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Synthetic Peptide-Based Enzyme-Linked Immunosorbent Assay for Human α-Fetoprotein

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α-Fetoprotein (AFP) is a good marker for the detection of several diseases such as hepatocellular carcinoma, gonadal germ cell tumor, gastric tumor, and Down's syndrome. In this study, we developed ELISA, using synthetic peptides corresponding to the epitopes of AFP. Five kinds of peptides were synthesized from AFP to produce antibodies in rats that recognize AFP in human plasma as well as amniotic fluid and do not cross-react with serum albumin. All five kinds of antibodies showed good reactivities with their peptide-keyhole limpet hemocyanin conjugates. Anti-synthetic peptide 1 (R-N-E-Y-G-I-A-S-I-L, 4-13) antibody, in particular, reacted well with AFP as well as synthetic peptide 1-KLH but not with human serum albumin. The binding affinity (Kd) was 2.7×10^{-9} M for peptide 1 and 6.8×10^{-8} M for AFP. The range for measurement of AFP was 10~1,000 ng/ml. The within-assay and between-assay coefficients of variance (CV) were 4.83% and 10.97%, respectively. In a sample of 31 sera and 33 amniotic fluids, there was a good correlation between AFP values determined in this assay and those in a commercial kit. These results indicate that the antibodies against synthetic peptides corresponding to the epitopes of AFP are highly specific to AFP and synthetic peptide-based ELISA would be useful for the measurement of human AFP.

Key Words: Synthetic peptide, ELISA, α-fetoprotein

INTRODUCTION

Among the cancer markers, α-fetorprotein (AFP) has been widely used for 30 years as a diagnostic marker for hepatocellular carcinoma[2,24]. In addition to the hepatocellular carcinoma, AFP is a useful marker protein to diagnose several diseases such as testicular germ cell tumor, gastric carcinoma, Down's syndrome and neural tube defects[1,6,28,29]. AFP-producing neoplasm has also been found in ovary, urinary tract systems, renal pelvis, stomach, pancreas and lung, although it is only rarely elevated[4,5,12,14,16].

AFP is a major embryonal serum protein produced by fetal liver cells, yolk sac cells and some fetal gastrointestinal cells[8]. After birth, the serum concentrations of AFP decrease drastically to levels which are barely detectable in nonpregnant adults except for the above tumors. In contrast, serum albumin, which is the major serum protein synthesized by the adult liver, increases from low levels early in development to high levels after birth and in adult life[20].

AFP belongs to a gene family that is most closely related to serum albumin[9,17,27]. There are several striking structural and functional similarities between AFP and albumin, including amino acid sequence, molecular weight and isoelectric point. The presence of common antigenic determinants within AFP and serum albumin has, moreover, made difficult the production of antisera which are monospecific to AFP.

For these reasons, we attempted to synthesize five kinds of peptides corresponding to the epitopes of AFP, to produce the anti-synthetic antibodies that recognize AFP with no cross-reactivity to serum albumin and to develop the synthetic peptide-based ELISA for human α-fetoprotein. The results of this assay show good correlation with the AFP levels measured in a commercial kit.

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MATERIALS AND METHODS

1. Peptide synthesis

Five kinds of peptides were synthesized by the Korea

Basic Science Institute (Seoul). These sequences were chosen as highly antigenic sites by computer prediction, according to the method of Hopp and Woods[19] and the peptides were also selected for the lack of homology with human serum albumin.

2. Antibody production

The synthetic peptide was covalently conjugated to maleimide-activated keyhole limpet hemocyanin (KLH) by 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) (Pierce, USA). The peptide (2 mg/500 μl) was mixed with KLH (2 mg/200 μl). The peptide-carrier mixture was added to 10 mg of EDC and this reaction continued for 2 hrs at room temperature. The conjugate was purified by gel filtration and the degree of conjugation was estimated by comparing the difference in absorbance at 280 nm. The purified conjugate emulsified with Freund's complete adjuvant was subcutaneously injected into adult Sprague Dawley rats (Daehanbiolink Co, Eumsung). Four weeks later, booster injection was done with freshly prepared emulsion of the conjugate and Freund's incomplete adjuvant. Blood was drawn from the rats, allowed to clot at 4℃, and the antiserum was recovered by low speed centrifugation.

