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Localization of α-Fetoprotein in Hepatoma Tissues by Immunofluorescence

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SUMMARY

By means of the indirect immunofluorescent technique with anti-α-fetoprotein antiserum, α-fetoprotein (AFP) was detected in the tumor cells from 9 of 10 patients with primary hepatocellular carcinoma. Even the patient with such a slight amount of serum AFP that it was undetectable by single radial immunodiffusion and Ouchterlony technique had AFP in the tumor cells.

AFP of tumor cells was localized in the following three areas: (*a*) cytoplasm, (*b*) cytoplasmic membrane, and (*c*) perinuclear zone. The distribution of the tumor cells containing AFP varied with the case and the specimen, and the number of these staining cells was one-fifth or less of the total.

AFP was sometimes found in the cytoplasm of Kupffer cells but was absent in connective tissues, bile ducts, and normal hepatic cells.

INTRODUCTION

Since Tatarinov (24) reported the presence of AFP,[1] an embryo-specific α_1-globulin, in sera from patients with primary liver carcinoma, the procedure for immunological detection of AFP in serum has been of great clinical value for the diagnosis of primary liver carcinoma. The reappearance of embryo-specific proteins in primary liver carcinoma and the significance of AFP in relation to the malignant process have been the subject of extensive investigations (1, 13, 22). AFP is the product of tumor cells; several recent investigators (9, 12, 16) have succeeded in detecting AFP in hepatoma tissues.

The purpose of this is to determine whether AFP is present in all tumor cells or only in certain cells and to determine its localization in the cell. The relationship between the localization of AFP in hepatoma tissue and the serum AFP levels or the histological classification of primary liver carcinoma will be described.

MATERIALS AND METHODS

Specimens. Thirty-eight autopsy specimens were obtained from 10 patients with primary hepatocellular carcinoma and 3 patients with liver carcinoma metastasized from the stomach. The classification of Edmondson and Steiner (8) was used for the histological gradings of primary liver carcinoma. Normal human livers were also obtained from patients who died of other diseases and from 8- to 20-week fetuses.

Preparation of Tissues. Tissue was prepared in 1 of 2 ways. Tissues were cut into small blocks and fixed in cold 95% ethanol. After dehydration with absolute alcohol, the blocks were passed through xylene and embedded in paraffin at 56°. Microtome sections, 4 mm thick, were cut from these blocks and floated on water at 40°. The sections were picked up and mounted on 1.0-mm-thick slides. Some tissues were frozen by putting them into test tubes placed in a Dry Ice-alcohol mixture at −70°. The frozen sections were cut in a cryostat at −20°.

Antisera. Rabbit γ-globulin was obtained by chromatography on a diethylaminoethyl cellulose column (14). Antisera were prepared by giving goats injections of a total of 10 mg of protein in complete Freund's adjuvant and repeating the same procedure 6 weeks later. γ-Globulin of these antisera was conjugated with FITC (Baltimore Biological Laboratories, Baltimore, Md.) by the method of Coons and Kaplan (6). Nonspecific fluorescence was absorbed with the use of acetone-dried liver powder. Rabbit antisera to AFP were prepared in our laboratory as reported previously (18).

Immunofluorescence Staining. Fluorescence staining was performed by the indirect method of Coons and Kaplan (6) with a slight modification. Sections were deparaffinized in xylene baths after cutting, hydrated through successive ethanol baths, and washed in several changes of cold PBS, pH 7.2. Then, sections were incubated with diluted anti-AFP antiserum for 30 min at 37° and washed in PBS for 30 min. Thereafter, they were stained with FITC-labeled anti-goat antiserum to rabbit γ-globulin for 2 hr at room temperature. They were again washed in PBS for 30 min, mounted in buffered glycerol, and examined with the Zeiss fluorescence microscope.

One of the serial sections was stained with hematoxylin and eosin for the identification of structural localization.

