Altered protein expression at early-stage rat hepatic neoplasia

Qiute Luo,1,3 Linda Siconolfi-Baez,2,3 Pallavi Annamaneni,4 Mark T. Bielawski,4 Phyllis M. Novikoff,4 and Ruth Hogue Angeletti1,2,3

Departments of 1Developmental and Molecular Biology and 2Biochemistry, 3Laboratory for Macromolecular Analysis, and 4Department of Pathology, Albert Einstein College of Medicine, Bronx, New York

Submitted 12 October 2006; accepted in final form 25 January 2007

HEPATOCELLULAR CARCINOMA (HCC) is one of most frequent causes of death by cancer worldwide, with no reliable diagnosis prior to late stages of disease and no cure except surgery (13, 34, 47, 56, 66). The development of HCC is invariably associated with liver damage resulting from chronic hepatitis, extensive alcohol intake, or toxins, sequentially leading to liver cirrhosis, dysplastic lesions, and finally invasive liver carcinoma (13). However, the mechanism of HCC initiation and progression and how specific lesions interact to produce its aggressive characteristic remain poorly understood. Thus there is some urgency to deepen understanding of the process of neoplasia and to elucidate biomarkers for early diagnosis as well as to identify potential targets for early therapeutic intervention. Proteomics approaches are now being employed in a variety of models to achieve these goals (18, 35, 40, 60–62, 78–80).

Rodent models of human cancer provide powerful tools to investigate cancer molecular biology and therapy (14, 18). Here, we used the highly reproducible rat liver cancer model, the resistant hepatocyte (RH) model, to study cancer development in a multistage process, with the successive appearance of distinctive cell populations (3, 14, 15). The RH model shows synchronous appearance of unique cell populations in well-defined stages that progress to increasing malignancy (14, 16, 17). Each cell type has a characteristic phenotype that can be analyzed by a variety of genetic, biochemical, and cytological techniques. Metastasis of the liver cancer to lung and other tissues has also been observed in this model. We initiated proteomic studies of liver containing preneoplastic nodules, which represent the earliest preneoplastic lesion visible on gross examination of the liver without prior immunohistochemical or histochemical staining. After an additional 6- to 8-mo period, a subset of preneoplastic nodules develops into larger neoplastic persistent nodules and then into hepatomas. Those nodules that do not progress into hepatomas redifferentiate into hepatocytes morphologically indistinguishable from normal hepatocytes (12).

A great deal of pioneering work has been done in proteomic analysis of hepatocellular carcinoma (HCC) (9, 35, 37–39, 41, 43, 55, 61, 79, 80). However, most work has focused on late-stage HCC (18) rather than early-stage HCC. To reduce the mortality due to liver tumor and metastasis, it is important to develop methods for early diagnosis and prognosis of HCC. The application of the RH resistant model will possibly contribute to identifying biomarkers for detection of liver cancer at the early stage.

The goal of the present study was to determine the feasibility of the RH model for providing insights into the development of protein expression differences that are correlated with specific stages of carcinogenesis and/or specific cell types. In this report, we show that differences in protein expression can be identified in the RH model at the preneoplastic nodule stage in the neoplastic process that reflect proteins associated with functions related to tumor development as well as to the extensive reorganization of the cytoskeleton known to occur during carcinogenesis.

MATERIALS AND METHODS

Materials. Immobilized pH gradient (IPG) buffer and IPG strips of pH 3–10, 4–5, 5–6, and 6–9 were obtained from Amersham Pharmacia Biotech (Uppsala, Sweden). The 12% SDS-PAGE gel was one of three options. The options are 4% SDS-PAGE, 10% SDS-PAGE, and 12% SDS-PAGE.
RH model. The RH model of liver cancer was reproduced as previously described (12, 14, 67). Figure 1A outlines the protocol to induce preneoplastic nodules and hepatomas. The model was reproduced in male Fischer 344 rats (Charles River Laboratories, Wilmington, MA) as follows: initiating carcinogen, DEN, at a dose of 200 mg/kg was injected intraperitoneally into rats; 2 wk after the injection, carcinogen, acetyaminofluorene was administered in a time-release pellet designed to release a total of 35 mg for 2 wk (Innovative Research of America, Miami, FL) that was inserted under the skin of the rat’s neck; 1 wk after insertion of the pellet, two-thirds surgical partial hepatectomy (PH) was performed. Nine days after PH (i.e., 5 wk and 2 days after initiation), rats were killed and liver tissue was removed for proteomic and morphological analysis. At this time point, multiple preneoplastic hyperplastic nodules are present throughout all the lobes of the liver. They are easily distinguishable from the surrounding normal liver because of their white color in contrast to the brown color of the normal liver, their spherical shape, and their essentially uniform size (~1 μm in diameter). Figure 1B illustrates pyronin-positive spherical preneoplastic nodules. Distinct multiple nodules in a section of the liver are evident especially when methyl-green pyronin (MGP) (48) staining is employed to highlight the nodules. Different profiles of nodule size are evident in the section because the nodules are sectioned at different levels. For protein extraction analysis, sections serial to this section were used for proteomic analysis. We estimate that about 70% of the hepatic cells in a section analyzed for proteins are derived from nodular cells. The nodules also express the glutathione S-transferase (GST)-P1, a human hepatoma marker (59, 70) (Fig. 1C). The rat nodules are histologically similar to early dysplastic lesions that appear in humans before HCC (36). Bile ductule/oval cells, which also proliferate in this model, are evident (Fig. 1B).

