HISTOCHEMICAL STUDY ON THE ACTIVITY OF THE ENZYMES IN HUMAN HEPATOMAS

HISANDO KOBAYASHI
Department of Clinical Pathology, Aichi Cancer Center Hospital

HISASHI TAUCHI
2nd Department of Pathology, Nagoya University School of Medicine
(Director: Prof. Hisashi Tauchi)

ABSTRACT

Ten cases of human hepatoma obtained at surgery and autopsy were studied enzyme-histochemically, and compared with gastric carcinoma, intrahepatic bile duct carcinoma and prostate carcinoma. The enzymes studied were glucose-6-phosphatase, acid phosphatase, alkaline phosphatase, succinic dehydrogenase, cytochrome oxidase, and peroxidase.

In conventional stained sections, case 1 was grade I and II hepatocellular carcinoma by the classification of Edmondson. Case 2 and case 3 were grade IV or more malignant than grade III, and the remaining were almost all grade III.

Case 1, in which all enzymes except alkaline phosphatase were histochemically demonstrated in hepatoma cells, could belong to such minimum deviation hepatoma. Acid phosphatase was demonstrated as more prominent granules in the hepatocellular carcinoma cells of all cases.

Succinic dehydrogenase was noticed to be very weak or negative except for case 1, but cytochrome oxidase was positive in all cases.

Glucose-6-phosphatase was histochemically demonstrated in all cases examined, but the activity was low except for case 1, and negative in control cancer cases.

Peroxidase was generally low in activity in all hepatomas except for case 1.

The intensity of glucose-6-phosphatase and peroxidase activities in the present human hepatomas may be related to a more detailed and accurate differentiation of hepatocellular carcinoma than the classification of Edmondson by conventional stained sections.

INTRODUCTION

Many biochemical studies on experimental animal hepatomas have been made. Recently, from a comparative study of the enzymes in animal liver tumors of different growth rates, Weber et al. stated that the glucose-6-phosphatase activity, a marker enzyme of hepatic parenchymal cells, was absent in rapidly growing hepatoma but remained at a relatively high level in slow growing ones. They suggested that the extent of enzymatic lesion could be correlated roughly with the
growth rate of hepatomas. More recently, Mochizuki et al. studied the microbodies and peroxidase activity of many different growth rate animal hepatomas. They also suggested that the number of microbodies and peroxidase activity of hepatoma cells could run parallel with the differentiation and growth rates of the carcinoma.

It is generally known that the various liver tumors differ not only in their histological structure but also in their biochemical aspects, both quantitatively and qualitatively.

However, as far as we know, no quantitative biochemical studies have as yet been conducted on the tumor tissue as such, and as for its histochemical properties, only occasional references have been made to the enzyme histochemistry of human malignant hepatoma.

Edmondson classified the hepatocellular carcinoma of human cases into 4 grades according to the degree of differentiation. This histological grouping was in parallel to their growth rate and metastasis.

A comparison between the morphology and metabolism of these tumors would bring a new understanding of the biology, and also of the subsequent treatment of these tumors.

Here, the findings of the histochemical study on succinic dehydrogenase, cytochrome oxidase, peroxidase, glucose-6-phosphatase, acid phosphatase, and alkaline phosphatase in 10 cases of human hepatocellular carcinoma will be presented to find out some metabolic aspects of these tumors.

MATERIALS AND METHODS

Seven surgical biopsy specimens of hepatocellular carcinoma and one of primary carcinoma of the liver by surgical hepatectomy were obtained from patients admitted to the Aichi Cancer Center Hospital. Two autopsy cases of primary hepatic carcinoma were examined within 24 hours after death. There was no overlap between the surgical and autopsy groups.

The patients, 9 males and 1 female, ranged from 36 years to 70 years in age. Seven cases out of 10 were associated with liver cirrhosis. In 7 cases, alpha fetoglobulin was present in the serum, and 2 cases had Australia antigen.

The tissue blocks of the tumor specimens were all taken so as include neighbouring normal hepatic tissue as well. From each block, fresh frozen sections were cut at 8 microns in a cryostat at −20°C for demonstration of enzymes. The enzymes studied were glucose-6-phosphatase, acid phosphatase, alkaline phosphatase, succinic dehydrogenase, cytochrome oxidase, and peroxidase. The techniques employed for the staining are listed in Table 1. Intensity of staining was usually estimated and graded, and that in the hepatoma was compared with that in hepatocytes in the normal portion of the liver.

