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Evaluation of Anti-AFP Monoclonal Antibodies as Immunodiagnostic Reagents for Hepatocellular Carcinoma

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To check the possible application of our anti-AFP monoclonal antibodies (MAbs) as immunodiagnostic reagents for hepatocellular carcinoma, ELISA and immunohistochemical assay were performed on the sera and liver biopsy specimens from the patients of hepatocellular carcinoma and other non-malignant hepatic disease. By non-competitive ELISA using anti-AFP MAbs, the highest incidence of AFP value was found only in the sera of hepatocellular carcinoma patients, i.e., more than 54% of patients had serum AFP levels of more than 500 ng/ml. By immunoperoxidase and indirect immunofluorescence techniques, anti-AFP MAbs were found to react with cytoplasm of hepatocellular carcinoma cells. However immunohistochemical reactivity to AFP in hepatocellular carcinoma cells was lower than that in non-neoplastic liver cells adjacent to the hepatocellular carcinoma. From these results with the similar findings from other studies, we suggest that AFP antigen is appropriate in the diagnosis assay (ELISA) but is not by immunohistochemical detection.

KEY WORDS: Alpha-Fetoprotein, Monoclonal Antibody, Hepatocellular Carcinoma

Alpha-fetoprotein (AFP) is a major plasma α_1-glycoprotein present in the early stage of developement in human and animals, which virtually disappears in adult life. Reappearance of AFP in adult serum often signals several malignant disease, particulary hepatocelluar carcinoma, teratoblastoma and gall bladder carcinoma (Rouslaliti and Seppala 1979; Brown *et al.*, 1992). Elevated serum AFP levels in amniotic fluid or maternal serum during pregnancy also have been found to be related to neural tube defects and other pregnancy disorders (Fuhrmann and Weitzel., 1985; Korner *et al.*, 1986; Macri *et al.*, 1992; Sundaram *et al.*, 1992). The AFP, therefore, has been regarded as one of the remarkable tumor-associated antigens, and the determination of AFP levels has been used in diagnosis of adult cancers associated with AFP as well as in prenatal diagnosis of certain pregnancy disorders (Rouslahti *et al.*, 1982; Chen *et al.*, 1984; Phillips *et al.*, 1992).

Immunological assay systems are of importance in characterizing the antigenic structure of AFP molecule and can allow the elucidation of molecular structure and properties, from which physiological function of this protein can be deducible (Mayforth, 1993). A major advance in molecular immunology is the production of monoclonal antibodies (MAbs) by somatic cell fusion technique (Kohler and Milstein, 1975), which leads to an enormous breakthrough in the ability of making large quantities of uniform and homogeneous antibody reagents (Liddel and Cryer, 1991).

In our previous study MAbs against human AFP were produced by hybridizing SP 2/0-Ag 14 mouse myeloma cells with spleen cells of Balb/c mice immunized with purified AFP. Two MAbs, D-

6 and E-2, were identified to be homogeneous by several criteria and to have high affinity constant to AFP (Kang *et al.*, 1993).

In this study, the evaluation of our Mabs as immunodiagnostic reagents for the sera and liver biopsy specimens from patients with hepatocellular carcinoma or other non-malignant hepatic diseases was performed by enzyme-linked immunosorbent assay (ELISA) and immunohistochemical methods.

Materials and Methods

Antibodies and Samples

The anti-AFP MAbs (D-6 and E-2) were produced and characterized as reported previously (Kang *et al.*, 1986; 1993). Tissue specimens and serum samples from normal donors and patients with a verified diagnosis were obtained from Dongsan Hospital, Taegu and stored at –20°C until use.

Enzyme linked Immunosorbent Assay

Non-competitive ELISA using monoclonal antibodies was performed in sera of 173 normal persons, 37 patients with hepatocellular carcinoma and 48 patients with other hepatic diseases. Wells in mcrotiterplate were coated with 100 μl of purified MAb (D-6, 1 μg/ml) in borate buffered-saline and incubated at 37°C for 2 hr, followed by blocking with 200 μl of 1% gelatin in PBS at 37°C for 1 hr. The wells were washed with PBS-Tween 20, and 100 μl of various concentrations of AFP or serum samples were added to the wells. After incubation at 37°C for 2 hr and subsequent washing, the wells were filled with 100 μl of alkaline phosphatase-conjugated antibody (E-2) and the plate was incubated at 37 °C for 2 hr. After another extensive washing, 100 μl of p-nitrophenyl phosphate (4 mg/ml in carbonate buffer, pH 10.0) was added as enzyme substrate, and the plate was incubated for 30 min at room temperature. The color development was stopped by adding 50 μl of 2 M NaOH, and the absorbance was read at 405 nm with a Titertek Multiskan plus (Kang *et al.*, 1988).