3. Immunoblotting

Immunoblotting was performed according to the method of Towbin *et al*[26]. Proteins from SDS-PAGE were transferred onto nitrocellulose membrane at 40V-constant condition overnight. After transfer, membrane was soaked in a blocking buffer (10 mM sodium phosphate, 150 mM NaCl, 0.05% Tween 20, 1% bovine serum albumin, pH 7.5) for 1 hr at room temperature and washed 3 times with fresh changes of wash buffer (10 mM sodium phosphate, 150 mM NaCl, 0.05% Tween 20, pH 7.5). Next, the membrane was incubated in diluted antiserum against peptide for 1 hr at room temperature. After wash, the membrane was incubated in diluted horseradish peroxidase (HRP)-labelled anti-rat IgG for 1 hr at room temperature and then washed again. A freshly prepared solution of peroxidase substrates, 4-chloro-1-naphthol and H_2O_2, was added to the membrane for the visualization of positive bands.

4. ELISA

Ninety-six well microplates were coated with 100 μl of 10 mM carbonate buffer (pH 9.6) containing various concentrations of AFP as standard or with 100 μl of diluted human samples including serum and amniotic fluid (Eulji General Hospital, Taejon) overnight at 4℃. The wells were washed 3 times with 200 μl of wash buffer (PBS containing 0.5 mg/ml of BSA and 0.05% Tween 20). A blocking buffer (1% BSA in PBS) was added, the plates were incubated for 1 hr at room temperature with gentle agitation, and then the wells were washed 3 times. 100 μl of diluted anti-peptide antiserum was added and the plates were incubated for 1 hr at 37℃. After wash, 100 μl of HRP-conjugated anti-rat IgG was added to the wells, the plates were incubated for 1 hr at 37℃ and then washed again. A freshly prepared solution of o-phenylenediamine and H_2O_2 was added to the wells, and the absorbances at 450 nm were determined by ELISA reader (BIO-RAD, USA).

5. Affinity measurement using ELISA: Free-capture mode

The determination of affinity constant using ELISA has previously been described[30]. The antigen at various concentrations was first incubated in solution with the antibody at constant concentration until equilibrium was reached. The concentration of free antibody was then determined by an indirect ELISA. The antigen at various concentrations (4×10^{-10} M to 2×10^{-7} M) was mixed with a constant amount of antibody (3×10^{-10} M), in 0.1 M potassium phosphate, 2 mM EDTA, pH 7.8, supplemented with 10 mg/ml BSA. After overnight incubation at 4℃, 150 μl of each mixture was transferred and incubated overnight at 4℃ into the wells of a microtiter plate previously coated with apo A-I (150 μl per well, at 1 μg/ml in 50 mM sodium carbonate, pH 9.6 overnight at 4℃), in which free antibody is captured by binding to antigen on the well. After washing in PBS supplemented with 0.5% Tween 20, the bound Igs were detected by adding rabbit Ig with specificity against rat IgG coupled with HRP and measuring the HRP activity retained in each well. Dissociation constant was calculated by the modified Scatchard equation[21].

RESULTS AND DISCUSSION

The clinical significance of human AFP as a marker in the diagnosis and monitoring of certain types of cancer as well as screening for neural tube defects has been exten-