As control, stomach cancer, intrahepatic bile duct carcinoma and prostate carcinoma were examined enzyme histochemically by the same procedure.
Table 1. Histochemical Determination of the Enzymes

<table>
<thead>
<tr>
<th>Enzymes</th>
<th>Staining technique</th>
<th>Substrate</th>
<th>pH</th>
<th>Time and temperature</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose-6-phosphatase</td>
<td>Wachstein &amp; Meisel (1957)</td>
<td>Disodium glucose-6-phosphate</td>
<td>6.7</td>
<td>15 min. 25°C</td>
</tr>
<tr>
<td>Acid phosphatase</td>
<td>Burstone (1958)</td>
<td>Naphthol AS-BI phosphate</td>
<td>5.2</td>
<td>30 min. 37°C</td>
</tr>
<tr>
<td>Alkaline phosphatase</td>
<td>Burstone (1957)</td>
<td>Naphthol AS-BI phosphate</td>
<td>9.0</td>
<td>60 min. 37°C</td>
</tr>
<tr>
<td>Succinic dehydrogenase</td>
<td>Nachlas et al. (1957)</td>
<td>Sodium succinate</td>
<td>7.6</td>
<td>30 min. 37°C</td>
</tr>
<tr>
<td>Cytochrome oxidase</td>
<td>Seligman et al. (1968)</td>
<td>Cytochrome C</td>
<td>7.4</td>
<td>30 min. 37°C</td>
</tr>
<tr>
<td>Peroxidase</td>
<td>Graham &amp; Karnovsky (1966)</td>
<td>Hydrogen peroxide</td>
<td>9.0</td>
<td>60 min. 37°C</td>
</tr>
</tbody>
</table>

Summary of the cases is shown in the Tables 2 and 3.

RESULTS

The results obtained are summarized in Table 4. Succinic dehydrogenase activity was generally weak or negative in the tumor cells studied except for case 1, in which the staining patterns of tumor cells were quantitatively similar to normal hepatocytes. In case 4, case 6, case 7 and case 8, there were scattered slightly positive carcinoma cells. Case 2, case 3, and case 10 showed complete absence of the enzyme activity in any carcinoma cells of a given tumor tissue. There were occasionally cells with faint enzyme activity in case 5 and case 9.

Cytochrome oxidase was relatively intensively noted in all cases. In case 1, case 2, and case 7, the grade of activity did not significantly vary among cells of a given tissue, and the activity was almost the same in grade as in normal liver cells. In the other 7 cases, enzyme activity varied remarkably from cell to cell. In the cases of stomach carcinoma, intrahepatic bile duct carcinoma, and prostate carcinoma, the tumor cells were weak in cytochrome oxidase activity and negative in succinic dehydrogenase activity.

Acid phosphatase activity of the all hepatoma cells appeared to be similar in grade as that of the surrounding non-neoplastic liver. They were distributed through the cytoplasm in a random manner. The positive granules were more prominent and larger in size, when compared with normal hepatic cells. The intrahepatic bile duct carcinoma and prostate carcinoma showed moderate enzyme activity. The stomach carcinoma had no activity.

Alkaline phoshatase activity was not demonstrated histochemically in the hepatic carcinoma cells, and to be limited only to the endothelial cells in the tumor tissue. The cancer cells of control tumor cases revealed no enzyme activity.

Glucose-6-phosphatase activity in case 1 was observed to be of the same intensity as the surrounding non-neoplastic liver, showing brownish coarse granules.
### TABLE 2. Case Summary

<table>
<thead>
<tr>
<th>Case age. (yrs)</th>
<th>Admission time and clinical sign.</th>
<th>History.</th>
<th>Examination</th>
<th>Therapy</th>
<th>Discharge and clinical course after discharge or autopsy</th>
</tr>
</thead>
<tbody>
<tr>
<td>44. Japanese male</td>
<td>June 8, 1970. Recurrent upper abdominal pain with rising of body temperature and weight loss of 6 kg.</td>
<td>PAS, INAH and Kanamycin for past 4 years, for pulmonary tuberculosis. Alcohol (++).</td>
<td>Liver; 3 fingerbreadths below the right costal margin. Liver scan 99 m Tc; diminished uptake, measuring 4.8 cm in diameter in the right lobe. Exploratory celiotomy; huge tumor measuring 5 cm in diameter in right lobe and a nodule in left lobe. Edmondson grading of hepatoma; grade III without cirrhosis.</td>
<td>On program of radiation and chemotherapy.</td>
<td>On Sept. 28, 1970, with gradual improvement of general condition including hepatic function. Lost for follow up study.</td>
</tr>
</tbody>
</table>

Liver; 4 fingerbreadths below right costal margin. Jaundice (−). Liver scan 99 m Tc; a large filling defect in the left lobe. Peritoneoscopy; cirrhosis with multiple cancer nodules in left lobe and few in right lobe. Edmondson grading of hepatoma; grade III with cirrhosis.

