A Cytokeratin-immunohistochemical Study of Hepatoblastoma

PETER VAN EYKEN, MD, RAF SCIOIT, MD, FRANCESCO CALLEA, PhD,
FRANS RAMAEKERS, PhD, G. SCHAART, AND VALEER J. DESMET, PhD

Six cases of hepatoblastoma (five epithelial, one mixed epithelial-
mesenchymal) were studied on serially cut cryostat sections, us-
ing a panel of monoclonal antibodies directed against individual
cytokeratins, vimentin, and desmin, in an indirect immunoper-
oxidase procedure. Embryonic and fetal-type tumor cells ex-
pressed the "hepatocellular" cytokeratins no. 8 and 18 but, sur-
prisingly, also expressed the "bile duct type" cytokeratin no. 19.
In addition, two cases had a number of tumor cells which were
also positive for the "bile duct type" cytokeratin no. 7. Cells
embedded in osteoid-like material were immunoreactive for vi-
mentin but also for cytokeratins no. 7, 18, and 19. Gel electroph-
oresis, and Western blotting of cytoskeletal extracts, con-
firmed the immunohistochemical data. The implications of these
findings for the histogenesis of hepatoblastoma are discussed in
this report. HUM PATHOL 21:302–308. © 1990 by W.B. Saun-
ders Company.

Hepatoblastoma is the most common hepatic tu-
mor of childhood with a well-characterized light
microscopic and ultrastructural appearance. The
epithelial component of hepatoblastoma can display
a spectrum of maturational changes, and the occasional
presence of bone, cartilage, or squamous epithelium
reflects the capacity of heterologous or divergent dif-
ferentiation of this tumor. Recently, Abenoza et al
have used immunohistochemistry in addition to elec-
tron microscopy to address some questions concern-
ing the histogenesis of hepatoblastoma. Based on
their findings, a scheme of cytodifferentiation was
proposed that partly explains the wide range of mor-
phologic components present in hepatoblastoma. Abenoza et al reported focal expression of a
54-kilodalton cytokeratin in embryonic and fetal-type tu-
mor cells in all 19 cases they studied, and positivity for
high molecular weight cytokeratin (65 to 67-
kilodalton) in areas of squamous differentiation.
Schmidt et al reported positive staining for cyto-
keratin (not otherwise specified) of neoplastic cells in
one case of hepatoblastoma. The exact pattern of cyto-
keratin expression of hepatoblastoma has not been
analyzed in detail.

Embryonic, fetal, and adult human hepatocytes
contain cytokeratin polypeptides no. 8 and 18 of the
catalog of Moll et al, whereas bile duct cells in adult
human liver express, in addition to cytokeratins no. 8
and 18, cytokeratins no. 7 and 19. It is generally
believed that the pattern of cytokeratin expression is
preserved during neoplastic transformation.

We applied a panel of monoclonal antibodies di-
rected against specific cytokeratin polypeptides to
cryostat sections of hepatoblastoma specimens to find
out (1) which cytokeratins are expressed by hepat-
oblastoma, (2) whether embryonic and fetal-type tu-
mor cells contain the same cytokeratins, and (3)
whether the pattern of cytokeratin expression of he-
patoblastoma corresponds to that of the "hepa-
tocellular" type.

In one case, gel electrophoresis and Western
blotting were performed to supplement and confirm
the immunohistochemical data.

MATERIALS AND METHODS

Two liver biopsy specimens (one surgical, one needle
biopsy) taken for diagnostic purposes, and four surgical
resection specimens from six children (three female, three
male) ranging in age from 1 month to 2 years were used for
this study. Four cases were from the Department of Pathol-
yogy, UZ Sint-Rafael, Leuven, Belgium; one case came from
the files of the Department of Pathology, University Hospi-
tal, Nijmegen, The Netherlands; and one case was col-
lected from the files of the Gianni Gaslini Institute, Genova,
Italy. All specimens were received fresh and a portion was
snap-frozen in liquid nitrogen-cooled isopentane. The re-
mainder was fixed in Bouin's fluid or formalin and rou-
tinely processed. Data on patients and specimens are sum-
marized in Table 1. The diagnosis of hepatoblastoma was
based on routine histologic examination using published
criteria. Cases two through six were diagnosed as ephe-
rial hepatoblastoma, case one was a mixed epithelial mes-
enchymal hepatoblastoma. The epithelial component of all
cases consisted predominantly of fetal-type tumor cells, but
a variable number of embryonic-type cells were always
present (Fig 1). "Small" or "anaplastic" cells were not
found. Case five contained some "macrotubular" areas,
as described by Gonzalez-Crussi et al. In the mixed ephe-
rial-mesenchymal hepatoblastoma, osteoid-like foci were
present. Areas of squamous differentiation, bone or car-
tilage were not found.

