Variation in Cytosolic Protein Expression between Human Colon Tumors That Differ with Regard to Differentiation Class

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Using a combination of two-dimensional gel electrophoresis, silver staining, and a 16-quadrant grid system, we established a set of composite patterns for the colon tumor cytosol proteins of well, moderately, and poorly differentiated tumors. These composite patterns were found to be characteristic of the three individual differentiation classes for colon tumors. The apparent relative molecular masses (Mr) of the resolved proteins ranged from 14,000 to 105,000 and their isoelectric points from pH 5.2 to 8.4. Although the vast majority of the proteins identified in the composite patterns were common, a comparison based upon these patterns revealed two qualitative and seven quantitative protein differences. The quantitative differences were identified by apparent Mr × 10^3/pI coordinates of 73/7.2 and 66/6.2. Quantitative differences were identified by Mr × 10^3/pI coordinates of 71/6.0, 59/6.7, 57/6.7, 56/5.4, 52/6.1, 30/5.8, and 18/6.2. These cytosolic differentiation marker proteins may facilitate the diagnosis, staging, and monitoring of human colon cancer.

Early detection of colorectal cancer is limited by the small number of useful tumor "markers" that are available (1) and the difficulty associated with detection of such cancer when few tumor cells (or their products) are present. The proliferative and metastatic potential of colorectal cancer cells (2–8) underscores the great need for more-accurate diagnostic and monitoring probes. The most clinically investigated tumor-associated antigen in cancer of the colon is CEA (9,10). Although CEA-based tests are now widely used in the diagnosis and evaluation of gastrointestinal and urogenital cancers, tests based upon the detection of CEA have not proven to be adequately sensitive, reliable, or specific to warrant their use for the screening of possible malignancy in an asymptomatic patient population (10,11).

In a comparison of moderately to well differentiated invasive colon adenocarcinomas by Tracy et al. (12), five major protein spots were detected in the tumor samples that were not detectable in normal samples. Tumor-associated antigenic markers have been detected in the cell cytoplasm and in the nuclei of mouse- and rat-tumor cell lines (the 70,000-Da NAg-2), as well as in specimens of human solid adenocarcinoma (13–15). In addition, the cytoplasm may contain proteins involved in signal transduction that account for regulatory differences between normal and neoplastic colon cells (16–18). Differences in the levels of expression of signaling proteins may serve as intracellular markers for cellular transformation and may account for the maintenance of transformed-cell growth rates and phenotypes (19). We have previously shown that cytosolic proteins and antigens of human colon-tumor cell lines can be correlated with growth rate and differentiation (20). Therefore, to determine whether cytosolic protein changes could be detected between stages of differentiation in human primary colon tumor specimens, we used two-dimensional polyacrylamide gel electrophoresis and silver staining. To verify that these proteins are products of colon cells and not derived from infiltrating neutrophils or adjacent cells of stromal origin, differentiation marker proteins have been identified in a series of human colon-tumor cell lines that have been highly characterized with regard to their biological characteristics (20–22).

Materials and Methods

Preparation of Clinical Specimens

A total of 16 clinical specimens ranging in pathological classification from poorly to well-differentiated adenocarcinoma of the colon were obtained from the University of Alabama Tissue Procurement Program in Birmingham, AL. Approval for the acquisition and study of these materials was granted by the Baylor College of Medicine Institutional Board for Human Research. Histological classifications of well, moderately, or poorly differentiated were provided by the Procurement Program. In addition, two normal specimens of human colon mucosa were analyzed. A series of nine human colon-tumor cell lines were kindly provided by Dr. Michael G. Brattain of the Bristol-Baylor Laboratory at Baylor College of Medicine. These cell lines have been characterized with regard to their rates of growth, extent of differentiation, mucin production, production of colonies in soft agarose, and tumorogenicity in athymic nude mice (20–23). Their expression of cytosolic proteins and antigens has been previously shown to correlate with their phenotypic characteristics (20).