Livers from eight male Fischer rats of the same age consisting of two different groups were used for this study. One experimental group of four rats was used for developing the RH model of liver cancer to stage 4 preneoplasia, and the second group consisted of untreated sham-operated control rats. All were killed on the same day. The RH protocol produces successive stages of liver cancer progression at predictable times as defined in the literature (12, 14, 67). If any step is omitted, the sequential process of cellular changes does not occur (49, 51, 53, 67). All animal procedures were approved by the Institutional Animal Care and Use Committee of the Institute for Animal Studies at the Albert Einstein College of Medicine.

Liver samples from RH model and control sham rat livers. The right lobe of liver was removed from RH rats 9 days after PH, when early-stage nodules appear. The nodule-enriched livers were quickly frozen in liquid nitrogen for proteomic analysis (14, 67). For histological and immunocytochemical analysis, liver tissue was either frozen in cold methyl-butane surrounded by dry ice or immersed in aldehyde fixatives (49, 51, 53). All liver samples were obtained at 9 days after PH (i.e., 5 wk and 2 days after DEN initiation) and were designated RH stage 4 nodules according to a staging naming system defined in our laboratory and based on the published literature (14, 17, 67). Liver samples from the same lobes were analyzed from four RH-model and four control rats.

Protein extract samples of controls and RH liver. The frozen liver tissue was cut into thin slices (30-μm thickness and ~20-mm diameter) under ~2°C using Cryostat (Belair Instrument, Fanwood, NY). The slices were suspended in sample buffer containing protease inhibitor cocktail (Sigma), 7 M urea, 2 M thiourea, 4% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate, silver nitrate, formaldehyde, and α-cyano-4-hydroxycinnamic acid were from Sigma (St. Louis, MO). Sequencing grade-modified trypsin was from Promega (Madison, WI). The packed reversed-phase column (15 cm × 0.2 mm ID) was from Micro-Tech Scientific (Sunnyvale, CA). Distilled water was deionized (18 MΩ) by use of the Milli-Q system from Millipore (Bedford, MA). Other reagents were from Fisher Scientific (Pittsburgh, PA).

Fig. 1. A: timeline of carcinogen protocol for induction of preneoplastic nodules and hepatomas in a resistant hepatocyte (RH) rat model. Five weeks after initiation of protocol, preneoplastic nodules develop which progress into metastatic hepatomas by 9 mo. This study investigates the liver at stage 4, i.e., liver with multiple preneoplastic hyperplastic nodules. DEN, diethylnitrosamine, initiating carcinogen; AAF, aminoacetylfluorene, a mitosis-inhibiting carcinogen; PH, partial hepatectomy. B: methyl-green pyronin. Multiple preneoplastic nodules are present in the liver and show strong pyronin staining (pink) in contrast to the surrounding hepatocytes. Oval/bile ductule proliferation is also evident (blue stain). C: immunolocalization of glutathione S-transferase (GST)-P1. A preneoplastic nodule is positive for GST-P1; no GST-P1 is present in hepatocytes.
the same buffer for another 15 min. The second-dimension electro-
phoresis was carried out until the bromophenol blue dye marker
reaches the bottom of the gel. Silver stain method was modified from
Ref. 65. The procedures are the same as described previously (45).

Image and statistical analyses. The gels were scanned as 16-bit
gray TIFF images with a ScanMaker III (Microtek Lab, Ontario, CA).
The image analysis expression data generation was performed by
applying the software package Investigator HT Analyzer Version 2.1
(Genomic Solutions) according to the manufacturer’s instructions.
Average volumes of the differentially expressed protein spots were
calculated after normalization using data of three gels obtained by
densitometric analysis. The maximal level of the protein spot volume
was defined as 100%. In the test of significance, the data were
subjected to stringent statistical analysis, and the t-test (19) was
applied to select the significant differences of spots. Statistical signif-
icance (P < 0.05) between nodule and normal (n = 3–5) was
determined by the software.