Control. Controls were treated first with preimmune rabbit serum instead of anti-AFP antiserum or with the anti-AFP antiserum absorbed with a purified AFP,[2] before staining with FITC-labeled goat antiserum to rabbit γ-globulin. The purified AFP was obtained from fetal tissues by preparative disc electrophoresis (23) based on the principle of Ornstein and

[1] The abbreviations used are: AFP, α-fetoprotein; FITC, fluorescein isothiocyanate; PBS, phosphate-buffered saline.

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[2] K. Hironaga, Pharmacy Department, Yamaguchi University School of Medicine, unpublished data. A single peak appeared in the α_1-globulin region when the purified AFP was separated by polyacrylamide gel electrophoresis.

Davis (19). Direct staining of sections with labeled antiserum to rabbit γ-globulin was also performed.

Detection of Serum AFP. Serum AFP was detected by the Ouchterlony gel-diffusion technique (20). The concentrations of serum AFP were determined by single radial immunodiffusion with the anti-AFP antiserum and 1.5% agar (Difco Specific Noble) in Veronal buffer, pH 8.6, with an ionic strength of 0.07 (17). In order to obtain greater sensitivity for the detection of AFP, we used fluoroelectroimmunodiffusion based on the immunoelectrosyneresis of Bussard and Huet (5). In this procedure, after immunoelectrosyneresis in agar gel, FITC-labeled antiserum to rabbit γ-globulin was placed in the anode well, and electrophoresis was performed under the same conditions as immunoelectrosyneresis. This technique permits the detection of 0.05 mg AFP per 100 ml serum. The AFP level of the standard serum was determined by planimetry of scans obtained by polyacrylamide gel electrophoresis of the serum (16); concentration of the standard serum was 428 mg/100 ml serum. From this serum, a series of 17 progressively diluted solutions was prepared for calibration (428 mg/100 ml → 0.015 mg/100 ml).

RESULTS

Localization of AFP. In sections of liver tissues which were caused to react with anti-AFP antiserum followed by fluorescence-labeled antiserum to rabbit γ-globulin, there was a bright fluorescence of tumor cells, although not all the tumor cells on the slide showed fluorescence. The fluorescence of tumor cells appeared in 3 forms: (*a*) diffuse, finely granular fluorescence of the cytoplasm (Fig. 1); (*b*) fluorescent line of the cytoplasmic membranes; and (*c*) brightly fluorescent line of the perinuclear zones (Fig. 2). The 1st type was the most common. The differentiating histological characteristic of the tumor cells showing fluorescence in comparison with those not showing fluorescence was the presence of acidophilic granular cytoplasm with a larger nucleus, but in many cases it was difficult to find any difference between the histological features of the tumor cells showing fluorescence and those not showing fluorescence.

Fluorescence was in many cases found in the cytoplasm, as well as in the cytoplasmic membrane, and the 1st type was undistinguishable from the 2nd type. In the specimens of Grade III carcinoma, diffuse, finely granular fluorescence was sometimes seen in the acidophilic cytoplasm of giant cells (Fig. 1*b*). The 3rd type of fluorescence was seen in 2 cases, in which also nuclei were larger and more hyperchromatic than those of the tumor cells showing no fluorescence and occupied a greater portion of the tumor cells (Fig. 2). In only 1 case could all 3 types be seen in the same specimen. The distribution of the tumor cells showing fluorescence varied with the case and the specimen. Sometimes there was a mass of tumor cells showing fluorescence (Fig. 3), but in many cases the tumor cells showing fluorescence were one-fifth or less of the total tumor cells. Sometimes fluorescent granules were found in the cytoplasm of Kupffer cells and round cells, but neither connective tissues, nor bile ducts, nor hepatic cells were fluorescent.

In sections of fetal liver, diffuse granular fluorescence was found in the cytoplasm of the hepatic cells (Fig. 4). One-third of the hepatic cells were fluorescent, and a number of them appeared to be binucleate. Although there were numerous hemopoietic islands in the parenchyma, no hemopoietic cells were fluorescent.

