F1-ATPase (complex V) were found to be overexpressed when evaluated by proteomics and Western blot analysis, respectively. These results suggested the preferential utilization of aerobic metabolism by normal tissue.

Data from colon cancer indicated the overexpression of mitochondrial transcription factor A [also known as mitochondrial transcription factor A (TFAM)]. TFAM is encoded by nuclear DNA and controls the synthesis of mitochondrial respiratory chain components (cytochrome c oxidase I-III, NADH dehydrogenase subunits 1–6, cytochrome b, and ATPase 6 and 8) by initiating the transcription of mtDNA (25, 29, 52, 71). An overexpression of TFAM supports the notion that in cancer cells mtDNA has been preserved, for TFAM stabilization requires the presence of intact mtDNA. Furthermore, cells depleted of mtDNA are characterized by the ab-
### Proteins differentially expressed in normal and cancerous colon tissues

<table>
<thead>
<tr>
<th>Name</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>NADH-ubiquinone oxidoreductase B18</td>
<td>Transfer of electrons from NADH to the respiratory chain; the immediate electron acceptor for the enzyme is believed to be ubiquinone.</td>
</tr>
<tr>
<td>β-subunit of F&lt;sub&gt;1&lt;/sub&gt;F&lt;sub&gt;0&lt;/sub&gt;-ATPase†</td>
<td>Component of mitochondrial ATPase.</td>
</tr>
<tr>
<td>Liver fatty acid-binding protein</td>
<td>Intracellular transport of longchain fatty acids and their acyCoA esters.</td>
</tr>
<tr>
<td>Soluble carrier family 5 Na&lt;sup&gt;+&lt;/sup&gt;-glucose cotransporter member 1</td>
<td>Transports glucose to the cell by sodium cotransport.</td>
</tr>
<tr>
<td>β₃-Subunit of Na⁺-K⁺-ATPase</td>
<td>Component of sodium-potassium-exchanging ATPase activity.</td>
</tr>
<tr>
<td>Eukaryotic translation initiation factor 4E</td>
<td>Recognizes and binds the 7-methylguanosine-containing mRNA “cap” during an early step in the initiation of protein synthesis and facilitates ribosome binding by inducing the unwinding of mRNA secondary structures.</td>
</tr>
<tr>
<td>Insulin-like peptide 5</td>
<td>May have a role in gut contractility or in thymic development and regulation; see text for more details.</td>
</tr>
<tr>
<td>Zn finger protein 272 (partial sequence)</td>
<td>Protein translation factor SUI1</td>
</tr>
<tr>
<td>Inducible cAMP early repressor type 1</td>
<td>Probable transcription factor activity.</td>
</tr>
<tr>
<td>Protein translation factor SUH homolog</td>
<td>Probably involved in translation.</td>
</tr>
<tr>
<td>Prefoldin subunit 1</td>
<td>Binds specifically to cytosolic chaperonin and transfers target proteins to it; binds to the nascent polypeptide chain and promotes folding in an environment in which there are many competing pathways for nonnative proteins.</td>
</tr>
<tr>
<td>Glutathione transferase M₄</td>
<td>Conjugation of reduced glutathione to a wide number of exogenous and endogenous hydrophobic electrophiles.</td>
</tr>
<tr>
<td>GTP-binding protein (Ral)</td>
<td>Belongs to the ERA/TRME family of GTP-binding proteins.</td>
</tr>
</tbody>
</table>