Protein identification. Protein spots were excised from the gel
before the destaining procedures (24) were carried out. The whole
protocol of protein in-gel digestion (11) was performed before
(45) except that two steps, protein reduction and alkylation, were
excluded in this experiment. The matrix-assisted laser desorption
ionization (MALDI) mass spectrometric analysis of supernatants from
in-gel digestion, and database searches were performed as before (45).
Some identifications were checked with mass spectrometry (MS)/MS
to screen the case, more than one protein in one spot. A reversed-
phase capillary HPLC column (15 cm × 320 μm ID) was attached to
the Elexis gradient system (Elexis Laboratories, Napa, CA). A 50-μl
sample loop was applied to load supernatant from an in-gel tryptic
digest of protein spot into the capillary column. The column was then
coupled to a LCQ MS (Finnigan Mat, San Jose, CA). A gradient for
column elution was developed over 40 min at a flow rate 5 μl/min.
The eluting peptides were ionized by electrospray ionization. The
specific peptide ions were automatically selected and fragmented by a
LCQ mass spectrometer (10). The mass spectrometer was set to
switch between the MS mode and the MS/MS mode. The selected
peptides were fragmented to generate a MS/MS spectrum, which contains
the sequence information for a single peptide and was compared by the
computer program Sequest (11) to predict spectra from sequence
database. This leads to the identification of the peptide corresponding
to the protein in the spot. Unambiguous protein identification was
attained in a single analysis by the detection of multiple peptides
derived from the protein. The MS/MS together with peptide mass
fingerprinting (PMF) forms an orthogonal check for the identification.

Western blot analysis. Protein sample preparation and electro-
phoresis were carried out as described before (71) transfer onto a
polyvinylidene fluoride membrane in 25 mM Tris and 190 mM
glycine containing 5% methanol at 4°C for 2 h at 160 mA (71). The rabbit polyclonal antibody against rat kallikrein-binding protein
(KBP), a serpin, was developed in the laboratory of Dr. Julie Chao
(Medical University of South Carolina, Charleston, SC) (6). The antigen-antibody complexes were visualized using the Western Light-
ning as recommended by the manufacturer PerkinElmer Life Sciences
(Boston, MA) and recorded with Hyperfilm (Amersham Biosciences,
Piscataway, NJ).

Microscopy of RH and control livers. For histology and for immu-
nolocalization studies, sections were prepared from the same RH and
control livers used for the proteomic studies. For histology, the
sections of RH and control livers were stained with MGP after fixation
in a mixture of paraformaldehyde and glutaraldehyde (51). MGP
staining was used instead of hematoxylin and eosin staining because
nodule cells contain higher levels of cytoplasmic ribosomes compared
with surrounding hepatic cells. Picrosin, a specific stain for RNA,
dramatically distinguishes all nodule cells in the section, including
those smaller foci of nodule cells sectioned at the periphery of the
spherical nodules. Moreover, hematoxylin and eosin staining of nod-
ules has previously been published (see Fig. 6 in Ref. 69). For
immunolocalization studies, the following antibodies were localized
in liver sections from RH and control rats according to previously
published methods (50–53): 1) von Willebrand factor (vWF) (Santa
Cruz Biotechnology, Santa Cruz, CA), a blood vessel marker (44); 2)
KBP (kindly provided by Dr. Chao), a serpin; 3) mannosidase II
(Babco, Berkeley, CA), a Golgi apparatus marker (52); and 4) GST-Pi
(StressGen, Victoria, Canada), a human hepatoma marker (59, 70).
Either aldehyde-fixed cryostat sections or nonfrozen aldehyde-fixed
sections (10 μm sections) were exposed first to a diluted primary
antibody (vWF, 1:50; KBP serpin, 1:100; mannosidase II, 1:200,
followed by fluorescently labeled second antibody (donkey anti-rabbit
or anti-mouse IgG-Cy 3, 1:200, Jackson Immunoresearch Laborato-
ries, West Grove, PA). Immunostained sections were also stained with
phalloidin-Alexa 488 to show actin filaments (Molecular Probes,
Eugene, OR). Coimmunolocalization of KBP serpin and mannosidase
II was also performed on RH and control liver sections by using a
mixture of second antibodies (Cy-3-labeled donkey anti-rabbit and
Cy-5 labeled donkey anti-mouse IgG for kallistatin and mannosidase
II, respectively) (Jackson Immunoresearch Laboratories). For local-
ization of GST-Pi, the second antibody was donkey anti-rabbit labeled
with horseradish peroxidase, visualized by incubation in diaminoben-
zidine substrate (52). Controls were performed at the same time
and consisted of sections not exposed to the primary antibodies, but
with all other procedures the same. Images of fluorescent-labeled sections
were examined with an inverted Nikon fluorescent microscope using a
×60 oil immersion lens with numerical aperture 1.4 Planapo objective
and a confocal microscope attached to a laser imaging system (Bio-Rad Laboratories, Hercules, CA) equipped with a krypt-
argon laser. Black level, gain and laser intensity. Kalman averaging,
excitation intensity, pinhole aperture, and Z-series analysis of
sections were performed as described elsewhere (50, 51).