Immunohistochemistry was performed on serially cut
cryostat sections using a panel of monoclonal antibodies
directed against various cytokeratins, vimentin, and des-
min. The antibodies, their source and specificity are
listed in Table 2. In all cases, a three-step indirect immu-
noperoxidase procedure was used. The primary antibodies
were applied for 30 minutes at room temperature. Mono-

From the Department of Pathology, UZ Sint-Rafael, Katho-
lijeke Universiteit Leuven, Leuven, Belgium; the Depart-
ment of Pathology, Gianni Gaslini Institute, Genova, Italy; and the Depart-
ment of Pathology, University Hospital, Nijmegen, The Nether-
lands. Accepted for publication August 5, 1989.

Peter Van Eyken is a research assistant of the Belgian National
Fund for Scientific Research.

Key words: hepatoblastoma, cytokeratins, histogenesis, immu-
nohistochemistry.

Address correspondence and reprint requests to Peter Van
Eyken, MD, Laboratorium voor Histo-en Cytochemie, Universitair
Ziekenhuis Sint-Rafael, Minderbroedersstraat 12, 3000 Leuven,
Belgium.

© 1990 by W.B. Saunders Company.
0046-8177/90/2103-0008$6.00/0

302
TABLE 1. Hepatoblastoma Cases Examined in This Study

<table>
<thead>
<tr>
<th>Case No.</th>
<th>Age (mo)/Sex</th>
<th>Specimen</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>12/M</td>
<td>SR</td>
</tr>
<tr>
<td>2</td>
<td>1/F</td>
<td>SB</td>
</tr>
<tr>
<td>3</td>
<td>16/F</td>
<td>SR</td>
</tr>
<tr>
<td>4</td>
<td>14/M</td>
<td>NB</td>
</tr>
<tr>
<td>5</td>
<td>24/M</td>
<td>SR</td>
</tr>
<tr>
<td>6</td>
<td>14/F</td>
<td>SR</td>
</tr>
</tbody>
</table>

Abbreviations: SR, surgical resection; SB, surgical biopsy; NB, needle biopsy.

clonal antibodies RCK105, M20, RGE53, CK18.2, A55-B/A2. RCK102, RKSE60 and RV202 were used as undiluted culture supernatant; the remaining antibodies were diluted 1:10. Incubation with the primary antibodies was followed by peroxidase-conjugated rabbit anti-mouse antiserum, and finally by peroxidase-conjugated swine anti-rabbit immunoglobulin. Controls, which were invariably negative, consisted of omission of the primary antiserum. In case one, sufficient material was available for additional biochemical analysis. SDS-polyacrylamide gel electrophoresis and immunoblotting of Triton X-100-extracted cytoskeleton preparations were done essentially as described by Ramaekers et al. and Broers et al.

RESULTS

The immunohistochemical staining results are summarized in Table 3. In non-neoplastic liver tissue (when included in the specimen), hepatocytes were positive for cytokeratins no. 8 and 18, whereas bile ducts and ductules expressed cytokeratin polypeptides no. 7, 8, 18, and 19.

Epithelial tumor cells, in all cases, stained positively for monoclonal antibodies anticytokeratin 8 and M20, both specific for cytokeratin no. 8 (Fig 2). The latter antibody usually produced a more intense staining. Fetal-type tumor cells with a granular cytoplasm were more strongly positive than “pale” fetal-type cells. In some areas, embryonic-type tumor cells were focally reactive, and displayed a paranuclear cytoplasmic positivity.

Both fetal and embryonic-type cells abundantly expressed cytokeratin no. 18, as evidenced by intense immunostaining with all three monoclonal antibodies directed against this polypeptide (Fig 3).