### Proteins increased in colon cancer tissue compared with normal colon tissue

<table>
<thead>
<tr>
<th>Name</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mitochondrial transcription factor 1</td>
<td>Involved in mitochondrial transcription regulation</td>
</tr>
<tr>
<td>Cytochromes oxidase subunit II†</td>
<td>Component of the terminal oxidase of the electron transport chain; catalyzes the 4-electron reduction of oxygen to water.</td>
</tr>
<tr>
<td>Lactate dehydrogenase (M)</td>
<td>Protein involved in the anaerobic enzymatic conversion of glucose to lactate or pyruvate, resulting in energy stored in the form of ATP.</td>
</tr>
<tr>
<td>p28 BAP31*</td>
<td>May play a role in anorectograde transport of membrane proteins from the endoplasmic reticulum to Golgi; may be involved in caspase-8-mediated apoptosis.</td>
</tr>
<tr>
<td>Glucosamine-6-sulfatase</td>
<td>Hydrolysis of the 6-sulfate groups of the N-acetyl-d-glucosamine 6-sulfate units of heparan sulfate and keratan sulfate.</td>
</tr>
<tr>
<td>Caspase-9†</td>
<td>Involved in the activation cascade of caspases responsible for apoptosis execution; binding of caspase-9 to Apaf-1 leads to activation of the protease that then cleaves and activates caspase-3; proteolytically cleaves poly(ADP-ribose) polymerase.</td>
</tr>
<tr>
<td>Apaf-1†</td>
<td>Oligomeric Apaf-1 mediates the cytochrome c-dependent autocatalytic activation of procaspase-9 (Apaf-3), leading to the activation of caspase-3 and apoptosis. This activation requires dATP. Oligomerizes upon binding of cytochrome c and dATP.</td>
</tr>
<tr>
<td>p53-induced protein SIP27</td>
<td>SIPs regulate p53-dependent apoptosis.</td>
</tr>
<tr>
<td>Neurofibromatosis type-2 protein isofrom Mer162</td>
<td>Novel alternatively spliced isoforms of the neurofibromatosis type-2 tumor suppressor are targeted to the nucleus and cytoplasmic granules.</td>
</tr>
<tr>
<td>Ubiquitin-conjugating enzyme 1</td>
<td>Has no ubiquitin ligase activity on its own. The UBE2V1/UBE2N heterodimer catalyzes the synthesis of noncanonical polyubiquitin chains that are linked through Lys&lt;sup&gt;65&lt;/sup&gt;. This type of polyubiquitination activates IKK and does not seem to involve protein degradation by the proteasome. Plays a role in the activation of NF-κB mediated by IL-1β, TNF, TRAF6, and TRAF2. Mediates transcriptional activation of target genes. Plays a role in the control of progress through the cell cycle and differentiation. Plays a role in the error-free DNA repair pathway and contributes to the survival of cells after DNA damage.</td>
</tr>
<tr>
<td>DNA excision repair protein ERCC-1</td>
<td>Structure-specific DNA repair endonuclease responsible for the 5’-incision during DNA repair</td>
</tr>
<tr>
<td>Alternative splicing product of P04895 or guanine nucleotide-binding protein G&lt;sub&gt;s&lt;/sub&gt;, α-subunit 1</td>
<td>G&lt;sub&gt;s&lt;/sub&gt; proteins are involved as modulators or transducers in various transmembrane signaling systems. G&lt;sub&gt;s&lt;/sub&gt; protein is involved in hormonal regulation of adenylate cyclase; it activates the cyclase in response to β-adrenergic stimuli.</td>
</tr>
<tr>
<td>Replication factor C 37-kDa subunit</td>
<td>The elongation of primed DNA templates by DNA polymerase-δ and ε requires the action of the accessory proteins proliferating cell nuclear antigen and activator 1</td>
</tr>
<tr>
<td>DNA mismatch repair protein MSH2</td>
<td>This mutation is associated with stomach, prostate, colon, cervical, skin, and endometrial cancers as well as polyps and histiocytoma; Gin&lt;sup&gt;87&lt;/sup&gt; in wild type but stop in mutant is associated with hereditary nonpolyposis colorectal cancer.</td>
</tr>
<tr>
<td>LIM/homeobox protein LHX2</td>
<td>Transcriptional regulatory protein involved in the control of cell differentiation in developing lymphoid and neural cell types (by similarity).</td>
</tr>
<tr>
<td>G protein G&lt;sub&gt;x&lt;/sub&gt; α-subunit 11</td>
<td>G proteins are involved as modulators or transducers in various membrane signaling systems. G&lt;sub&gt;x&lt;/sub&gt; acts as an activator of phospholipase C.</td>
</tr>
<tr>
<td>G protein α-subunit 14</td>
<td>G proteins are involved as modulators or transducers in various transmembrane signaling systems.</td>
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</table>