Chemotherapy and radiotherapy. Died on May 3, 1971, with weight loss of 12 kg. for 4 months. At autopsy: Liver; 3,300 g weight. Cirrhosis (+). occupied by tumor nodules on about 90% of the organ. Both lungs; numerous metastatic nodules.

6. March 2, 1970. Abdominal swelling for 2 months. At age 51, “jaundice”. In 1932, gastrectomy for peptic ulcer. Family history; brother died of alcohol cirrhosis, and father and mother of liver cirrhosis. 46 year old wife has also suffered from liver cirrhosis. Alcohol (−).


No therapy. Died on March 12, 1970, with progressive jaundice, and weakness, complicated by abdominal swelling and bloody stool. At autopsy; 3,000 ml. bloody ascites. Liver; Cirrhosis (+). 1,900 g weight. Almost replaced by tumor nodules. A nodule of right lobe, hemorrhagic rupture (+), portal vein, occluded by tumor mass. Tumor; (Edmondson grading) hepatocellular carcinoma grade III. Hyaline body (+) in some cells.


Liver; 5 fingerbreadths below the right costal margin. Icterus (−). Spider angiomata (+). Splenomegaly (−). Ascites (−). Liver scan 99 m Tc; a large space-occupy lesion in the right lobe. Edmondson grading of hepatoma; grade III, occasionally grade II, with cirrhosis.

Chemotherapy and radiotherapy. Infusion of 30 mg. Mitomycin C into the right hepatic artery. On Dec. 25, 1970, with marked improvement of general condition. About 3 months later, he was admitted, because of developing pruritus, fatigue, upper abdominal pain, vomiting, and jaundice. Expired on April 26, 1971 without improvement on radiotherapy. Autopsy was not performed.


Chemotherapy. Expired on Sept. 27, 1970, with progressive jaundice, weakness and rapid downhill. An autopsy was not carried out.
<table>
<thead>
<tr>
<th>Case age</th>
<th>Admission time and clinical sign</th>
<th>History</th>
<th>Examination</th>
<th>Therapy</th>
<th>Discharge and clinical course after discharge or autopsy</th>
</tr>
</thead>
</table>
TABLE 3. Results of Laboratory Tests of Serum in Patients with Hepatoma.

<table>
<thead>
<tr>
<th>Determination, normal range</th>
<th>Patient NO.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
</tr>
<tr>
<td>Biochemical studies</td>
<td></td>
</tr>
<tr>
<td>Total protein, 6.5 to 8.0 g/100 ml</td>
<td>6.8</td>
</tr>
<tr>
<td>A/G, 1.2 to 2.0</td>
<td>1.0</td>
</tr>
<tr>
<td>Urea N, 8 to 20 mg/100 ml</td>
<td>12.8</td>
</tr>
<tr>
<td>Total bilirubin, 0.2 to 1.0 mg/100 ml</td>
<td>0.7</td>
</tr>
<tr>
<td>Direct bilirubin, 0 to 0.2 mg/100 ml</td>
<td>0.4</td>
</tr>
<tr>
<td>GOT, 8 to 40 units</td>
<td>87</td>
</tr>
<tr>
<td>GPT, 5 to 35 units</td>
<td>130</td>
</tr>
<tr>
<td>Alkaline phosphatase, 2.7 to 10.0 King-Armstrong units</td>
<td>5.4</td>
</tr>
<tr>
<td>Cholesterol, 150 to 250 mg/100 ml</td>
<td>221</td>
</tr>
<tr>
<td>LDH, 100 to 350 units</td>
<td>285</td>
</tr>
<tr>
<td>BSP test, 4.5%</td>
<td>3.1</td>
</tr>
<tr>
<td>Immunological tests</td>
<td></td>
</tr>
<tr>
<td>Alpha-fetoglobulin</td>
<td>-</td>
</tr>
<tr>
<td>Australia antigen</td>
<td>-</td>
</tr>
</tbody>
</table>

ENZYME HISTOCHEMISTRY OF HUMAN HEPATOMAS
### Table 4. Histochemical Study on the Enzyme Activity of Human Hepatomas

