Immunohistochemistry of Hepatocellular Carcinoma Associated with Cirrhosis

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ABSTRACT

Tumor cell marker antibodies were used to analyze ten cases of hepatocellular carcinoma associated with cirrhosis. Clinically, eight of these cases gave a history of chronic alcoholism and the other two of hepatitis B virus infection. Formalin-fixed, paraffin-embedded sections from these cases were screened with antibodies against alpha fetoprotein (AFP), hepatitis B surface antigen (HBsAg) and carcinoembryonic antigen (CEA) using the peroxidase antiperoxidase and avidin-biotin immunoperoxidase procedures. Three cases were positive for AFP, four for HBsAg, and three for CEA; two cases had both HBsAg and CEA. Alpha fetoprotein was present only in the cytoplasm of tumor cells in three cases. Hepatitis B surface antigen, on the other hand, was present in the cytoplasm of hepatocytes in cirrhotic areas and, in one out of the four cases, was also present in hepatocellular carcinoma cells. Carcinoembryonic antigen was seen in three cases; it was present on the surface and in the cytoplasm of proliferating ducts within the cirrhotic areas and between cell surfaces of individual tumor cells in two cases.

The presence of different markers was not related to the microscopic appearance of the tumors. In one case, positivity for AFP was of diagnostic help in a tissue sample obtained by needle biopsy. The avidin-biotin immunoperoxidase procedure was more sensitive than the peroxidase antiperoxidase (PAP) technique in the pathological assessment of autopsy specimens. Our findings are in agreement with those of other reports and indicate that AFP and HBsAg are the most commonly found markers in hepatoma associated with cirrhosis, and that CEA staining is variable and probably non-contributory.

Introduction

Hepatocellular carcinoma is uncommon in the United States and accounts for less than one percent of all malignancies. Eight percent of hepatocellular carcinomas occur in livers with preexisting cirrhosis, postnecrotic cirrhosis being the most common predisposing condition. Alcoholic cirrhosis is highly...
prevalent in this country; however, only 3.2 percent of alcoholic cirrhosis is complicated by hepatocellular carcinoma. 

In the past decade, with the use of newer immunologic techniques, a wide range of different antigen systems such as alpha-fetoprotein, alpha-1-antitrypsin, hepatitis B antigens, and carcinoembryonic antigen have been demonstrated in formalin-fixed paraffin-embedded tissues of patients with hepatocellular carcinoma. Additional tumor markers have been investigated in the serum; among the new markers investigated are: \( \gamma \)-glutamyl transpeptidase isozyme, \( 5' \)-nucleotide phosphodiesterase isozyme-\( V \), hepatitis B antigens, and carcinoembryonic antigen. \( \gamma \)-glutamyl transpeptidase isozyme, \( 5' \)-nucleotide phosphodiesterase isozyme-\( V \), hepatitis B antigens, and carcinoembryonic antigen. However, false positivity has been observed with the majority of these markers.

The purpose of this study was to evaluate the presence of the most commonly studied tissue markers in hepatocellular carcinoma by means of two different immunoperoxidase procedures.

Material and Methods

Tissue from ten cases of hepatocellular carcinoma associated with cirrhosis was obtained from the Pathology files of the James A. Haley Veterans Hospital, Tampa, Florida. The oldest paraffin-embedded tissue was 11 years and most were between one and two years old. Nine Caucasian patients and one Black patient were included in this study. All patients were male with an age range from 24 to 85 years. Clinically, eight cases gave a history of chronic alcoholism and two of hepatitis B virus infection. In one patient, the diagnosis was made on a needle biopsy, and in all cases, autopsy material was available.

Immunoperoxidase Techniques

These techniques involved the use of antibodies and the enzyme peroxidase. Two immunoperoxidase procedures were used,—the peroxidase anti-peroxidase (PAP) and the avidin-biotin immunoperoxidase (ABC) technique.