Specimens of colon tumors and normal colon mucosa were individually minced and suspended in 50 mmol/L tris(hydroxymethyl)methylamine:25 mmol/L KCl:5 mmol/L MgCl₂ buffer, pH 7.4 (TKM buffer), containing, per liter, 1.0 mmol of phenylmethylsulfonyl fluoride and 0.1 mmol of leupeptin at 4 °C. Cell membranes were disrupted by homogenization in a Tissumizer® (Tekmar, Cincinnati, OH) for a total of 60 s. The cytosolic supernatant was collected after centrifugation at 25,000 × g for 20 min, followed by high-speed centrifugation at 95,000 × g for 4 h to remove nuclei, mitochondria, membranes, and endoplasmic reticulum. The clear cytosolic supernatant fraction was precipitated by the addition of four volumes of ethanol at −20 °C. After 16 h the precipitate was collected by centrifugation, redissolved in O'Farrell's lysing buffer (24), and adjusted to a final concentration of 5.0 mg of protein per milliliter.

Human colon-tumor cell lines were detached from their culture flasks by scraping, collected by centrifugation at 500 × g for 10 min, and washed three times by resuspension in phosphate-buffered saline containing 1.0 mmol of phenylmethylsulfonyl fluoride and 0.1 mmol of leupeptin per liter.

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followed by centrifugation (500 × g, 10 min). Cells were suspended in 10 volumes of TKM buffer containing 1.0 mmol of phenylmethylsulfonyl fluoride and 0.1 mmol of leupeptin per liter. A cytosol fraction was prepared as described above.

Two-Dimensional Gel Electrophoresis and Silver Staining

Two-dimensional gel electrophoresis was a modification of the method of O’Farrell et al. (24). The 100 × 2 mm cylindrical isoelectric focusing gels were cast in 200-μL disposable glass micropipets. Cytosolic proteins, 100 μl in lysis buffer, were underlayered beneath a fourfold dilution of the lysis buffer. Before isoelectric focusing 2 μL of a 10 g/L solution of crystal violet was placed at the anode end of each gel. Proteins were separated according to their isoelectric point by NEPHGE (24, 25) for 3200 V · h (16 h at 200 V). Gels were extruded from the tubes and equilibrated for 15 min (24) before transfer to preformed 100 g/L acrylamide/SDS slab gels (1.5 × 100 × 140 mm). The second dimension of electrophoresis was run in SDS-containing buffer according to the procedure of Laemmli (26). Electrophoresis was performed at 20 mA per gel until the bromphenol blue dye front came within 0.5 cm of the bottom of the gel. Gels were fixed in methanol/water (1/1 by vol) for 16 h. We used the procedure of Wray et al. (27) for silver staining of the gels.

Grid Development and Alignment

A modification of the grid system used by Narayan et al. (28) and Comings (29) was necessary to accommodate the protein patterns typical of extracts of colon-tumor cytosols. Four major polyproteins standards of known molecular mass (Mr) and isoelectric point (rabbit muscle actin, 43 000/5.6; bovine serum albumin, 67 000/6.0; horse skeletal muscle myoglobin, 17 900/6.8, 7.3; and sperm-whale myoglobin, 17 500/7.7, 8.1) were co-electrophoresed as internal standards. Bio-Rad Laboratories’ low-molecular-mass standards (phosphorylase b, 92 500; bovine serum albumin, 66 200; ovalbumin, 45 000; carbonic anhydrase, 31 000; soybean trypsin inhibitor, 21 500; and lysozyme, 14 000) were co-electrophoresed in the second dimension. Division of the photographed gel into 16 quadrants and apparent molecular mass × 10−3/isolectric point assignments were based upon the migration of the internal standards as determined by a least squares fit of the relationship between their log Mr and distance of migration. In addition, a constellation of 50 common human colon-tumor proteins was identified on or near the designated grid lines in all gels examined. We adjusted minor variations in the isoelectric focusing or second-dimension electrophoresis for each gel by aligning the grid lines with proteins of the constellation.