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Case 1</th>
<th>T.M.</th>
<th>S.T.</th>
<th>C.M.</th>
<th>T.O.</th>
<th>M.T.</th>
<th>S.T.</th>
<th>S.E.</th>
<th>M.K.</th>
<th>A.M.</th>
<th>S.M.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose-6-phosphatase</td>
<td>+++</td>
<td>+</td>
<td>±</td>
<td>~</td>
<td>++</td>
<td>±</td>
<td>~</td>
<td>++</td>
<td>±</td>
<td>~</td>
<td>+</td>
</tr>
<tr>
<td>Succinic dehydrogenase</td>
<td>+++</td>
<td>-</td>
<td>-</td>
<td>±</td>
<td>~</td>
<td>+</td>
<td>±</td>
<td>~</td>
<td>±</td>
<td>~</td>
<td>~</td>
</tr>
<tr>
<td>Acid phosphatase</td>
<td>+</td>
<td>+</td>
<td>±</td>
<td>~</td>
<td>+++</td>
<td>+</td>
<td>±</td>
<td>~</td>
<td>+++</td>
<td>+</td>
<td>~</td>
</tr>
<tr>
<td>Alkaline phosphatase</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Peroxidase</td>
<td>+++</td>
<td>-</td>
<td>+</td>
<td>±</td>
<td>+</td>
<td>-</td>
<td>++</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Cytochrome oxidase</td>
<td>+++</td>
<td>+++</td>
<td>++</td>
<td>~</td>
<td>±</td>
<td>~</td>
<td>++</td>
<td>~</td>
<td>+++</td>
<td>++</td>
<td>~</td>
</tr>
</tbody>
</table>
in the cytoplasm. This enzyme activity was occasionally strongly observed in the perinucleus of tumor cells. The activity was uniform in all tumor cells in a given tissue of case 1. In all other cases, the activity was lower in grade, compared with normal liver tissue. In case 4, case 5, case 6, and case 10, a picture of moderate activity was noted. In case 10, tumor cells showed scattered marked activity. Case 2, and case 9 revealed the presence of slight activity. The activity in case 3, case 7, and case 8, was minimum in grade, but there were scattered positive cells with marked activity. The glucose-6-phosphatase activity varied remarkably in grade from cell to cell. The cancer cells of stomach carcinoma, intrahepatic bile duct carcinoma, and prostate carcinoma showed complete absence of enzyme activity.

The peroxidase activity of all cases except case 1 was generally lower in grade. The activity in case 1 appeared to be normal. In case 7, activity was slightly low in grade. The demonstrable activity varied moderately among cells of a given tissue. Case 3 and case 5 showed moderately low activity. The activity in case 4 was faint. Case 2, case 6, case 9, and case 10 revealed complete absence of activity. The other cancer cases showed no peroxidase activity.

**DISCUSSION**

The histochemical features of normal liver tissue have been summarized by Wachstein\textsuperscript{26,27} and by Novikoff et al.\textsuperscript{15} Histochemical and biochemical features of highly undifferentiated rat hepatoma included a deficiency of multiple enzymes.\textsuperscript{14,18,19,20} It was maintained, moreover, that similar alteration in metabolism of the liver might actually precede the overt neoplasm. However, these early enzyme defects were found to reflect an alteration of cellular population from hepatic to biliary ductal element.\textsuperscript{10} Subsequent studies on well differentiated rat hepatoma failed to reveal similar enzyme abnormalities.\textsuperscript{12,18,23}

Although the incidence of human primary hepatic cancer is relatively high in Japan, no histochemical study on the enzymatic activity of these tumors has been made. The enzymes examined in the present study were glucose-6-phosphatase, acid phosphatase, alkaline phosphatase, succinic dehydrogenase, cytochrome oxidase, and peroxidase.

In case 1, which showed histologically low grade malignancy, there was an almost complete maintenance of all enzymes. Mitochondrial enzymes such as succinic dehydrogenase and cytochrome oxidase appeared unchanged. There may be no indication of a shift in oxidative metabolism from the citric acid to the pentose shunt. Glucose-6-phosphatase, which is a marker enzyme of the hepatic cells and localized in the endoplasmic reticulum\textsuperscript{7}, was near normal. The endoplasmic reticulum in case 1 could be intact. So the hepatic carcinoma of this case may have other drug metabolizing enzymes, which are associated with endoplasmic reticulum\textsuperscript{22}. From the above, this human case could belong to such minimum
deviation hepatoma described by Potter\textsuperscript{21}).

In all cases of hepatic carcinoma, alkaline phosphatase was not demonstrated in cancer cells, but noted only in the endothelial cells. The absence of this enzyme in the canaliculi may indicate the failure of the hepatoma cells, deprived of its access to excretory ducts, to excrete enzyme carried to it\textsuperscript{22}).