RESULTS

2-DE maps for liver tissue proteome. Two-dimensional
electrophoresis (2-DE) maps were constructed for the proteomes of normal and stage 4 nodule rat liver tissues, a
prerequisite for subsequent comparative proteomic studies.
The 2-DE maps for total extracts of sham and stage 4 nodule
rat liver tissues are shown in Fig. 2, representing a compre-
prehensive view of the major proteins differentially expressed in
normal and stage 4 nodule rat liver tissue. However, broad-
range IPGs do not provide the resolving power needed for
separating proteomes from complex organisms like eukaryotes
(76). The challenge of analyzing a complicated proteome is
better addressed by the use of very narrow-range IPG gels for
better resolution and efficient separation. In this research, not
only broad pH 3–10 IPG strips were used, but also narrow-
range pH 4–5, pH 5–6, and pH 6–9 IPG strips, since more
protein sample (at least 5 times higher than broad IPG) can be
loaded into narrow pH strips, which can significantly increase
the identification of proteins with low copy numbers in cell
culture tissue. Fig. 3 clearly shows a typical example. Car-
boxylesterase 2 could not be discriminated in the broad pH IPG
strip (left). However, in the narrow-range IPG strip, it is easy
to locate, identify, and confirm.

Protein expression differences identified from 2D gel spots.
PMF is a highly sensitive method for the identification of proteins from acryl amide gels (29). Table 1 lists proteins
identified using PMF and LC-MS/MS. All listed proteins meet the
current stringent criteria for positive identifications (45,
81). The proteins in the table are identified as upregulated or
downregulated from sham-operated control to nodule-enriched
neoplastic liver. Under the conditions used in these experi-

AJP-Gastrointest Liver Physiol. • VOL 292 • MAY 2007 • www.ajpgi.org
ments, three identified protein spots present in normal liver were undetectable in nodule-enriched liver. Conversely, eight identified protein spots were detected exclusively in 2D-PAGE of nodule-enriched liver. This was somewhat surprising in that the tissue used in these preliminary studies contain mixed populations of cells, e.g., hepatocytes, bile ducts, endothelial cells, and neoplastic nodular cells, among others. It is possible that the amounts of some of these proteins were below the limits of detection in the gels in which they were absent, or that their mobilities had been altered by a posttranslational processing event. However, in the case of vimentin, its expression solely in preneoplastic nodule-enriched liver, and not in normal liver, is indeed consistent with other studies of liver tumors vs. normal liver tissue (31, 59, 70).

Figure 4 shows the details of analysis of protein differences by 2D-PAGE and mass spectrometry. In this example, a number of lightly stained spots are detected in experimental tissue, but not controls. Two protein spots are greatly increased in nodule-enriched liver compared with control liver (Fig. 4A). These were identified by PMF to be cytokeratin 8, known to be altered in cancers (75), and actin-related protein 3 a protein name (Table 1). The MS/MS spectrum and SEQUEST analysis of one of the cytokeratin 8 peptides are also shown in Fig. 4B, exemplifying the analytical procedures used in these experi-

Fig. 2. Representative 2-DE maps of the control (left) tissue and preneoplastic nodules (right) of rat liver are demonstrated. For each gel, 130 μg of total proteins were subjected to 2-DE separation, and gels (12%T) were stained and exposed to silver stain as described in MATERIALS AND METHODS. 2D gel reproducible error is <20%. Labeling nos. 01, 05, 09, 11, and 18 correspond to nos. in Table 1.

Fig. 3. Representative example of zooming-in gel in improving protein separation resolution. A broad range of immobilized pH gradients (left) is contrast to a very narrow range (right) in which separation resolution is significantly enhanced.
ments. Other difference spots were also noted in this figure but were either mixed populations of polypeptides, below the level of detection, or otherwise outside the criteria for unambiguous identification.

Differences in expression of a serine protease inhibitor (serpin). A region of 2D-PAGE (pH 3–10) shows what appears to be extensive modification of possibly a single polypeptide in control untreated sample (Fig. 5 inset) that is not detected in experimental sample. Although each of the spots in the parallel tracks encircled was only lightly silver stained, in-gel digestion followed by PMF and bioinformatics searches demonstrated the sites and types of modifications will be carried out using LCQ analysis as well.

Western blot analysis. To confirm that the serpin polypeptide was indeed diminished in nodule-enriched rat liver and to determine whether all molecular forms, both unmodified and posttranslationally modified, were involved, Western blot analysis of the tissue was carried out (Fig. 6). Approximately two-thirds of serpin is present in the apparently posttranslationally modified form in control untreated rat tissue. The strongly immunoreactive spot in the center of the panel (Fig. 6, top, arrow) appears to be unmodified serpin. This polypeptide was not identified in the silver-stained 2D-PAGE experiments, most likely because the pattern in this region of the electrophoretic separations was very complex, even in narrow-range isoelectric point (pI) gels. All forms of serpin appear to be greatly diminished in nodule-enriched liver (bottom). Future studies of the sites and types of modifications will be carried out using immunoaffinity purified polypeptides.