Proteins are organized chronologically as they appeared in the text with the exception of GTP-binding protein, which was identified as described in the text but not discussed in this study. ERAITRME, Escherichia coli ras-like GTPase/RNA modification GTase; BAP31, B cell-activated protein 31; Apaf-1, apoptotic peptidase-activating factor 1; SIP27, stress-inducible protein; UBE, ubiquitination-dependent endocytosis motif; TRAF, TNF receptor-associated factor. *Proteins identified as overexpressed by two-dimensional gel electrophoresis and confirmed by Western blot analysis. †Proteins tested only by Western blot analysis.
ence of detectable TFAM protein levels but with normal levels of TFAM transcript (28, 45). When one of the mtDNA-encoded proteins, cytochrome c oxidase subunit II (a component of complex IV), was evaluated by Western blot analysis, its expression was found to be two times higher in cancer than in the normal colon (Fig. 2). These results confirmed the presence of intact mtDNA in cancer cells and supported the operativity of TFAM in cancer cells as an initiator of the transcription at the mtDNA level. These results (relatively lower expression of NADH-dehydrogenase B18 and the β-subunit of F0F1-ATPase (b-subunit)) might force tumor cells to find a pathway other than β-oxidation/oxidative phosphorylation to provide ATP.

An indication that this shift had occurred was provided by the low expression of FABP [liver-type (L-)FABP] and the high expression of lactic dehydrogenase (LDH). Although the precise physiological functions of FABPs remain uncertain, several roles have been proposed. Among them are 1) enhancing the uptake of fatty acids into cells and facilitating their transport to intracellular organelles, 2) protecting enzymes and membranes from the effects of free fatty acids and their acyl-CoA derivatives, 3) maintaining a large intracellular pool of fatty acids for rapid mobilization, and 4) targeting fatty acids to specific metabolic pathways (for reviews, see Refs. 9 and 35). The low expression of FABP in colon cancer is in agreement with published data describing a significant decrease in FABP mRNA abundance in experimentally induced colon cancers of rats (17) and in FABP protein expression in transformed human colon mucosa (69). A FABP expression change has been reported to occur in other situations. Animals fed a poor-fermentable fiber diet (fibers protect against colon cancer) significantly increased the FABP mRNA level in exfoliated colonocytes during tumor development (19), whereas a loss of adipocyte-type FABP during bladder carcinoma progression has been also described (18).

Enterocytes contain both intestinal (I-) and L-FABPs (36) aside from another, immunologically distinguishable FABP that is associated with the microvillus membrane (68). Although the mRNAs specifying I- and L-FABP are among the most abundant sequences that accumulate in the small intestinal epithelium (8, 37), it is unclear why the intestine requires the expression of two cytosolic FABPs. Gang and Ockner (34) have shown that in the intestine, luminally derived fatty acids are largely reesterified in the enterocyte to form triacylglycerol, whereas fatty acids derived from the intestinal blood supply are utilized for energy production and phospholipid biosynthesis (75). One attractive but unproven hypothesis is that the two FABPs contribute to this metabolic compartmentalization. If this hypothesis is correct, then the low expression of L-FABP in tumor colonocytes could reinforce the concept that these cells do not perform β-oxidation owing to a lack of normal supply of fatty acids via the low expression of L-FABP.

The overexpression of LDH found in cancerous tissue might reflect the stimulation of ATP production by glycolysis to overcome the decreased ATP formation by oxidative phosphorylation (Table 1). Of interest, based on the proteomic data, a shift in the LDH isoenzyme pattern was also observed. LDH is a tetramer constituted by the combination of the M and H isoforms. The LDH expressed in cancerous tissue was identified as type 5, constituted only by M subunits. This isoform has a low affinity for its substrates and operates essentially toward the formation of lactate from pyruvate, working optimally under anaerobic conditions. These findings are in agreement with other studies (16, 79) performed with intestinal tumors and adenocarcinomas.

Combining these results (relatively lower expression of NADH-dehydrogenase B18 and the β-subunit of F0F1-ATPase and higher expression of cytochrome c oxidase IV subunit II), it could be hypothesized that tumor cells shifted from β-oxidation as their main energy source to glycolysis because of an imbalanced stoichiometry between mtDNA- and nuclear DNA-encoded proteins, resulting in a dysfunctional oxidative phosphorylation. This hypothesis may provide the molecular basis for the “respiratory impairment” of cancer cells postulated by Warburg (79) more than 80 years ago to explain the invariably high ratio of fermentation to respiration in cancer metabolism. However, our results extend this observation to compartments other than mitochondria, i.e., the bloodstream side of colonocytes (vide infra under lower capacity to control cation cycling).