Acid phosphatase, a marker enzyme of lysosomes, was demonstrated in the tumor cells of all cases studied. The distribution of acid phosphatase in the peribiliary granules was well illustrated in the normal liver but not in the tumor. The acid phosphatase positive granules were prominent and larger in size in the hepatic carcinoma cells, compared with normal hepatic cells. The prominence of these lysosome granules may represent an adaptation of the hepatoma to the increased work required to excrete into blind canaliculi\textsuperscript{4}).

Succinic dehydrogenase was noted to be very weak or negative in the present cases studied except for case 1. However, cytochrome oxidase activity, which plays an important role in biological function and respiration and is closely associated with the membrane system of mitochondria, was clearly seen in hepatoma cells. The discrepancy in attitude between the two enzymes may indicate an abnormal respiration of tumor cells by molecular structural alteration of their mitochondria as compared with normal hepatic cells.

The most interesting finding is the presence of glucose-6-phosphatase activity in the tumor cells, though the activity varied from case to case. Except for case 1, glucose-6-phosphatase, a marker enzyme of the hepatic cells\textsuperscript{28}), associated with endoplasmic reticulum\textsuperscript{7}), was lower in activity in all cases. Generally, it is considered that cancer cells lose or diminish the enzymes of specialized function characteristic of the normal tissue of origin, and increase the enzyme activity for their proliferation or growth\textsuperscript{9}). In some animal hepatomas, there was demonstrated complete absence of glucose-6-phosphatase\textsuperscript{13}). Recently, however, Weber \textit{et al.}\textsuperscript{13, 30} reported that in a comparative study of the enzymes in animal hepatomas of different growth rates, glucose-6-phosphatase activity in slowly growing hepatomas was one-third the normal value, and decreased further in more rapidly growing tumors with no activity in the fastest growing hepatomas. Activity of glucose-6-phosphatase of the present cases did not run parallel with morphological grade of differentiation of tumor except for case 1. From the above, it is thought that the biological character or grade of differentiation of hepatomas may be suggested more clearly by the histochemical finding of the glucose-6-phosphatase activity than by routine stained sections. In the hepatoma cells with weak or faint activity of glucose-6-phosphatase, the enzyme which channels glucose-6-phosphate into nucleoprotein synthesis may be highly increased\textsuperscript{13, 29}). The tumor cells with maintained activity of glucose-6-phosphatase could have mature endoplasmic reticulum or function to release glucose from cells to the blood stream\textsuperscript{13}). This enzyme activity of the tumor cells may be correlated with the clinical course of the patient, and problem of selective toxicity of the cells. From the complete absence of glucose-
6-phosphatase in control cancer cases, and the maintenance of it in all examined hepatomas, the histochemical study of this enzyme could be utilized to prove the tumors to be liver cell in origin.

The peroxidase activity except for case 1 was generally decreased. The peroxidase is normally contained in microbodies of hepatic parenchymal cells as catalase or hydrogen peroxide-producing oxidase\(^{15}\).

Examination of microbodies of transplantal animal hepatomas with a wide spectrum of growth rates was performed by Mochizuki et al.,\(^{11}\) and they reported that catalase activity of hepatomas roughly correlated with hepatoma growth rates, and the fine structural features of microbodies often correlated with enzyme activity. The fast-growing hepatomas contained only a few small microbodies, usually without crystallloid. On the other hand, microbodies of hepatomas with intermediate growth rates were more abundant and larger than those of fast-growing tumor, and generally had crystallloid. Slowly growing hepatomas had abundant and large microbodies. The peroxidase was considered to be correlated with special function of certain cell types.

The peroxidase activity in the present human hepatomas did not always run parallel with morphological grade of differentiation in H-E. section, except for case 1. However, histochemical study of the peroxidase could provide more detailed information on the differentiation and function of hepatomas.

REFERENCES


---

**LEGENDS TO FIGURES**

**FIG. 1.** Glucose-6-phosphatase in hepatoma of case 1. Activity not significantly varied between the cells.

**FIG. 2.** Glucose-6-phosphatase activity in hepatoma of case 5 varied remarkably from cell to cell.

**FIG. 3.** Succinic dehydrogenase activity in hepatoma of case 1 at least equally prominent as in normal hepatocytes.
FIG. 4. Dark staining area reveals succinic dehydrogenase activity in normal liver cells. No staining area shows absence of enzyme activity in hepatoma of case 2.

FIG. 5. Cytochrome oxidase activity in hepatoma of case 2 relatively intensively shown.

FIG. 6. Peroxidase activity in hepatoma of case 7 varied moderately between cells.

FIG. 7. Acid phosphatase granules in hepatoma of case 1 seen distributed through cytoplasm randomly.

FIG. 8. Alkaline phosphatase activity in hepatoma of case 1 shown only in endothelial cells. Complete absence of the enzyme in hepatoma cells.