Immunolocalization of serpin, mannosidase II and vWF in control and noduled livers. In control liver (Fig. 7A, inset), colocalization of serpin and mannosidase II revealed the following: 1) serpin staining in sinusoidal endothelial cells; 2) serpin staining in hepatocytes in a structure at the trans aspect of the Golgi apparatus and in the endoplasmic reticulum; and 3) mannosidase II in the Golgi apparatus. Serpin and mannosidase II did not colocalize in the same compartments of the Golgi apparatus. Serpin is found in a distinct Golgi-associated compartment situated at the trans aspect of the Golgi apparatus, and this structure does not have the Golgi marker, mannosidase II (58, 59, 70, 72).

Table 1. Comparison of proteins identified with altered expression in normal and stage 4 nodule rat liver tissue

<table>
<thead>
<tr>
<th>No.</th>
<th>Accession No.</th>
<th>Protein Identified</th>
<th>Spot Intensity (Nodule/Normal)</th>
<th>Molecular Mass, kDa (Experimental/Theoretical)</th>
<th>Experimental/ Theoretical, pI</th>
<th>Peptide Number (Matched/Measured)</th>
<th>Coverage, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>01*</td>
<td>gi: 39923720</td>
<td>Aflotoxin BI aldehyde reductase</td>
<td>36/37</td>
<td>7.08/6.8</td>
<td>27/15</td>
<td>32</td>
<td></td>
</tr>
<tr>
<td>02</td>
<td>gi: 4139951</td>
<td>Annexin V</td>
<td>33/36</td>
<td>4.94/9.4</td>
<td>22/12</td>
<td>69</td>
<td></td>
</tr>
<tr>
<td>03</td>
<td>gi: 19424284</td>
<td>Carboxylesterase 2</td>
<td>55/63</td>
<td>5.45/5.6</td>
<td>11/8</td>
<td>16</td>
<td></td>
</tr>
<tr>
<td>04</td>
<td>gi: 6680606</td>
<td>Cytochrome C</td>
<td>41/45</td>
<td>5.25/3.3</td>
<td>22/19</td>
<td>55</td>
<td></td>
</tr>
<tr>
<td>05*</td>
<td>gi: 121749</td>
<td>Glutathione S-transferase π 2</td>
<td>25/24</td>
<td>6.76/9.9</td>
<td>13/5</td>
<td>38</td>
<td></td>
</tr>
<tr>
<td>06</td>
<td>gi: 6677381</td>
<td>Retinoblastoma binding protein 4</td>
<td>50/52</td>
<td>4.74/9.9</td>
<td>43/8</td>
<td>35</td>
<td></td>
</tr>
<tr>
<td>07</td>
<td>gi: 23821785</td>
<td>UMP-CMP kinase</td>
<td>25/22</td>
<td>5.7/5.7</td>
<td>22/8</td>
<td>41</td>
<td></td>
</tr>
<tr>
<td>08</td>
<td>gi: 2078001</td>
<td>Vimentin</td>
<td>48/45</td>
<td>4.8/4.7</td>
<td>47/28</td>
<td>60</td>
<td></td>
</tr>
<tr>
<td>09*</td>
<td>gi: 17621520</td>
<td>KBP serpin</td>
<td>57/47</td>
<td>4.3/5.3</td>
<td>19/9</td>
<td>27</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>gi: 204261</td>
<td>α-2-Micro globulin precursor</td>
<td>21/21</td>
<td>4.7/5.4</td>
<td>44/13</td>
<td>59</td>
<td></td>
</tr>
<tr>
<td>11*</td>
<td>gi: 13929030</td>
<td>Sulphotransferase, phenol preferring</td>
<td>35/36</td>
<td>6.0/6.1</td>
<td>44/12</td>
<td>21</td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>gi: 1526539</td>
<td>14-3-3 zeta</td>
<td>1.5</td>
<td>29/28</td>
<td>4.6/4.7</td>
<td>55/19</td>
<td>62</td>
</tr>
<tr>
<td>13</td>
<td>gi: 62659235</td>
<td>Actin-related protein 3</td>
<td>2.6</td>
<td>46/48</td>
<td>5.6/5.6</td>
<td>31/16</td>
<td>48</td>
</tr>
<tr>
<td>14</td>
<td>gi: 309090</td>
<td>Actin beta</td>
<td>21.4</td>
<td>39/32</td>
<td>5.4/5.2</td>
<td>39/19</td>
<td>59</td>
</tr>
<tr>
<td>15</td>
<td>gi: 71766</td>
<td>Annexin III</td>
<td>2.8</td>
<td>36/37</td>
<td>5.6/6.0</td>
<td>22/12</td>
<td>37</td>
</tr>
<tr>
<td>16</td>
<td>gi: 13519897</td>
<td>Adenine phosphoribosyltransferase</td>
<td>1.7</td>
<td>24/20</td>
<td>5.7/6.2</td>
<td>15/9</td>
<td>53</td>
</tr>
<tr>
<td>17</td>
<td>gi: 2037342</td>
<td>Cytochrome C</td>
<td>5</td>
<td>53/53</td>
<td>5.6/5.5</td>
<td>31/21</td>
<td>48</td>
</tr>
<tr>
<td>18*</td>
<td>gi: 50814</td>
<td>Elongation initiating factor 1 γ</td>
<td>1.7</td>
<td>43/51</td>
<td>5.3/6.3</td>
<td>20/10</td>
<td>24</td>
</tr>
<tr>
<td>19</td>
<td>gi: 6981672</td>
<td>Tropomyosin</td>
<td>6.7</td>
<td>32/29</td>
<td>4.6/4.7</td>
<td>34/14</td>
<td>40</td>
</tr>
<tr>
<td>20</td>
<td>gi: 8393197</td>
<td>C-reactive protein</td>
<td>0.19</td>
<td>31/26</td>
<td>4.6/4.9</td>
<td>18/6</td>
<td>31</td>
</tr>
<tr>
<td>21</td>
<td>gi: 20837134</td>
<td>Golgi reassembly stacking protein</td>
<td>0.03</td>
<td>53/47</td>
<td>4.6/4.7</td>
<td>19/7</td>
<td>22</td>
</tr>
</tbody>
</table>