The immunofluorescent staining was specific for AFP, failing to stain normal human liver obtained from patients with other diseases (Fig. 5) or liver carcinoma metastasized from the stomach except for 1 positive case in which AFP was detected in the serum. Specificity of immunofluorescent reaction was demonstrated by the negative results obtained by treatment with preimmune rabbit serum or with anti-AFP absorbed with purified AFP before staining with FITC-labeled goat antiserum to rabbit γ-globulin. Direct staining of the section with labeled antiserum to rabbit γ-globulin was also nonreactive.

Relationship between Fluorescence and the Serum AFP Levels. Table 1 presents the appearance of fluorescence, the serum AFP levels, and the histological gradings of primary liver carcinoma. Serum AFP was detected by single radial immunodiffusion in 8 of 10 patients with primary hepatocellular carcinoma. In 2 patients in whom serum AFP could not be detected by single radial immunodiffusion, a trace amount of serum AFP was detected by

Table 1

Relationship between localization of AFP and levels of serum AFP or histological gradings of primary hepatocellular carcinoma

Patient	Sex	Age	Histological findings	Serum AFP level (mg/100 ml)	Fluorescence of tumor cells		
					Cytoplasm	Cytoplasmic membrane	Perinuclear zone
J. W.	M	61	Grade IV anaplasia	26	+[a]	+	–
S. M.	M	74	Grade IV anaplasia	Trace	–	–	–
Y. N.	M	39	Grade III trabecular	121	+++	+	–
S. K.	M	61	Grade III trabecular	2.7	+	+	+
S. H.	F	56	Grade II tubular	24.0	+++	+	–
T. N.	M	29	Grade II trabecular	13.0	+	+	–
Y. Y.	F	50	Grade II trabecular	6.0	+	±	–
K. Y.	M	63	Grade II trabecular	1.6	+	–	–
H. H.	M	56	Grade II trabecular	Trace	+	–	–
T. D.	M	51	Grade II trabecular	0.5	–	±	++

[a] –, no fluorescence; ±, equivocal fluorescence; +++, widespread fluorescence.

fluoroelectroimmunodiffusion. Nine of 10 patients having AFP in their sera had specific fluorescence in the tumor cells. There was no correlation between the levels of serum AFP and the occurrence of fluorescence in the tumor cells, except that widespread fluorescence in the cytoplasm appeared to be more frequent in 1 patient with high levels of AFP. One patient who had no fluorescence in the tumor cells had a histological diagnosis of Grade IV carcinoma and a trace amount of serum AFP as found by fluoroelectroimmunodiffusion. If a greater number of specimens taken from various parts of hepatoma had been examined, fluorescence might have been detected in some of the tumor cells.

Relationship between Fluorescence and the Histological Gradings of Primary Liver Carcinoma. Six patients had a histological diagnosis of Grade II carcinoma, 2 patients had Grade III, and 2 had Grade IV. Diffuse, finely granular fluorescence of the cytoplasm of tumor cells appeared to be more common in both Grade II and Grade III carcinoma. However, no correlation was found between the occurrence of fluorescence and the histological gradings of primary liver carcinoma by Edmondson and Steiner (8).

DISCUSSION

By means of the indirect immunofluorescent technique with anti-AFP antiserum, we have demonstrated fluorescence in tumor cells. This reaction is specific for AFP because of the specificity of antiserum, the positive inhibition test, and the failure to stain normal hepatic cells. So that a good supply of antiserum to AFP could be obtained, rabbits were immunized with a supernatant (soluble in 34% saturated ammonium sulfate) of serum from a patient with primary liver carcinoma (18), since antiserum prepared with immunization against fetal serum contained several other fetal or tissue-specific antibodies (4). As judged by the complete absence of fluorescence in cell nuclei and connective tissue, the convenient paraffin embeddings could be utilized in the preparation of sections for detecting AFP without solubilization of AFP in tissue and destroying their antigenicity. The superiority of paraffin embedding has also been reported by Engelhardt *et al.* (9).