An interesting consequence of the shift from β-oxidation to glycolysis by tumor colonocytes is that an accumulation of one of the main fuels, butyrate, may ensue. Considering that butyrate plays a role in histone acetylation (11), it is possible that higher concentrations of butyrate may be required to induce the transcriptional activation of the Bax gene, as reported by Mandal et al. (47), thereby inhibiting proliferation and inducing apoptosis. In this regard, short-chain fatty acids in the colon are effective at reducing cancer incidence (12, 21, 77). This effect has been explained through several potential mechanisms: inhibition of histone deacetylases (39), induction of apoptosis (7, 40), modulation of gene expression (10), and induction of cell arrest (78).

Buttgereit and Brand (15) documented that in mammalian cells, macromolecular biosyntheses are the most sensitive processes to energy supply, followed by calcium and sodium.
cycling. The rate of protein and polynucleotide synthesis decreases to 40% when respiration is inhibited by 30% (15). If indeed in tumor tissue there is an overall lower capacity for ATP production, then several processes should be affected: 1) a decline in macromolecule biosynthesis should be observed (accompanied or not by an increased proteolysis); 2) a lower capacity to regulate sodium/potassium cycling; and 3) a minor impact on other processes relatively less dependent on ATP, such as apoptosis.

Decline in macromolecule biosynthesis. Regarding the first scenario, an average of 167 ± 10 protein spots was identified in gels from normal colon tissue, whereas a consistently lower number (105 ± 12, P < 0.05) was obtained from colon cancer samples.2 This difference in protein expression may represent halted protein synthesis, as indicated above, without excluding a simultaneous increased proteolytic activity (48, 64, 72).

Lower capacity to control cation cycling. The differing Na+ and K+ concentrations inside and outside of eukaryotic cells are maintained largely by an antiport protein known as Na+-K+-ATPase. Na+-K+-ATPase catalyzes ATP-dependent transport of three sodium ions out and two potassium ions into the cell per pump cycle, thereby generating a transmembrane sodium gradient. The activity of Na+-K+-ATPase is involved in the control of cellular pH, osmotic balance, and Na+-coupled transport of nutrients such as amino acids and vitamins into cells. Another transporter, the sodium-glucose cotransporter (SLC5A1), takes advantage of this ion gradient by efficiently cotransporting glucose (or galactose) and sodium into cells. As a consequence of cotransporter activity, glucose becomes more concentrated inside the cell, which it then exits, down its concentration gradient, via a passive uniport glucose transporter (GLUT5 or GLUT2). This mechanism allows the intestine to collect glucose/galactose from digested food and then release it into the bloodstream. The overexpression of SLC5A1 found in normal colonocytes (Table 1) is indicative of appropriate expression of Na+-K+-ATPase and by an adequate ATP supply. In colon tissue, oxygen consumption decreases by about 30% in the presence of the Na+-K+-ATPase inhibitor ouabain (6), indicating that a significant fraction of ATP serves to power Na+-K+-ATPase. The lower ATP capacity by colon cancer tissue may have a significant impact on this process. The lower expression of SLC5A1 (present study) would lead to a lower entry of glucose and sodium. Intracellular sodium (among other factors) modulates the transcription of both components of Na+-K+-ATPase (i.e., the α- and β-subunits), increasing the levels of these mRNAs and proteins (46, 49, 81). At early stages of cancer, less expression (number of pumps) of Na+-K+-ATPase would be required to match the lower SLC5A1 expression to spare ATP while preserving sodium homeostasis. From our proteomic data, we found no detectable expression of the Na+-K+-ATPase β1-subunit in cancer tissue, although a considerable amount was observed in matched control tissue. No data on the Na+-K+-ATPase α-subunit was obtained from either cancer or normal tissue owed to the underrepresentation of high-molecular-mass proteins and/or proteins with extreme pI in 2-D GE (82). Similarly, in other types of cancer, lower protein expression of the β-subunit of Na+-K+-ATPase (with a constant or lower expression of the α-subunit) has been reported: renal clear cell carcinoma (53), oncogenic transformation of Madin-Darby canine kidney (MDCK) cells with Moloney sarcoma virus (MSV-MDCK; Ref. 55), or early stages of bladder cancer (32).