Results

Figure 1 shows representative silver-stained isoelectric focusing/SDS two-dimensional polyacrylamide gels for each of the three stages of differentiation of human colon cancer examined. The pH gradient of the first-dimension gel was linear from 5.2 to 8.4. The linear Mr range in the second-dimension SDS-containing gel was from 14 000 to 105 000 Da. Duplicate patterns obtained for each human colon-tumor sample revealed a high degree of consistency in pattern complexity and silver staining. Spots that were observed in each differentiation class at least four out of eight times were counted. An analysis of complexity (number of protein spots) revealed the following order for specimens: well > moderately > poorly differentiated tumors. Those spots that appeared in four out of eight patterns of each differentiation class and that exhibited changes between differentiation classes (well to moderate, or moderate to poor) were designated "composite spots." Figure 2 shows the computer illustration of the composite patterns for each differentiation class.

The composite patterns were used as an aid in the selection of qualitative and quantitative differences. Critical analyses of the photographs ruled out apparent quantitative and qualitative changes attributable to run-to-run variation or minor shifts in migration. Density changes of less than two units on an arbitrary scale of one to four were excluded from consideration as quantitative changes, to remove the influence of minor variations in stain density between experiments. On the basis of these guidelines, we selected two qualitative and seven quantitative differences.

Well-differentiated tumors expressed a protein with an Mr/pI value of 73/7.2, whereas the other differentiation classes did not. Five of eight moderately differentiated tumors expressed a protein with an Mr/pI value of 66/6.2, which was not detected in the other differentiation classes. These qualitative changes, indicated on the figures with black arrows, are summarized in Table 1.

The quantitative changes observed between differentiation classes of specimens of human primary colon tumor are also indicated on the figures. Table 2 summarizes the quantitative differences. A protein with an Mr/pI value of 71/6.0 was expressed in 11 of 16 moderately and well-differentiated tumors. The three proteins with an average Mr/pI value of 56/5.4, 30/5.8, and 18/6.2 were expressed at high density in all well-differentiated specimens, expressed with less intensity in moderately differentiated tumors, and were not observed in poorly differentiated colon tumors. Similarly, the silver staining intensity of the protein doublet with an Mr/pI value of 59/6.7 decreased in going from well to poorly differentiated tumors. A protein with an Mr/pI value of 52/6.1 appeared most dense in the moderately differentiated tumors, less intense in the poorly differentiated tumors, and the least dense in the well-differentiated tumors. The doublet with an Mr/pI value of 57/6.7 exhibited the most complex quantitative changes. Well-differentiated tumors expressed a very faint staining pattern, poorly differentiated specimens exhibited a more intense staining of the basic component, and the greatest expression for both components was observed in moderately differentiated colon tumors.

Discussion

Changes in cytosolic protein constituents expressed by colon tumors may occur as a result of reversion to the expression of earlier developmental types of cellular products, the expression of silent genes in a more ectopic manner, the arrest of immature cells in an early stage of their normal differentiation, or unusual cell-cycle populations present in actively proliferating groups of cells (30–35). Of 475 proteins mapped in human colon-tumor specimens, 50 common cytosolic proteins were identified in the majority of the two-dimensional silver-stained patterns we examined. These proteins are common, basal constituents of the class-specific composite patterns that were developed (Figure 2) and were used as internal markers to aid in the assignments of apparent isoelectric point and molecular mass. The 16-quadrant grid system, utilizing Mr/pI values provided by known standards, was superimposed upon each gel and aligned with the constellation of composite colon-protein spots.

Seven quantitative and two qualitative changes were
observed. These distinctions were identified in at least four out of eight sample patterns for each differentiation category. Although further identification of these differentiation markers must await further biochemical and immunological characterization, we compared molecular masses and pIs for those changes observed in this study with those reported in the literature in an effort to provide potential identifications.

Those differentiation marker proteins that exhibit some similarity to proteins already described in the literature include the quantitative changes observed at $M_r$/pI values of 52/6.1 and 30/5.8. Although different in molecular mass and pI values from the values of 42,000 and 5.3-5.5 reported for cellular and skeletal-muscle actins (36), this system a cytosolic protein with an $M_r$/pI of 52/6.1 underwent a quantitative change and migrated in close proximity to internal-standard actin. Transformed cells have been shown to express aberrant actin species and cytoskeletal proteins (37–40).