With monoclonal antibodies anticytokeratin 19 and A55-B/A2, specific for cytokeratin no. 19, a uniformly positive staining of embryonic-type tumor

TABLE 2. Monoclonal Antibodies Used in This Study

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Specificity</th>
<th>Source</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Monoclonal anticytokeratin (glanular epithelia)</td>
<td>7</td>
<td>Amersham International, Buckinghamshire, England</td>
<td>11, 22</td>
</tr>
<tr>
<td>RCK 105</td>
<td>7</td>
<td>FCS Ramaekers, Ramaekers, Nijmegen, The Netherlands</td>
<td>11, 18</td>
</tr>
<tr>
<td>Monoclonal anticytokeratin 8</td>
<td>8</td>
<td>Amersham International, Buckinghamshire, England</td>
<td>11, 28</td>
</tr>
<tr>
<td>M 20</td>
<td>8</td>
<td>FCS Ramaekers</td>
<td>11, 24</td>
</tr>
<tr>
<td>Monoclonal anticytokeratin (simple epithelia)</td>
<td>18</td>
<td>Amersham International, Buckinghamshire, England</td>
<td>11, 23</td>
</tr>
<tr>
<td>RGE 53</td>
<td>18</td>
<td>FCS Ramaekers</td>
<td>11, 25</td>
</tr>
<tr>
<td>CK 18.2</td>
<td>18</td>
<td>FCS Ramaekers</td>
<td>11, 26, 27</td>
</tr>
<tr>
<td>Monoclonal anticytokeratin 19 (LP 2K)</td>
<td>19</td>
<td>Amersham International, Buckinghamshire, England</td>
<td>11, 28</td>
</tr>
<tr>
<td>A55-B/A2</td>
<td>19</td>
<td>U. Karsten, Berlin-Buch, GDR</td>
<td>11, 12, 29</td>
</tr>
<tr>
<td>RCK 102</td>
<td>5 and 8</td>
<td>FCS Ramaekers</td>
<td>11, 26, 27</td>
</tr>
<tr>
<td>RKSE 60</td>
<td>10</td>
<td>FCS Ramaekers</td>
<td>11, 30</td>
</tr>
<tr>
<td>Monoclonal antidesmin</td>
<td>desmin</td>
<td>Boehringer Mannheim, Mannheim, West Germany</td>
<td>51</td>
</tr>
<tr>
<td>Monoclonal antivimentin</td>
<td>vimentin</td>
<td>Labsystems Oy, Helsinki, Finland</td>
<td>32</td>
</tr>
<tr>
<td>RV 202</td>
<td>vimentin</td>
<td>FCS Ramaekers</td>
<td>13</td>
</tr>
</tbody>
</table>

* Specificities of antibodies directed against cytokeratin are given as numbers of the catalog of human cytokeratin of Moll et al.
TABLE 3. Summary of Immunohistochemical Results

<table>
<thead>
<tr>
<th></th>
<th>Fetal Areas</th>
<th>Embryonal Areas</th>
<th>Mesenchymal Areas*</th>
<th>&quot;Osteoid&quot; Areas*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cytokeratin no. 7</td>
<td>–</td>
<td>+†</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>Cytokeratin no. 8</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Cytokeratin no. 18</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Cytokeratin no. 19</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Cytokeratin no. 10</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Vimentin</td>
<td>–</td>
<td>+‡</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Desmin</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

* Present in one specimen only.
† In two specimens.
‡ Focal positivity.

cells was found. In two cases, clusters of tumor cells were more strongly positive than surrounding cells (Fig 4, left). Fetal-type tumor cells also stained for cytokeratin no. 19, but stained less strongly than did embryonic-type cells (Fig 4, right). Fetal-type tumor cells with a pale appearance were sometimes only very weakly labeled, and some cells were not labeled at all. Embryonic-type tumor cells in the center of "macrotrabeculae" (case no. 5) stained intensely for cytokeratin no. 19 (Fig 5).