*Proteins for nos. 01, 05, 09, 11, and 18 were identified in broad pI range (3–10) (see Fig. 2). These values are from posttranslation modified serpin in normal liver tissue.
In noduled liver (Fig. 7B, and inset), colocalization of serpin and mannosidase II revealed the following: 1) serpin staining in cytoplasm of nodular cells diffusely distributed; 2) no serpin staining in either sinusoidal endothelial cells (although a few cells express low levels) or in endothelial cells within in the nodules; 3) no serpin in Golgi apparatus, in trans Golgi compartment, in ER of nodular cells and surrounding hepatocytes; and 4) mannosidase II in Golgi apparatus of nodular cells and surrounding hepatocytes. The Golgi apparatus of the nodule cells and surrounding hepatocytes are similar to that of normal hepatocytes in that they express mannosidase II activity; however, differences in Golgi morphology are evident (Fig. 7). Immunolocalization of vWF, a marker for capillaries and blood vessels, was also carried out to determine whether neoangiogenesis occurs at an early stage of liver cancer in which an angiogenesis inhibitor (i.e., serpin) is reduced or nonfunctional. Figure 7, C and D, shows vWF localization of in normal and nodule-enriched rat liver, respectively. In normal liver, vWF staining is confined to the portal vein and no branches of the vein extend into the liver parenchyma. However, in noduled liver, a prominent vWF-positive blood vessel network has formed. vWF-positive branches are evident emanating from the portal vein. The branches are evident at the periphery of the nodules as well as branches of the network extending into the nodule (Fig. 7D).

**DISCUSSION**

Identification of a protein in a proteomics experiment is only the first step in understanding the possible significance of the
discovery (1, 18). After literature searches, cross-validation of the protein’s presence is helpful, as is determination of cellular localization and investigation of biological relevance. This process is very time consuming, and extensive studies must often be carried out on each individual protein identified in the experiments. Two identified protein spots functioned as internal controls, confirming observations made previously by histochemical methods that show them to be greatly increased in preneoplastic nodule-enriched liver compared with control liver. Cytokeratin 8, known to be altered in cancers (75), is upregulated in preneoplastic nodule-enriched liver (Table 1). GST-Pi polypeptide is also present in preneoplastic nodule-enriched liver. This protein is also expressed in human hepatomas and frequently used as a marker for diagnosis of HCC (59, 70). High levels of this GST isoform are known to be localized in preneoplastic nodules (see Fig. 1C) as well as in hepatomas, with low levels in proliferating bile ductules in contrast to normal hepatocytes (49, 51, 53).

Many polypeptides related to the organization of the cytoskeleton and cellular architecture, metabolic state, and regulation were identified in this study. Changes in expression were observed in a large number of cytoskeleton-associated proteins and other polypeptides associated with cellular architecture. Of these, cytokeratins 8 and 19, for example, have been noted in other studies with other model systems (37, 53, 75). Shape changes known to accompany tumorigenesis reflect rearrangement in the cytoskeleton and its associated proteins (8). In preneoplastic nodules of the RH rat model, we have previously found a major change in the cell architecture and polarity of the nodular cells from the background hepatocytes reflected by the in situ localization of actin and by alterations in cell shape and arrangement (49, 51, 53). This change in architecture had been observed in earlier histology studies (8). The nodular cells are grouped together to form acini or glandular-like structures. This acinus arrangement is in contrast to the background hepatocytes, which are organized into linear cords of contiguous cells with one aspect (basal) of the hepatocytes facing the sinusoids and the other (apical) facing the bile canaliculus. In normal hepatocytes, actin is present underneath the entire plasma membrane with higher concentrations in the bile canaliculus. The apical-basal polarity and the hepatic cord arrangement characteristic of normal hepatocytes are not seen with nodular cells. This observation strongly supports the notion that components of the cytoskeleton have been altered after the carcinogen treatment. Beyond changes in cell shape.

