Technical Report

Pretreatment of Serum Using a Microplate for Fractional Determination of Low Concentrations of α-Fetoprotein

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Summary An immunological pretreatment method using a microplate was developed for fractional determination of low concentrations of α-fetoprotein (AFP) in serum. This method allows simultaneous partial purification and concentration of AFP from many serum samples. The pretreated sample can then be subjected to lectin-affinity electrophoresis (LAE) coupled with detection by antibody-affinity blotting. More than 60% of the AFP was recovered by the partial purification from serum containing less than 100 pg/I of AFP, and the specific activities for the total protein was raised more than 20-fold. There was no bias or shift in AFP composition between the untreated and partially purified samples. By the present pretreatment method, AFP at a concentration of as low as 10 μg/I in serum was concentrated and detected by the LAE coupled with the antibody-affinity blotting.

Key words: partial purification, antibody-affinity blotting, glycoprotein, sugar chain, diagnosis of hepatocellular carcinoma

Introduction

Alpha-fetoprotein (AFP) is a well-characterized carcinofetal glycoprotein possessing a complex-type asparagine-linked sugar chain1). Serum AFP has been measured exclusively for diagnosis and surveillance of malignant diseases such as hepatocellular carcinoma2). However, the serum concentration of AFP also increases in non-malignant diseases during hepatic regeneration3). Also, AFPs of different origins display different microheterogeneities in the carbohydrate moiety4,5), although the structure of the protein moiety and immunoreactivity are identical. The fractional determination of AFP-glycoform, based on the affinity of sugar chains of AFP with certain lectins, is useful for early diagnosis of hepatocellular carcinoma and for distinction between benign and malignant liver diseases6-8). Taketa et al.9 developed an excellent method for fractional determination of serum AFP-glycoform by lectin-affinity electrophoresis (LAE) coupled with detection by antibody-affinity blotting. However, separation of AFP-glycoforms is not sufficient when serum is subjected to phytohemagglutinin-E4 (PHA-E4)-LAE without moderate dilution. Also, it is difficult to quantify each separated fraction when the AFP concentration in serum is low (< 50 μg/I)9). Detecting such minor fractions is of interest to pathological physiologists. This led us to develop a partial purification method using a microplate based on an immunological reaction as a pretreatment for serum to be subjected to fractional deter-
mination of AFP by LAE.

**Materials and Methods**

Reagents not specified were of analytical grade from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Serum samples, after measurement of AFP concentrations, were stored at -20°C until use. AFP-free serum was a pool of sera containing less than 2 μg/l of AFP. Serum containing 125I-AFP (Baxter Inc.; Deerfield, IL) was prepared by mixing serum with AFP of 50 μg/l and a 1/10 volume of 125I-AFP (0.5 mCi/l).

The antibody-coated microplate was prepared by adding rabbit anti-human AFP polyclonal antibody (Cosmo-bio Co. Ltd.; Tokyo, Japan) diluted to 10 mg/l with 50 mM carbonate buffer, pH 9.5, to each of microplate wells (0.2 ml/well) (Module plate, MaxiSorp; Nunc; Roskilde, Denmark), and keeping then kept at 4°C for 24 h or longer (up to 6 months). Antibody-coated membranes and lectin-contained agarose gel films were prepared as described by Taketa et al.9)

The antibody-coated microplate was washed with 0.2 ml of 10 mM HCl, followed by washing with 0.3 ml of 10 mM phosphate-buffered saline, pH 7.4, containing 0.01% of Tween 20 (Bio-Rad Lab.; Richmond, CA). A sample (0.2 ml) was added to the microplate, and then incubated for 4 h at 25°C with gentle shaking. After the solution was discarded, 0.2 ml of 10 mM HCl was added and incubated for 5 min at 25°C, followed by a transfer to a test tube and neutralization with 0.1 M NaOH. After evaporation of the mixture with a centrifugal concentrator (Sakuma Industries Ltd.; Tokyo, Japan), the residue was redissolved with an appropriate volume of 25 mM sodium barbiturate buffer, pH 8.6, (SBB) containing bromophenol blue (0.5 g/l) and subjected to LAE.

We modified the method of LAE and antibody-affinity blotting described by Taketa et al.9) without affecting the detection limit. Briefly, 5 μl of sample was applied to agarose gel (10 g/l, 1 mm thickness) containing 0.05% PHA-E₄ (Honen Co.; Tokyo, Japan) and electrophoresis was done at 5°C with SBB (15 V/cm, 60 min). After the migration, the agarose gel was covered with an antibody-coated membrane soaked in 20 mM Tris-HCl-buffered saline, pH 7.5 (TBS), followed by overlapping with filter paper pads and loading with a weight. After blotting, the membrane was washed twice with 10 ml of a wash buffer (0.2 M α-α-methylglucopyranoside, 0.05% Tween 20 in TBS) and incubated with 10 ml of peroxidase-conjugated rabbit anti-human AFP polyclonal antibody (Dako-immunoglobulins; Copenhagen, Denmark) diluted 100-fold with TBS containing 0.05% Tween 20. The membrane was washed twice with 10 ml of the wash buffer, followed by incubation with TBS containing 0.02% 3,3'-diaminobenzidine tetrahydrochloride, 0.02% H₂O₂ and 0.06% NiCl₂. After color development, the membrane was washed with water and dried. Stained bands on the membrane were scanned at 610 nm with a densitometer (Cliniscan, Helena Lab.; Beaumont, TX).