The PAP method uses three antibodies: a primary antibody specific against the antigen to be located, a secondary antibody against immunoglobulins of the animal species used to produce the primary antibody, and the tertiary antibody. The secondary antibody serves as a link between the other two antibodies. The tertiary antibody is against peroxidase; the enzyme peroxidase used in the assay and the tertiary antibody form a PAP complex. The peroxidase enzyme and, therefore, the original antigen are visualized via a chromogenic hydrogen donor substance in the presence of hydrogen peroxide.

The avidin-biotin method is based on the ability of the egg-white glycoprotein avidin to bind nonimmunologically four molecules of the vitamin biotin. Two antibodies and a reagent are used. The primary antibody is specific for the antigen to be located. The second antibody conjugated to biotin is directed against the primary antibody. The reagent is a complex of peroxidase conjugated against the primary antibody. The reagent is a complex of peroxidase conjugated biotin and avidin. Avidin will serve as a link between the secondary antibody and the complex. The peroxidase enzyme will then be visualized as with the PAP method.

Antisera against alpha fetoprotein, hepatitis B surface antigen, and carcinoembryonic antigen were obtained from commercially available sources and used as primary antibodies in both procedures. In addition, antisera against hepatitis B core antigen and keratin obtained from calluses were used. Details of the PAP staining for proce-
dure, as used in our laboratory, have been previously reported.³

For the ABC technique, sections were deparaffinized. After blocking endogenous peroxidase activity with three percent hydrogen peroxidase and nonspecific background staining with incubation of normal goat serum, the sections were serially incubated for 20 to 40 minutes with appropriate dilutions of the primary rabbit antibody, biotinylated goat anti-rabbit immunoglobulin‡ and avidin-biotin-peroxidase complexes.§ Between incubations, the sections were washed in phosphate buffer saline (PBS) 0.01 M, pH 7.6. The sections, after the final incubations, were developed in six mg of 3'3 diaminobenzidine and 0.01 percent hydrogen peroxidase in 10 ml PBS for 10 minutes.

The primary antisera was replaced by normal rabbit serum in the section used as controls.

Results

The results are summarized in table I. The correlation between serum and tissue positivity was excellent for HBsAg with the PAP technique. Unfortunately, the ABC technique for the detection of HBsAg in tissue could not be applied because at the time of the study the appropriate primary antibody to be used in this system was unavailable.

The PAP technique was found to be poorly sensitive for the detection of tissue AFP, and negative results were obtained in all 10 cases studied, some of them with high serum AFP concentrations. The ABC technique appeared to be more sensitive for tissue AFP detection; when it was applied to our 10 cases, three were found positive. However, there was not a close correlation between serum and tissue AFP detection when using this technique since one case (#9) with a positive serum AFP yielded a negative tissue reaction with the ABC technique. In the detection of CEA antigen, both the PAP and ABC techniques gave comparable results (table I).

Four cases were positive for hepatitis B surface antigen, three for AFP, and three for CEA. In two cases, both antigens HBsAg and CEA were found. Although the reaction for keratin was uniformly negative in all cases of hepatocellular carcinoma, this reaction was predictably positive in bile ductules of cirrhotic areas. It should be noted, however, that the antisera used against keratin was prepared against calluses and not against Mallory bodies. It should be

<table>
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<th>Case</th>
<th>Age/Years</th>
<th>Race</th>
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<th>PAP↑</th>
<th>Alpha-fetoprotein Serum</th>
<th>ABC§</th>
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All patients have been autopsied. ND = Not done. *Hepatitis B surface antigen. †Peroxidase anti-peroxidase method. ‡Carcinoembryonic antigen. §Avidin-biotin immunoperoxidase technique.
also noted that the same keratin antiserum reacted positively in two cases of cholangiocarcinoma studied in our laboratory. The reaction for hepatitis B core antigen was negative in all ten cases. Hepatitis B surface antigen was present in four cases in scattered hepatocytes within the cirrhotic area (figure 1); in one of the cases, HBsAg was present in the cytoplasm of some hepatocellular carcinoma cells.