A protein with an $M_r$/pI of 66/6.2 was observed as a qualitative difference in five of eight moderately differentiated tumors. Correlations between proteins with an apparent molecular mass of 60,000 and protein kinase C substrates (the beta subunit of Initiation Factor 2 and a 62,000-Da/pI 6.2–6.4 substrate in EL4 mouse thymoma cells) as well as transformed cell characteristics have been reported by several investigators (41–43). Previous reports for proteins with similar molecular masses have included a cancer-associated factor derived from plasma membrane (44), an oncofetal protein derived from plasma of tumor-bearing rats (45), and a glycoprotein secretory component with immunocytochemical properties characteristic of the grade and stage of large-bowel cancer (31).

The presence of a protein with an $M_r$/pI value of 73/7.2 in well-differentiated tumors, but absent in moderately and poorly differentiated tumors, provides a potentially valuable marker for the differentiated phenotype. The quantitative change observed at an $M_r$/pI value of 30/5.8 may correspond to the 26,500-Da subunit of the anionic form of glutathione S-transferase identified in human placenta (46). The amount of glutathione S-transferase has been shown to increase with the development of resistance to mitomycin C in colon-tumor cell lines (47).

Thus, a series of potentially valuable differentiation marker proteins has been described here for the analysis of colon adenocarcinoma specimens. The development of immunological and cDNA probes based upon these protein...
Fig. 2. Composite drawings showing the protein constituents consistently observed in % of patterns representing each differentiation class. See the legend to Figure 1 for the conditions of electrophoresis.

Table 1. Qualitative Differences in Colon Protein Spots between Two-Dimensional Gel Patterns of Clinical Specimens from Differently Differentiated Tumors

<table>
<thead>
<tr>
<th>$M_x \times 10^{-3}$</th>
<th>pI</th>
<th>Quadrant</th>
<th>Diff. class</th>
<th>Prevalence</th>
</tr>
</thead>
<tbody>
<tr>
<td>73</td>
<td>7.2</td>
<td>C</td>
<td>Well</td>
<td>4/8</td>
</tr>
<tr>
<td>66</td>
<td>6.2</td>
<td>F</td>
<td>Moderate</td>
<td>5/8</td>
</tr>
</tbody>
</table>

Table 2. Quantitative Changes in Colon Protein Spots between Two-Dimensional Gel Patterns of Clinical Specimens from Differently Differentiated Tumors

<table>
<thead>
<tr>
<th>$M_x \times 10^{-3}$</th>
<th>pI</th>
<th>Quadrant</th>
<th>Differentiation rank</th>
<th>Prevalence</th>
</tr>
</thead>
<tbody>
<tr>
<td>71</td>
<td>6.0</td>
<td>B</td>
<td>Mod. &gt; Well</td>
<td>6/8</td>
</tr>
<tr>
<td>59</td>
<td>6.7</td>
<td>F</td>
<td>Well &gt; Mod.</td>
<td>7/8</td>
</tr>
<tr>
<td>57</td>
<td>6.7</td>
<td>F</td>
<td>Mod. &gt; Poor &gt; Well</td>
<td>7/8</td>
</tr>
<tr>
<td>56</td>
<td>5.4</td>
<td>E</td>
<td>Well &gt; Mod.</td>
<td>8/8</td>
</tr>
<tr>
<td>52</td>
<td>6.1</td>
<td>F</td>
<td>Mod. &gt; Poor &gt; Well</td>
<td>7/8</td>
</tr>
<tr>
<td>30</td>
<td>5.8</td>
<td>J</td>
<td>Mod. &gt; Well</td>
<td>6/8</td>
</tr>
<tr>
<td>18</td>
<td>6.2</td>
<td>N</td>
<td>Well &gt; Mod.</td>
<td>5/8</td>
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</table>

distinctions should be useful in sensitive, quantitative assays to aid in the classification of clinical colon tumor specimens and for the analysis of regulatory mechanisms that may be specifically related to individual differentiation phenotypes. Of particular interest are the opportunities to correlate the presence or absence of these molecules with the development of metastases (66/6.2) or with resistance to a specific drug (30/5.8). The value of these markers for these purposes must await the development of immunological and cDNA probes and their application in sensitive assays to a larger sample population.

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References