Fig. 5. Identification of a serine protease inhibitor. A: matrix-assisted laser desorption ionization time of flight mass spectrum of trypsin-digested peptides in a serpin-containing spot. Top: 2D-PAGE of difference spots. All spots in the parallel tracks shown in the inset in the gel image at left (control) contain kallistatin. Right gel is from an experimental sample. The isoelectric points of these protein spots range from 4.2 to 5.5 (left to right), and the relative molecular weights from 50,000 to 68,000 Da (bottom edge to top edge). B: tandem mass spectrum of a tryptic peptide from the kallistatin-containing spots. The sequence is highlighted in the text below the spectrum.

Fig. 6. Western blot of 2-DE gels with polyclonal antibody against serpin. Top: normal rat liver tissue homogenate. Charge train of modified serpin and unmodified serpin (arrow) is revealed in gel. Bottom: homogenate of stage 4 liver. None to low levels of serpin are detected in gel.
and tissue morphology, rearrangements in the actin cytoskeleton have been associated with tumor cell migration (64).

Others of the proteins identified have complex functions. One of the 14-3-3 family proteins was increased in expression in nodule-enriched liver. These proteins can be involved with signaling, apoptosis, or interaction with integrins or transcription factors, and have been suggested to be a part of the cellular scaffold as well (57, 73, 74). Annexins constitute a family of proteins that exhibit Ca\(^{2+}\)-dependent binding to phospholipids and are implicated in multiple cellular interactions, such as membrane trafficking, transmembrane channel activity, inhibition of phospholipase A2, and mediation of cell-matrix interactions. The biological function of annexin V (lipocortin V) is not known, although its properties include inhibition of phospholipase A2, vascular anticoagulation, and binding of glycosaminoglycans (22). A proteomic study in normal liver did not find annexin V in parenchymal hepatocytes but did find the protein in an unidentified population of small hepatic cells (60, 61). The finding of annexin V in the liver with early nodules suggests that annexin V may be associated with populations of hepatic cells not usually present in normal liver and may indicate the beginning of the early pathology of liver cancer. Annexin V has been shown to play a role in early apoptosis. Both 14-3-3 and several annexins have been identified in proteomic studies of a human hepatocellular carcinoma cell line (43, 60, 61). Because of the complexity of their functions, orthogonal biological experiments need to be carried out to understand what precise role these and other proteins may play in development of cancer (1, 33).

Of the proteins that are present in normal liver, but are not detectable or are at very low levels of expression in nodule-enriched liver, the most striking is serpin, also known as kallistatin (6, 77). Serpin has been implicated as an inhibitor of angiogenesis and tumor growth. Angiogenesis plays an important role in tumor growth, invasion, and metastasis (2, 20, 25, 28). This process is not only a prerequisite for tumor growth
but is also a major influence on the metastatic spread of malignant cells (3). The polypeptides responsible for the parallel “charge-train” of spots in the 2D gels were identified both by the peptide mass mapping and MS/MS as serpins. This pattern had been identified previously in normal rat liver (21). However, this pattern was not detectable in multiple samples analyzed from liver enriched in nodules. This observation is consistent with proteomics studies of other model systems in which maspin, another serpin, was found to be present at lower levels in human hepatocellular carcinomas with respect to human liver cell line and in hepatoma cells treated with antiserum epidermal growth factor receptor sequence (78, 79). Maspin was not identified in the present experiments. Immunoblot analysis of both sample sets confirmed that both apparently modified and apparently unmodified serpin polypeptides are affected. Reduction of serpin expression is implicated in the onset of angiogenesis (4–6, 23, 30, 46, 54, 82). Immunolocalization studies of serpin paralleled the proteomic findings (i.e., presence of serpin in normal liver and low levels in nodule-enriched liver).

The observations of serpin expression differences by Western blotting and immunohistochemistry implies its important role in neoangiogenesis. Furthermore, serpin localization in the normal liver in contrast to the nodule-enriched liver, was present in a structure at the trans aspect of the Golgi apparatus and not in the mannosidase II-positive Golgi apparatus of normal hepatocytes or nodule cells. One function of the Golgi apparatus is the sequential addition of specific carbohydrate moieties (e.g., galactose, mannose, sialic acid) to amino acids of the proteins. Specific moieties are added in specific compartments of the Golgi apparatus (e.g., either in cis, medial, or trans). The nature of the serpin-positive structure needs to be more precisely defined to determine whether this structure is a part of the Golgi apparatus and therefore likely to function in glycoprotein processing or is a separate structure related spatially to the Golgi apparatus with another function (e.g., secretion). The absence of serpin in Golgi apparatus, trans Golgi compartment, and ER of nodule cells may indicate that major alterations in synthesis and processing of specific proteins and/or carbohydrates occur early in carcinogenesis. Nodule cells showed diffuse localization of serpin in the cytoplasm indicating that it may be a soluble protein. However, no serpin was evident in cytoplasmic membrane-bounded organelles (e.g., Golgi apparatus, trans Golgi structure, ER) that function in protein synthesis, glycosylation, and secretory processing. Whereas in hepatocytes of the normal liver, serpin localization is consistent with normal processing, the serpin localization in nodule cells and in hepatocytes from nodule-enriched liver indicates abnormal processing of the protein. Low levels of serpin may be present in the nodule-enriched liver but the protein may not be functioning because serpins are likely polymerized during inhibiting process, resulting in serpin aggregation (23). Our studies have detected differences in serpin levels and in posttranslational modifications between normal liver and nodule-enriched liver. The immunolocalization data is consistent with posttranslational modifications revealed by 2D-PAGE and supports the reduction of serpin, an angiogenesis inhibitor, in the noduled liver.