The differences in the mean values between the two groups, and the Y-intercept and slope of the regression lines, were assessed by Student’s t-test.

**Results**

Figure 1 shows the recovery of AFP in serum by partial purification. For serum AFP at a concentration of up to 100 μg/l (20 ng/well), the AFP recovery was more than 60%, and purification was more than 20-fold with respect to the total protein in serum. The same recovery of radioactive counts was obtained in an experiment using serum containing 125I-AFP (not shown). When serum AFP was more than 100 ng/l, the recovery was less than 50% and decreased with the increase of the amount of AFP applied. The maximum amount of recovered AFP was 20 ng per well. When AFP was less than 5 μg/l in serum, in spite of the partial purification, AFP bands were not detected by the differentiation test. As shown in Fig. 2, intact serum (lane B) did not show a complete separation pattern for the AFP fraction on PHA-E₄-LAE, i.e., the migration of P4 and P5 bands was faster.
Fig. 1 Recovery of AFP in serum by partial purification
The partial purification is described in "Materials and Methods". AFP was immunologically assayed.

Fig. 2 Representative separation patterns of PHA-E\textsubscript{a}-LAE
Each band was numbered in the order of migration speed\textsuperscript{9}, according to the pooled control serum containing all AFP bands (A, diluted 500-fold with SBB). The patient's serum (90,000 µg/l AFP) was diluted 300-fold with AFP-free serum (B) or SBB (C), and sample B was further treated by the partial purification (D).

Fig. 3 Comparison of AFP subfraction percentage found with the partial purified sample (\(y\)) and the untreated sample (\(x\))
The patient's serum was fractionated by PHA-E\textsubscript{a}-LAE directly (50–200 µg/l of AFP) or after dilution with SBB (200–350,000 µg/l of AFP) in parallel with the partial purification, and the percentage of the subfraction of P4+P5 was compared.

\[ y = 0.88x + 6.1, \quad r = 0.97, \quad S_y \cdot x = 4.9, \quad n = 20. \]

Discussion
The AFP in serum could not be completely recovered by the partial purification (Fig. 1). Therefore, a partial purification procedure that recovers all AFP-glycoforms equally from serum is necessary for the AFP quantitative differentiation test. The direct evidence for glycan integrity during the purification process was difficult to obtain, because a particular glycoprotein in a crude sample cannot be analyzed without purification except by using lectins. Immunological denaturation of AFP does not appear to take place upon partial purification as the percent recovery in immunoassay was the same as that measurement of \(^{125}\text{I}-\text{AFP}\). There was no bias or shift in the AFP subfractions (P4+P5) between the untreated serum (or diluted with SBB) and
partially purified samples (Fig. 3). Furthermore, specific antibody to a particular AFP-glycoform has not been reported6). Hence, we concluded that the anti-AFP antibody used was specific for AFP regardless of its glycoform and no change in the affinity of the AFP sugar chain to the lectins occurs during the partial purification. Consequently, this partial purification can be used in the AFP differentiation test by LAE.

A large quantity of glycoprotein and sugar relative to AFP in serum is thought to compete with AFP for the reaction of lectin. This may be due to incomplete separation when serum is directly subjected to LAE. Therefore, a method in which most of proteins in serum are concentrated, e.g., an ultrafiltration technique, may not be useful. By contrast, partial purification using a microplate, based on the principle of the antigen-antibody reaction, is a relatively simple procedure for simultaneously concentrating AFP in a large number of serum samples. By applying this method to LAE coupled with detection by antibody-affinity blotting, at least 10 µg/l of AFP in serum could be concentrated, separated and detected.

In general, serum AFP is moderately (< 50 µg/l) elevated in benign, but not malignant, hepatic disorders. However, the clinical significance of the differentiation of low levels of AFP in serum for early detection of hepatocellular carcinoma has recently been reported7,8). Furthermore, it is now possible to determine whether the AFP is of yolk sac or liver cell origin7); in some cases this may be necessary to distinguish liver metastases from AFP-producing tumors. By a combination use of our pre-treatment method and the AFP differentiation test, it will become feasible to analyze more exactly low levels of AFP in serum.

References

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