Tumor cells of cases no. 1, 4 and 5 were unreactive with both monoclonal antibodies specific for cytokeratin no. 7. In cases two and three, a small number of embryonic-type cells close to fibrous septa were weakly positive. In case no. 2, cells arranged in a layer around vessels coursing through the tumor were more strongly immunoreactive for cytokeratins nos. 8, 18, and 19 than surrounding cells (Fig 6, left), and some of them expressed cytokeratin no. 7 (Fig 6, right). These cells were unlikely to represent bile duct cells in ductal plate configuration entrapped in the tumor, since adjacent nontumoral parenchyma contained "mature" portal tracts with a centrally located bile duct. Monoclonal antibody RCK 102 reactive with cytokeratins no. 5 and 8 produced a uniform staining of epithelial tumor cells. No staining was observed with monoclonal antibody RKSE60, directed against cytokeratin no. 10. Some cells in mature fibrous septa, or occasionally in vessel walls, were positive with monoclonal antibodies M20, RGE53, CK18.2 and RCK102. Fetal-type tumor cells did not express vimentin, but embryonic-type tumor cells displayed focal positivity (Fig 7). In case no. 5, cells in the center of macrotrabecular areas were strongly positive for vimentin. Staining for desmin was negative in the epithelial areas.

Cryostat sections of case no. 1 also contained primitive mesenchymal cells and osteoid-like mate-

FIGURE 2. Predominantly embryonal area of hepatoblastoma showing paranuclear positivity of tumor cells for cytokeratin no. 8. (Indirect immunoperoxidase technique, counterstained with Mayer's hemalum, magnification × 356.)

FIGURE 3. Tumor cells both in fetal (top) and embryonal (bottom) areas strongly express cytokeratin no. 18. (Indirect immunoperoxidase technique, counterstained with Mayer's hemalum, magnification × 156.)
rial. The former cells were unreactive with all antibodies directed against cytokeratins and desmin, but stained positively for vimentin. Some of the cells embedded in the osteoid-like material were immunoreactive for vimentin, and some were also labeled by monoclonal antibodies RCK105, CK18.2 (recognizing cytokeratins no. 7 and 18, respectively), and by monoclonal anticytokeratin 19 and A53-B/A9 directed against cytokeratin no. 19 (Fig 8). Immunoblotting assays were performed on Triton X-100-extracted frozen sections of a hepatoblastoma (case no. 1), containing cytokeratins no. 8, 18, and 19, as concluded from the immunohistochemical staining procedures. Figure 9 illustrates these results, and shows that immunoblots of case no. 1 detect the unexpected presence of cytokeratin no. 7, next to the expected occurrence of cytokeratins no. 8 and 18. Cytokeratin no. 19 was detected as an extremely faint band in these immunoblots (asterisk in Fig 9), probably due to a low concentration of this cytoskeletal constituent in the specimens examined.

**DISCUSSION**

The immunoreactivity of epithelial cells of hepatoblastoma for cytokeratins confirms previous reports. However, the use of a panel of monoclonal antibodies directed against individual cytokeratins allowed a more detailed analysis of the pattern of cytokeratin expression. Both fetal and embryonic type tumor cells expressed cytokeratins no. 8 and 18. This was not an unexpected finding, since these two cytokeratins are present in normal embryonic, fetal, and adult liver parenchymal cells, and are the first cytokeratins to appear during embryonic development. However, the detection of cytokeratin no. 19 in the epithelial tumor cells of hepatoblastoma was surprising. In normal adult human liver, this cytokeratin polypeptide is restricted to bile duct cells. Also, in embryonic and fetal liver, only cells of the ductal plate express cytokeratin no. 19.
In two cases, a few embryonic-type tumor cells were also positive for cytokeratin no. 7, which in adult human liver is found exclusively in bile duct cells.\textsuperscript{15,14,16} During development of the intrahepatic bile ducts, cytokeratin no. 7 only appears in cells of the ductal plate after 20 weeks of gestation.\textsuperscript{17} The observed immunoreactivity for cytokeratins no. 7, 8, 18 or 19 is almost certainly due to the actual presence of these polypeptides in tumor cells, since at least two different monoclonal antibodies directed against an individual cytokeratin were used and yielded comparable results. Furthermore, in one case, the presence of cytokeratins no. 8, 18 and 19 was confirmed by Western blotting. It is not clear why cytokeratin no. 7 was detected by biochemical means, while immunohistochemistry failed to reveal cytokeratin no. 7-positive tumor cells in this same case. It is possible that the specimen used for biochemical analysis still contained

\textbf{FIGURE 7.} Predominantly embryonal area of hepatoblastoma showing numerous vimentin-positive cells. (Indirect immunoperoxidase technique, counterstained with Mayer's hemalum, magnification $\times 150$.)