Proteomic analysis has identified one protein that is an inhibitor of angiogenesis, namely KBP serpin, that is detected in low levels in nodule tissue compared with normal liver by proteomics. Moreover, immunocytchemistry has validated the proteomic data and showed localization to specific hepatic cells and subcellular organelles. In addition, these studies provide possible mechanisms for the differential expression of serpin in normal liver and early stage of liver carcinogenesis. At an early stage of liver cancer, a decrease in angiogenesis inhibitors may shift the normal physiological balance of angiogenesis factors resulting in the proliferation of new blood vessels. Our studies provide direct evidence that neoangiogenesis occurs at an early stage of liver carcinogenesis well before hepatoma development (49). In an earlier study, measurement of blood flow in rat liver with early-stage nodules was investigated and found to be reduced compared with the surrounding liver as measured by infusion of microspheres; however, the study did not demonstrate neoangiogenesis in relation to either nodules or surrounding liver (68). Whether expression of other anti-angiogenesis proteins are downregulated in all cell types or only in specific cells will be studied in future experiments. The modifications that result in the multiplicity of protein spots will also be analyzed with immunoaffinity purified protein.

A major hindrance to understanding the process of carcinogenesis is the paucity of specific markers for new populations that appear in the initiation and promotion stages of the disease, and that may differ from those at advanced stages. The RH rat provides a model in which the progression to tumors passes through synchronous stages, each well characterized in morphology and immunohistochemistry. Early-stage neoplastic lesions as manifested by preneoplastic hyperplastic nodules were selected for these initial studies because nodules can be distinguished without staining or other treatment that would interfere with protein analysis. Furthermore, early dysplastic nodules in human HCC and preneoplastic nodules in rat liver that represent early-stage neoplasm have similar hyperplastic morphology (36). These features enable future detailed studies of the differences in protein expression using laser capture microdissection before proteomic analysis for nodules cells as well as other hepatic cells type (e.g., stromal cells, bile ductule/oval cells). Since changes in stromal cells as well as tumor cells are thought to be important, the ability to dissect and enhance these differences is essential. This is further underscored by some of the findings in the present study. High levels of GST-Pi are localized exclusively in preneoplastic nodule cells, and low levels of GST-Pi are found in proliferating bile ductules (51, 53). Cytokeratin 19 has been demonstrated by immunofluorescence microscopy to be localized only in bile ductule cells (53).

Protein expression patterns are not necessarily equivalent to mRNA expression patterns (7, 27). Although the present studies are a prelude to more intensive proteomics analysis, some comparison can be made to results from published microarray studies of hepatocellular carcinoma and carcinogen-induced hepatoma. The levels of vimentin mRNA were found to be diminished in two such studies (32, 42), corresponding to the reduction in vimentin polypeptide found in our experiments. Jeong and colleagues (32) also observed a downregulation in levels of 14-3-3 mRNA. However, this group noted a downregulation of expression of cytokeratin 19 mRNA. This is in contrast to what is observed in our present study and in other histological and proteomic studies. Addition of other proteomic technologies to the methods used in the present work will include laser capture microdissection of individual cell
types, multidimensional chromatographic separations to improve the resolution and dynamic range of detection, and quantitative mass spectrometry procedures. The studies will also be expanded to stages of the RH model that appear before and after the nodules, so that a larger set protein expression patterns will be generated to discriminate important functional changes in the development of liver cancer. In summary, our studies have shown the feasibility of investigating a liver carcinogenesis model with properties similar to human liver cancer progression by proteomics technology. The goal is to discover alterations in protein composition and levels and posttranslational modifications between normal and liver cancer stages with the possibility of finding liver cancer markers.

ACKNOWLEDGMENTS

The authors thank Dr. Julie Chao of the Medical University of South Carolina for the gift of serpin antiserum and Michael Cammer, Analytical Imaging Center, for expert assistance in confocal microscopy.

GRANTS

This study was supported by grants from the National Institutes of Health: CA-055011 (R. H. Angeletti), CA-06576 (P. M. Novikoff), and P30 KD-41296 (Marion Bessin Liver Center of the Albert Einstein College of Medicine).

REFERENCES