AFP was detected in the tumor cells from 9 of 10 patients with primary hepatocellular carcinoma. Even the patient with a trace amount of serum AFP undetectable by single radial immunodiffusion had bright fluorescence in the tumor cells, which suggests that the immunofluorescence technique is more sensitive for detection of AFP than the precipitin tests. However, this inference seems contradictory to the report of Goussev *et al.* (12), who found no AFP in the tumor cells obtained from 2 patients with low levels of serum AFP. The reason for these differences is not clear at the moment. The authors believe that anti-AFP should be diluted as much as possible for detection of small amounts of AFP in hepatoma tissues; also, specimens should be obtained from various parts of the hepatoma material. This principle has been observed throughout the present study.

AFP has been detected in the serum in 28 to 90% of the patients with primary liver carcinoma (2, 3, 10, 18, 21, 25). The lowest limit of sensitivity with the Ouchterlony and single radial immunodiffusion techniques, which are most commonly used now for the detection of serum AFP, was about 0.5 mg/100 ml. In the present study, the immunofluorescent technique revealed the localization of AFP in tumor cells obtained from a patient with primary liver carcinoma in whom the Ouchterlony technique failed to detect serum AFP. Fluoroelectroimmunodiffusion was also sensitive enough to yield positive reactions in sera negative both by the Ouchterlony technique and the single radial immunodiffusion. These observations suggest that, in the majority of patients with primary hepatocellular carcinoma, AFP is present in the serum.

AFP of tumor cells was localized in 3 forms, whether or not these localizations represent the site of active synthesis cannot be determined. The tumor cells showing diffuse, finely granular fluorescence of the cytoplasm seem to synthesize AFP because the pattern of localization of AFP in the cytoplasm of these cells is similar to that of the cytoplasm of fetal liver, which is known to produce AFP (11, 26). The distribution of these tumor cells is even throughout the hepatoma tissue. Interesting results have been reported by Engelhardt *et al.* (9), who used immunofluorescence techniques to detect AFP in hepatoma tissue. In their investigations, specific AFP localization was observed in a small number of tumor cells near the capillaries and blood vessels. They suggested that these cells were synthesizing AFP. Although we could not observe similar findings in hepatoma tissue, further observations are necessary for the understanding of AFP synthesis in tumor cells.

AFP was present in the perinuclear zone of the tumor cells with a small amount of AFP in the serum in 2 cases. The reason for this particular localization of AFP is unclear. The nuclei of cells containing AFP are larger and more hyperchromic than those of the tumor cells containing no AFP. If AFP was synthesized in these cells and secreted into the blood, AFP synthesis might depend on the state of undifferentiation of the tumor cells. This hypothesis is also supported by the observations that diffuse, finely granular fluorescence was seen in the acidophilic cytoplasm of giant tumor cells from patients with Grade III carcinoma. However, there is no evidence that such tumor cells are similar to the hepatoblast (7).

In this study, it has been demonstrated that tumor cells contain different amounts of AFP and that not all tumor cells contain AFP. This strongly suggests that AFP is not a structural component or a marker of tumor cells. It has been proposed that the embryo-specific α-globulin, fetuin, promotes the growth of mammalian cell culture (15). Whether or not AFP serves as a stimulator for the growth of tumor cell is not yet known.