Immunohistochemical Analyses

Paraffin sections of formalin-fixed tissue specimens from human hepatoma or hepatitis were deparaffinized and stained with hematoxylin and eosin.

For peroxidase anti-peroxidase staining the deparaffinized sections were immersed for 30 min in methanol containing 3% hydrogen peroxide for inhibition of endogeneous peroxidase activity. Sections were incubated with goat serum for 20 min at room temperature to minimize any nonspecific binding. They were then incubated in monoclonal anti-AFP antibody (clone E-2) for 20 min at room temperature. And then they were reacted with anti-mouse IgG for 20 min at room temperature. Sections were then icubated for 20 min at room temperature in horse raddish peroxidase mouse anti-peroxidase (PAP) complex diluted 1:20 in PBS. The activity of peroxidase revealing the amount of antibody E-2 bound to tissue sections was detected by adding reaction mixture (0.027% 3-amino-9-ethylcarbazole-0.067% N,N-dimethyloformamide-0.12% H_2O_2 dissolved in 10 mM acetate buffer, pH 5.2). The washed sections were then lightly counterstained with Hematoxylin and mounted with glycerin jelly (Falin *et al.*, 1983).

For immunofluorescene staining tissue specimens were frozen in OCT-cryogel and prepared the sections (3-5 μm thick) with Cryocut Cryostat microtome (American Optical Model 851c). The tissue section was fixed by immersion in PBS containing 4% paraformaldehyde for 20 min at room temperature. These sections were incubated with 5% bovine albumin in PBS for 1 hr at 37°C to minimize any nonspecific binding. They were then reacted with anti-AFP (E-2) for 1 hr at 37°C, followed by washing the slide with PBS. The samples were then visualized by treating with FITC-conjugated rabbit anti-mouse IgG (Sigma Chemical Co.) for 30 min. Flurorgraphy was performed using Fluorescence Photomicrography system (Nikon Ophiphot).

Results and Discussion

To determine the AFP value of serum in clinical

practice, we assayed 173 sera from normal human, 37 sera from hepatoma patients and sera from patients of other liver diseases by non-competitive ELISA. As shown in Fig. 1, the AFP values in sera of healthy subjects were less than 10 ng/ml. The highest incidence of AFP value was found only for sera of primary hepatocellular carcinoma patients, *i.e.*, more than 54% of patients had serum AFP levels more than 500 ng/ml. However, most of other serum samples from patients with gastrointestinal carcinoma or other non-malignant hepatic diseases like hepatitis and liver cihrrosis showed AFP levels less than 15 ng/ml. Therefore, it can be said that higher serum value of AFP is strongly correlated with hepatocellurar carcinoma, and screening of serum AFP levels is essential in diagnosis of this malignancy.

In general hematoxylin and eosin (H & E) staining method for liver biopsy specimen from hepatoma patients was used for routine diagnosis for hepatoma. In H & E stain (Fig. 2A), hepatocellular carcinoma cells showed clear cell forms with increase in size, large nucleus, vacuolated appearance due to abundance of fat, and irregular cell organization. However, these data could not be correlated with the degree of morphological differentiation of hepatocellular carcinoma, primary tumor or metastatic foci.

Recently, several tumor markers which are carcinoembryonic antigen (CEA) and AFP are applied to detect hepatocellular carcinoma (Iomoto *et al.*, 1985; Koelma *et al.*, 1986). To check the reactivity of anti-AFP Mab to hepatocellular carcinoma biopsy specimen, PAP staining was performed. AFP-positive cells of hepatocellular carcinoma biopsy section displayed cytoplasm containing red granules (Fig. 2B). It appeared as lobular and disarray in fatty cirrhotic hepatocyte (Fig. 2B), and well differentiated pseudoglandular cells (Fig. 2C). Unexpectedly non-neoplastic hepatocytes adjacent to hepatocellular carcinoma cells showed positive to anti-AFP (data not shown). And positive reaction was also detected in tissues from liver cirrhosis patient (Fig. 2D), but negative in tissues from hepatitis. Indirect immuno-fluorescence of hepatocellular carcinoma tissue stained with anti-AFP Mab followed by FITC-anti-mouse IgG showed similar pattern as of PAP staining (data not shown).