However, we cannot exclude that in addition to a low expression of the Na+-K+-ATPase β-subunit, changes in α-isof orm expression and/or isoform distribution might have occurred. Recently, it has been observed that there is a switch from the α1-subunit (normal mucosa) to the α3-subunit (well to moderately differentiated human colon cancer) with essentially preservation of the activity [ratio of activity of α3/β to α1/β = 0.81 (63)]. Although it is known that several α/β combinations are functional, α3/β complexes have a significant lower turnover number (about one-fifth to one-fourth) and lower affinity for sodium (8 and 25 mM for α1/β and α3/β, respectively (26)). Thus, by switching to the α3/β combination, Na+-K+-ATPase can be transformed to a system of decreased energy supply (13, 54), a system typical of tumor cells, with an activity that matches that of SLC5A1 expression.

The lower expression of the Na+-K+-ATPase β-subunit might lead to reduced Na+-K+-ATPase activity, which, if sustained, might result in events that favor the progression of cancer. This is based on the crucial role that intracellular sodium homeostasis, regulated by Na+-K+-ATPase, plays in the development of tight junctions and induction of polarity in epithelial cells (54). Espineda et al. (32) showed that in E-cadherin-expressing MSV-MDCK cells, expression of the β-subunit induced the formation of junctional complexes (e.g., tight junction and desmosomes), significantly reducing the motility and invasiveness of these cells (55). These studies revealed that the β-subunit of Na+-K+-ATPase might be involved in the mechanisms leading to the suppression of invasiveness of carcinoma cells. It was suggested that increased β-subunit expression might reduce the invasiveness of tumor cells, thus reducing the chance of tumor spread and, consequently, its recurrence.

In this regard, to evaluate the probability of metastasis, we compared our proteomic profiles with a gene set considered as a predictor of both metastasis and poor clinical outcome (56). This set was derived from the gene expression profiles of adenocarcinoma metastases of a variety of cancer versus unmatched primary adenocarcinomas. In our study, only one protein (of the 17 gene signatures) reported in Ref. 56 was found to be upregulated (elongation factor 4E-like 3), and, more importantly, this was found in normal instead of cancer tissue. This indicated that the colon cancer cases that were studied in the present work (despite the fact of having a low expression of the β-subunit of Na+-K+-ATPase) had a low probability of metastasis, at the moment of sample collection, without excluding the involvement of other factors in the overexpression of elongation factor 4E-like 3.

Apoptosis. Proteins differentially expressed in colon cancer were BAP31, caspase-9, Apatf-1, and p53-dependent stress-inducible protein 27 (SIP27). BAP31 is ubiquitously expressed, involved in mechanisms of the regulation of apoptosis (51). BAP31 is highly enriched at endoplasmic reticulum membranes (14), where it forms both a homo- and hetero-oligomer with closely related BAP29 (14). BAP31 is a substrate of caspase-8 and can associate with procaspase-8 iso-

\[2\text{ The majority of the proteins were originated from colonocytes. For this comparison, multiple spots from the same protein (probably with various posttranslational modifications) were counted once. The actual numbers of individual spots were 345 and 283 for normal and cancer tissues, respectively.} \]
forms in response to apoptotic signaling/stimuli by the model oncogene E1A (14). The expression of BAP31 protein was confirmed by Western blot analysis performed on six of the eight cancer samples (Fig. 2). In agreement with our proteomic results, BAP31 protein levels were significantly higher in neoplastic tissue specimens than in normal tissue.

SIP27 is involved in the regulation of p53-related apoptosis, and its expression is dependent on p53 (73). SIP is a proapoptotic protein dependent on p53 activation, and it has been reported to be overexpressed in a variety of tumors. On the basis of this evidence, it was postulated that it acts as stress-induced gene with antitumor properties (73). The SIP overexpression in our samples of cancer suggests that p53 is functionally active.

Overexpression of proapoptotic BAP31 and p53-induced SIP27, in addition to caspase-9 and Apaf-1, might indicate that the mechanisms of apoptosis at these stages of cancer are still present and, probably, responsive to apoptotic stimuli.

This assumption is supported by the coexpression of neurofibrilosis type-2 protein (NF-2), which efficiently works as a tumor suppressor protein (3, 66) (Table 1). Taken together, these data suggest that colon cancer localized or confined to the intestinal wall may have an active defense mechanism against tumor progression. This underlines the importance of detecting cancer at these stages to favor apoptosis through therapeutic approaches. Results of clinical trials published in last decade line with these results.