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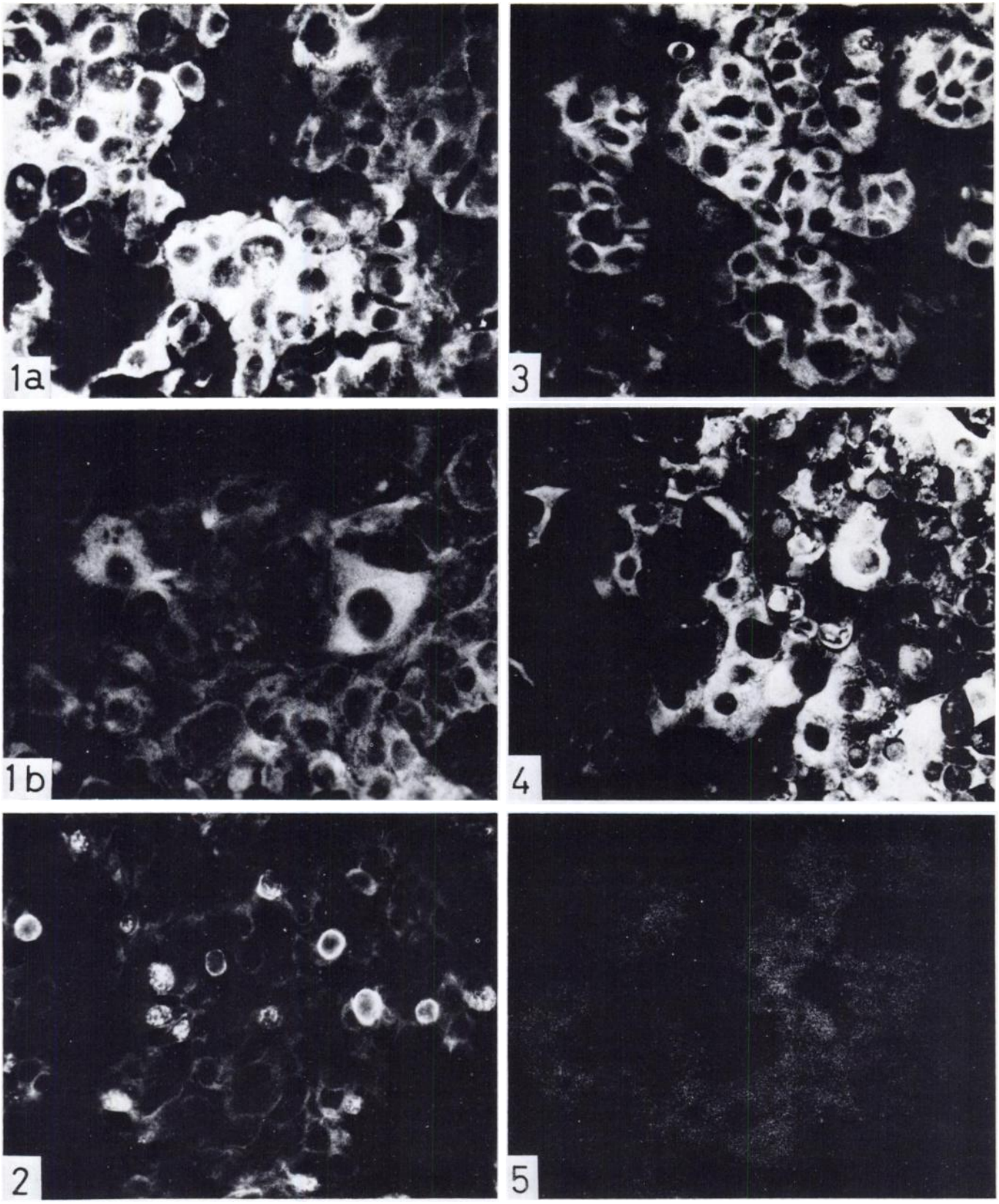

Fig. 1 Section of a primary hepatocellular carcinoma (Patient Y. N.). *a*, diffuse, finely granular fluorescence of cytoplasm; *b*, the giant-sized cytoplasm was fluorescent; nuclei and connective tissues were nonreactive. × 650.

Fig. 2. Section from Patient T. D. A brightly fluorescent line of the perinuclear zone was found. Cytoplasm and cytoplasmic membranes had equivocal fluorescence. × 650.

Fig. 3. Section from Patient S. H. Fluorescence was found in a mass of tumor cells. × 650.

Fig. 4. Section of fetal liver. Diffuse, finely granular fluorescence of cytoplasm was found. Some of the hemopoietic cells had equivocal fluorescence. × 650.

Fig. 5. A section of hepatic cirrhosis. The hepatic cells and the connective tissue appeared dark with no fluorescent granules. × 650.