Immunohistochemical results from patients with hepatocellular carcinoma or other malignant liver diseases were summarized in Table 1. As shown in Table 1, positive reaction to AFP was found to be 30% by PAP method and 32% by immuno-fluorescence method in hepatocellular carcinoma tissue sections analyzed. Still positive rate to AFP in non-neoplastic region of same tissues was more

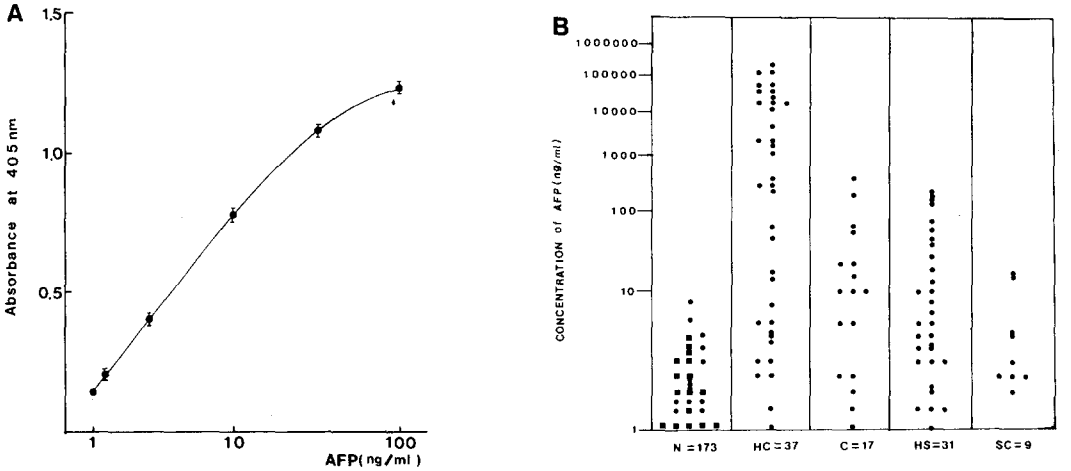

Fig. 1. Determination of serum AFP concentrations measured by ELISA. (A) Standard curve for AFP. (B) Human serum AFP concentrations measured by ELISA. Sera were collected from the following patients. N: Normal, H: Hepatocellular carcinoma, C: cirrhosis, Hs: Hepatitis, S: Stomach carcinoma. ■ : ten patients, ● : one patient.