Although these results on apoptosis might seem promising, they should be interpreted with caution, for not all caspases have been evaluated (i.e., initiators 4 and 5 and effectors 3, 6, and 8), and none of their activities were tested. This is important because as the correlation between transcript and protein levels might be misleading, the association between protein levels and activities might not be direct for critical events (such as posttranslational modifications and substrate and effector concentrations, among others) need to be considered for a comprehensive understanding of the operativity of a process.

Cell Cycle and Signaling

In this study, ubiquitin-conjugating E2 enzyme variant (UEV; Table 1) was found overexpressed in colon cancer tissues. This finding could be related to the role that this protein plays in in vitro differentiation and cell cycle (20). UEV constitutes a new subclass of proteins (originally named CROC-1) similar in sequence and structure to the ubiquitin-conjugating enzymes known as E2 enzymes (42, 60). UEV protein is downregulated upon differentiation of HT29-M6 cells (a tumor cell line derived from HT29); the constitutive expression of exogenous human UEV in these cells inhibits their capacity to differentiate upon confluence and causes both the entry of a larger proportion of cells in the division cycle and an accumulation in G2-M, accompanied by a profound inhibition of the mitotic kinase cdk1. Thus the fact that UEV has been found in tumor tissue suggests that at these stages of colon cancer (without evidence of extraorgan involvement), proliferation and cell differentiation might have been halted. More experimental evidence needs to be sought to confirm this possibility.

Proteins Related to Normal Functions of Colon Tissue

Several proteins related to colon functions were found differentially expressed in normal tissue. Of interest, insulin-like peptide 5 (INSL5), a member of the insulin/relaxin family, was also found overexpressed in the normal colon. Although the role of this protein is unknown, it could be speculated that it plays a role in gut motility based on the sequence homology with relaxin-3. INSL5 has been recently reported to be the ligand for G protein-coupled receptor 142 (GPCR142) based on the partial overlapping tissue expression profiles of INSL5 and GPCR142 (especially in the colon) combined with their high-affinity interaction. This recent finding will help to understand the role of INSL5 and those of other relaxin-related peptides.

Other proteins related to transcription (Zn finger protein and inducible cAMP repressor protein), translation (protein translation factor SU11), and posttranslational (prefoldin) events and detoxification of xenobiotics/endogenous metabolites (glutathione transferase) were also found to be differentially expressed in normal colon tissue (Table 1).

Bearing in mind that these proteins were found in “normal colon tissue” (see definition under footnote 1) but still originated from cancer patients, it could be speculated that this tissue may already harbor molecular differences compared with normal mucosa from subjects with no evidence of colonic disease. If so, then some of these proteins could be used as biomarkers for colon cancer risk.

Conclusions

Our methodology permitted us to make unique observations. First, there is a marked difference in the expression of important, nonabundant proteins between normal and cancer colon mucosa. This difference could be exploited to develop new diagnostic strategies, as recently pointed out (83). One of the most relevant issues in proteomic analysis is that without cell fractionation or protein enrichment, abundant proteins (in general, housekeeping and structural proteins) are the most likely ones to be resolved and identified (58, 59). Accordingly, two important enzymes implicated in tumor growth by promoting angiogenesis, inducible cyclooxygenase-2 and nitric oxide synthase (inducible nitric oxide synthase), found to be upregulated in human colorectal cancer by a different experimental approach (22, 23) were not observed in our study. However, the experimental modifications introduced in this study allowed us to detect a significant fraction of differentially expressed proteins, especially some low-abundance ones (e.g., G proteins) and some membrane-bound ones (e.g., NADH-ubiquinone reductase B18 subunit), with profound implications for our understanding of cancer metabolism.

Second, these samples (from colon cancer at early or intermediate stages, all confined to the colon wall without metastasis) show the expression of proteins that are involved in mechanisms of defence against cancer, suggesting that a therapeutic strategy should be based on this differential biochemistry.

Finally, this study supports the evidence of a preference by cancer tissues to utilize glycolysis, as originally hypothesized by Warburg (79). This observation has been explained through a limited availability of substrates to mitochondria (62) or by a selective repression of the expression of the β-subunit of
ATPase (27), although our results indicate that more than one mitochondrial component may be altered during the progression toward tumorigenesis.

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