\textbf{FIGURE 8.} Cells embedded in osteoid-like material clearly express cytokeratin no. 18. (Indirect immunoperoxidase technique, counterstained with Mayer's hemalum, magnification $\times 400$.)
some nontumoral parenchyma, including cytokeratin no. 7-positive bile ducts and ductules (although a cryostat section showed only tumor). However, the discrepancy between the biochemical and immunohistochemical findings could also be explained by tumor cell heterogeneity, with cytokeratin no. 7-positive tumor cells being present only in the specimen used for biochemical analysis. It is also possible that the tumor cells expressed cytokeratin no. 7 at a level too low to be detectable by immunohistochemistry.

The pattern of cytokeratin expression of epithelial cells of hepatoblastoma thus corresponds to the cytokeratin pattern of intrahepatic bile duct cells, or cells of the embryonic ductal plate. The presence of bundles of intermediate filaments in embryonic-type cells of hepatoblastoma has repeatedly been reported in ultrastructural studies. Since conspicuous bundles of cytoplasmic intermediate filaments are a normal feature of intrahepatic bile duct cells, but not of parenchymal cells, their presence in embryonic-type tumor cells might be related to the expression by these cells of “bile duct type” cytokeratins. In this respect, it is interesting to note that embryonic-type cells were sometimes more strongly positive for cytokeratin no. 19 than fetal-type cells. Further evidence for “ductular differentiation” was found in case no. 2. In this specimen, tumor cells more strongly positive for cytokeratins no. 8, 18, and 19 than the surrounding cells, and also positive for cytokeratin no. 7, were arranged in a layer around vessels, resembling the ductal plate structures found during normal development of the intrahepatic bile ducts. Tumor cells were unreactive for cytokeratin no. 10. Abenoza et al reported expression of this high molecular weight cytokeratin in hepatoblastoma, but only in areas of squamous differentiation. The positive staining for vimentin of primitive mesenchymal and some embryonic-type tumor cells also confirms their report.

Cells embedded in the osteoid-like areas of mixed hepatoblastoma were reported to be positive for vimentin, epithelial membrane antigen, cytokeratin, and S-100. In the present study, some of these cells were immunoreactive for vimentin and cytokeratins no. 7, 18, and 19, confirming the hypothesis of Abenoza et al that these cells are not merely osteoblasts.

Our findings raise some questions concerning the histogenesis of hepatoblastoma. It is generally believed that the pattern of cytokeratin expression of a given cell is maintained during neoplastic transformation. If so, the presence of the “bile duct type” cytokeratins no. 7 or 19, in addition to the “hepatocyte” cytokeratins no. 8 and 18, in tumor cells of hepatoblastoma would imply that they arise from a cell with a bile ductular, rather than a hepatocellular phenotype. In this respect, it should be noted that Tsao and Grisham have demonstrated that clonally derived cell lines of chemically transformed, cultured rat liver epithelial cells can give rise to mixed hepatoblastoma, indicating that these cells represent “stem cells” capable of divergent morphologic differentiation. The cultured rat liver epithelial cell line used by these authors is thought to be derived from bile ductular cells. It is also conceivable that, in man, hepatoblastoma arises from a “stem cell” with a “ductular” cytokeratin profile. However, we realize that our findings do not constitute unequivocal evidence in favor of this hypothesis. Indeed, Gould has warned against several hazards in tracing the histogenesis of tumors on the basis of patterns of intermediate filament expression. Numerous genetic and epigenetic factors can modulate the phenotype of a malignant cell. We have previously shown that some hepatocellular carcinomas, although thought to originate from
hepatocytes, can also express cytokeratins no. 7 and/or 19.30

Finally, in the histogenetic scheme of Abenosa et al, the cytokeratin and vimentin-positive "small" cell is considered to be the precursor of embryonic-type cells.9 No "small" cell areas were found in our material. It would be of interest to investigate the detailed pattern of cytokeratin expression of this "small" cell type in order to find out whether it also expresses cytokeratins no. 19 or 7.

Acknowledgment. The authors gratefully acknowledge the technical assistance of Suzanne Taucelmann, the secretarial assistance of Agnes Goethuys, and the photographic assistance of Michel Rooseleurs. They are indebted to Dr Karsten, Berlin-Buch, GDR, who generously supplied the monoclonal antibody A53-B/A2.

REFERENCES