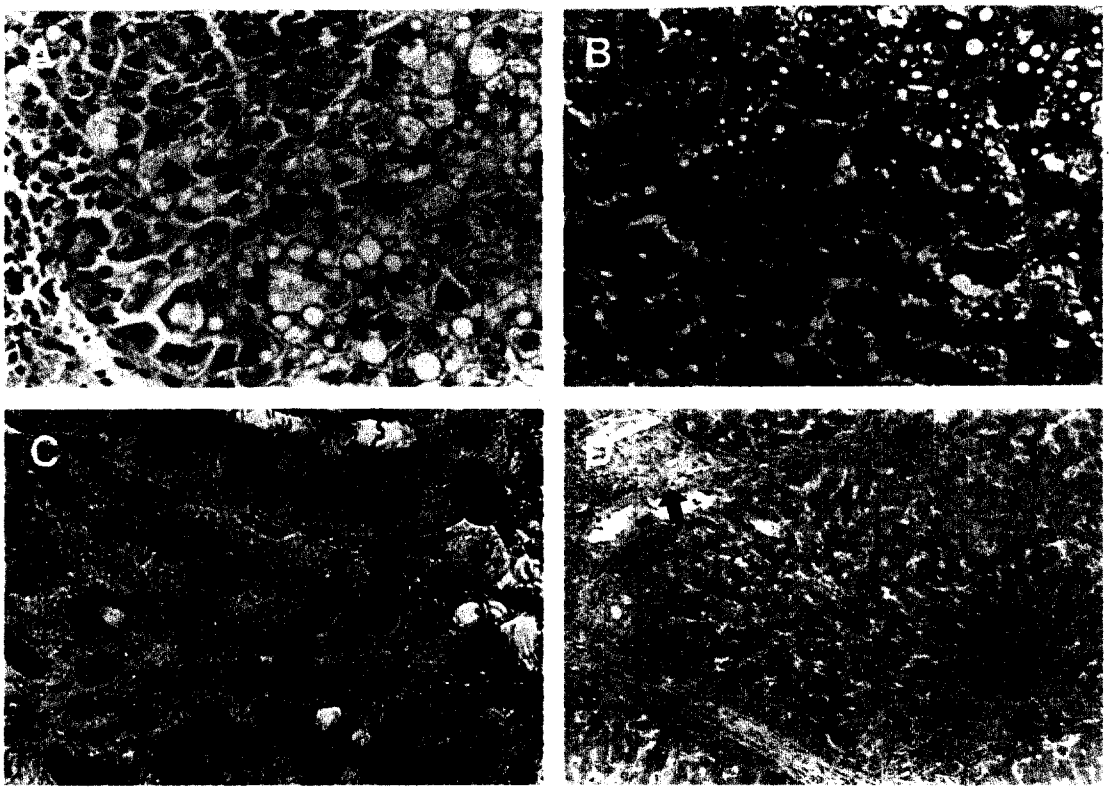

Fig. 2. Histological appearance of liver biopsy specimen stained with hemtoxylin and eosin (A), and immunoperoxidase-hematoxylin stain (B-D). A-C: Tissues from hepatocellular carcinoma patients; D: tissue from liver cirrhosis patient. (A) Hepatocellular carcinoma cells showing dysplasia and pleomorphism (× 100). The closed arrow indicates the clear-cell form showing a vacuolated appearence due to abundance of fat (➡), and the open arrow indicates the nuclei displaced to the periphery by large fat globule (⇨). (B) Positive reaction to AFP showing fatty cirrhotic hepatocyte (➡) and lobular disarray (⇨, × 100). (C) Positive reaction to AFP showing well-differentiated pseudoglandular hepatocyte (➡, × 400). (D) Positive reaction of cytoplasm to AFP in cirrhosis (➡) and thick fibrous band (⇨, × 100). All the tissue sections were formalin-fixed samples.

Table 1. AFP reactivity in immunohistochemical analyses of hepatocellular carcinoma and other liver deseases stained by immunoperoxidase and indirect immunofluorescence

Type of Tissue	Reactivity by	
	Immunoperoxidase	Indirect immunofluorecence
Hepatocellular carcinoma (HCC)	30% (11/37*)	32% (12/37)
Non-neoplastic region in HCC	40% (15/37)	43% (18/37)
Liver cirrhosis	23% (4/17)	29% (5/17)
Hepatitis	6% (2/31)	3% (1/31)
Stomach carcinoma	0% (0/9)	0% (0/9)

*No. of patients. For each biopsy case, three sections of the tumor were examined.

than 40%, which indicates that different immunoreactivities were displayed according to the positions of tissue sections. This result was in accordance with Koelma's (1986) and Ganje's (1988) studies.

Hepatocellular carcinoma cells were reported to contain AFP, CEA and ferritin heterogeneously. By immunohistochemical analyses using PAP, ferritin was detected in 70% of the cases, AFP was in 50% and CEA in 30% and some cells displayed two or more antigens simultaneously (Imoto, 1985). The distribution of these markers were confirmed to vary from tumor to tumor and from cell to cell in hepatocellular carcinoma even when malignant cells have similiar morphological appearance as reported by Thung *et al.* (1979) and Watanabe *et al.* (1983).

Immunohistochemical results indicate that AFP synthesis rate in cancer tissue is not correlated with AFP deposition in cells because AFP is secretory, not membrane bound, although the synthetic rate of AFP in hepatocellular carcinoma is high. Thus, we suggest that AFP antigen can be applied in the diagnosis of hepatocellular carcinoma by ELISA but is not appropriate in the diagnosis by immunohistochemical detection.

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항-AFP 단일클론 항체를 이용한 간암진단 효과의 검토

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항-AFP 단일 클론 항체를 생산한 다음, 이를 이용한 noncompetitive ELISA 방법으로 정상인과 간암 및 그 밖의 간질환 환자의 혈청내 AFP농도를 측정해 본 결과 간암진단 방법으로는 혈청 AFP농도 측정이 필수적임이 확인 되었다. 또한 간암 및 그 밖의 간질환 환자의 조직에 대한 항-AFP-항체의 반응성을 immunoperoxidase방법과 indirect immunofluorescence방법으로 검정해 본 결과, 간암조직세포 및 일부 간질환 조직세포에서 항-AFP항체에 대해 양성반응을 나타내었다. 그러나 그 반응성의 정도는 간암조직세포에서 보다 간암조직 주위의 비신생 간세포에서 더욱 높았다. 그러므로 간암 진단에 있어서 AFP항원을 면역조직화학적으로 검정하는 방법은 적합하지 않았다.