Alpha-fetoprotein (AFP) was present in three cases and only within the hepatoma cells (figure 2) and not within the cirrhotic area. In one case, the positivity for AFP was of diagnostic help; in this particular case, a 53-year-old white male presented with the chief complaint of a 16-pound weight gain in 1.5 weeks and upper epigastric pain. Physical examination disclosed an enlarged liver and ascitis. Laboratory data showed hypoglycemia and a high alkaline phosphatase. The other laboratory data were normal or only slightly elevated. Numerous nodules were present on chest x-ray and liver scan. During laparoscopy, a needle biopsy was obtained of a nodular liver. The biopsy revealed an undifferentiated carcinoma in a cirrhotic liver, and this tumor was strongly positive for AFP, confirming the diagnosis of hepatocellular carcinoma. Serum for AFP came back positive days later with a very high titer. The presence of alpha-fetoprotein was only detected by the ABC procedure. The same tumors failed to stain with the use of the PAP technique.

Carcinoembryonic antigen was seen in three cases, and it was located in the surface and cytoplasm of ductules within the cirrhotic areas. In two of these three positive cases, it was seen staining the canaliculi between tumor cells (figure 3).

Discussion
An increasing number of commercially available antibodies that reveal a wide range of different hepatocellular carcinoma antigens in fixed embedded tissue have become available. The age of the paraffin blocks does not affect the immunoperoxidase reactivity; however, the initial fixation does.17 Alpha-fetoprotein, since its discovery in 1963, has been the subject of numerous studies and has shown, rather consistently, to be a good marker for hepatocellular carcinoma and an even better reliable marker for tumor growth.6 In the fetus, alpha-fetoprotein is synthesized in the yolk sac and liver. In tissues, it has
been demonstrated in non-tumorous hepatocytes, hepatocellular carcinomas and yolk sac tumors. The presence of alpha-fetoprotein in germ cell tumors of testis or ovaries indicates differentiation toward yolk sac tumor which has a worse prognosis than embryonal carcinomas. Its presence in a given hepatocellular carcinoma, as seen in our series, may be focal or diffuse, and its presence in histological sections of hepatocellular carcinomas in this country has been reported.
in previous studies to be as low as 35 percent,\textsuperscript{18} much lower than the percentage of its positivity in serum. In one of our cases (Case #9), although the tissue reaction for alpha-fetoprotein was negative, both biopsy and autopsy samples, the serum level was 3,600 ng per ml. Elevated serum levels have been reported in pregnancy, hepatitis, cirrhosis and in tumors of the gastrointestinal tract.\textsuperscript{7,11,30}

Carcinoembryonic antigen is synthesized by the fetal intestine and is so ubiquitous that it has a limited value, except as a general epithelial tumor marker. It has been reported in patients with hepatocellular carcinoma in serum and tissues\textsuperscript{15,18} and also in a variety of non-malignant hepatic conditions.\textsuperscript{15} In this study, it was seen in non-tumoral proliferating bile ducts and in canaliculi but not in tumor cells as has been previously reported.\textsuperscript{18}

Hepatitis B virus has been considered to be an etiologic agent of hepatocellular carcinoma; however, as in our series, in the United States owing to the large number of alcoholic cirrhosis associated with hepatocellular carcinoma, this clinical association is less striking than in Asian and African series.\textsuperscript{12}

A popular marker for hepatocellular carcinoma is alpha-1-antitrypsin. It has been demonstrated in the liver within neoplastic and non-neoplastic cells, regardless of the presence of genetically determined AAT deficiency.\textsuperscript{10} At present, the development of monoclonal antibodies by hybridoma techniques will most likely yield still more specific antibodies; recent studies using monoclonal antibodies developed against hepatocarcinoma tumors of guinea pigs\textsuperscript{1} give hope for the use in the not distant future of monoclonal antibodies, not only for diagnostic purposes, but for the treatment of hepatocellular carcinoma.

In summary, data obtained from the use of hepatocellular tumor markers are presented. Our findings indicate that AFP and HBsAg are the most commonly found markers in hepatocellular carcinoma associated with cirrhosis and that CEA staining is variable and probably non-contributory. The ABC technique, as previously reported,\textsuperscript{5} is more sensitive than the PAP technique in the assessment of tissue samples.

References

HEPATOCELLULAR CARCINOMA