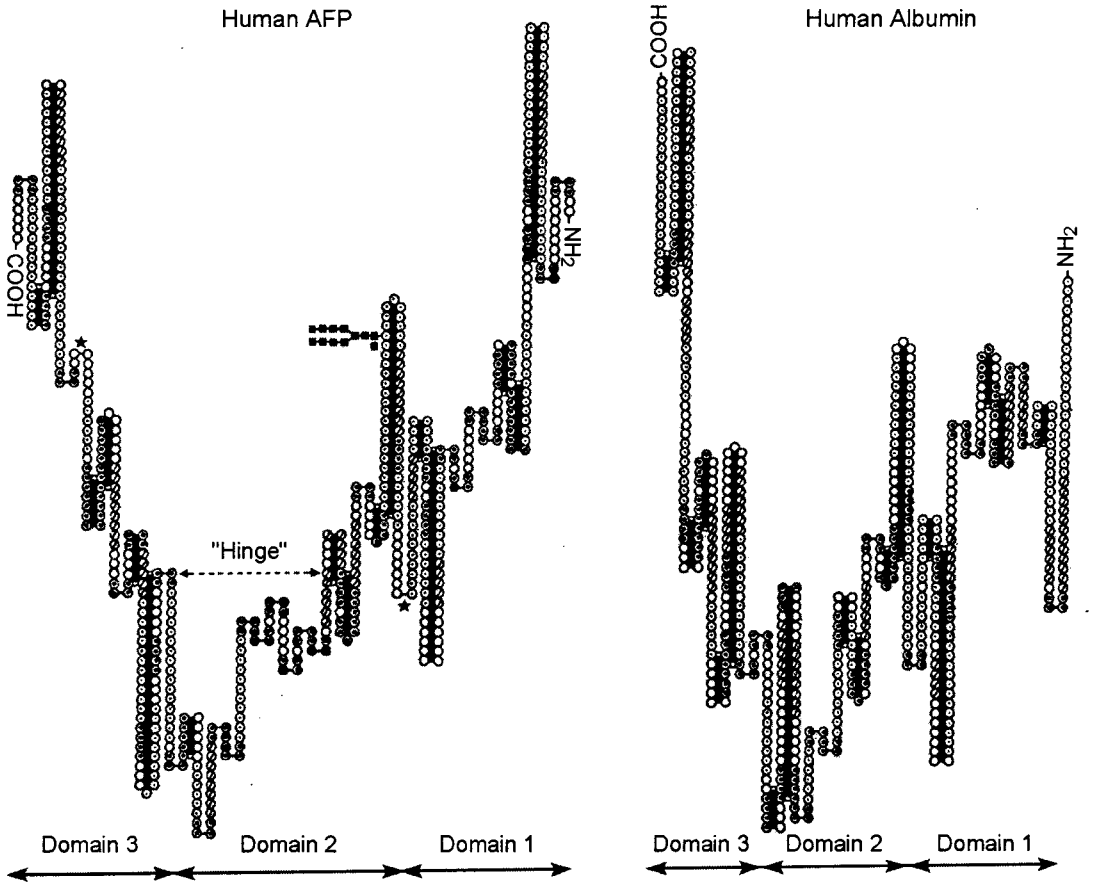

Fig. 1. Molecular configurations of human AFP and human serum albumin based on the secondary structures (Morinaga *et al.*, 1983). The amino acid residues participating in the formation of α-helices, β-sheets, β-turns and random coils are indicated by ⊙, ⊘, ◉ and ○, respectively. The loops formed by disulfide bonding are filled in black. Stars indicate extra turns introduced in human AFP at amino acid residues 195-198 and 504-507. Black squares represent the carbohydrate residues attached to asparagine-232 in AFP.

Table 1. Amino acid sequences and masses of synthetic peptides corresponding to human AFP epitopes

Peptides	Sequences	Masses
Peptide 1	R-N-E-Y-G-I-A-S-I-L (4-13)	1135.3
Peptide 2	E-K-P-T-G-D-E-Q-S-S-G (56-66)	1134.1
Peptide 3	L-F-Q-V-P-E-P-V-T-S (119-128)	1116.3
Peptide 4	M-T-P-V-N-P-Q-V-G-Q (471-480)	998.1
Peptide 5	I-N-L-V-K-Q-K-P-Q-I (535-544)	1179.4

sively reviewed[7]. AFP has been one of the first tumor-associated antigens proven useful in the diagnosis and evaluation of therapy of hepatocellular carcinoma and testicular germ cell tumors[3,25,28].

In the previous study, we have produced polyclonal and monoclonal antibodies and developed ELISA for human AFP[31,32]. However, these assays have certain limitations. One of the several problems in the development of AFP assay is that it is not easy to obtain large amounts of highly purified AFP and antibody specific to AFP due to the similarities of the overall structures of AFP and serum albumin[9,17,27]. Many studies have been directed to show the relationship between AFP and serum albumin. Primary, secondary, and tertiary structural aspects of AFP appear similar to the three-domain concept proposed for serum albumin (Fig. 1). The primary sequence of AFP departs most widely from serum albumin in the first 135 amino acid residues, with about 42% of the remaining 590 residues of the human proteins being identical. For this reason, we synthesized five kinds of peptides corresponding to the epitopes of AFP and lacking in homology with serum albumin (Table 1), and also produced antibodies that react with AFP in human plasma as well as amniotic fluid and have good specificity and sensitivity against AFP, using synthetic peptides.

The two most important advantages of anti-peptide an-

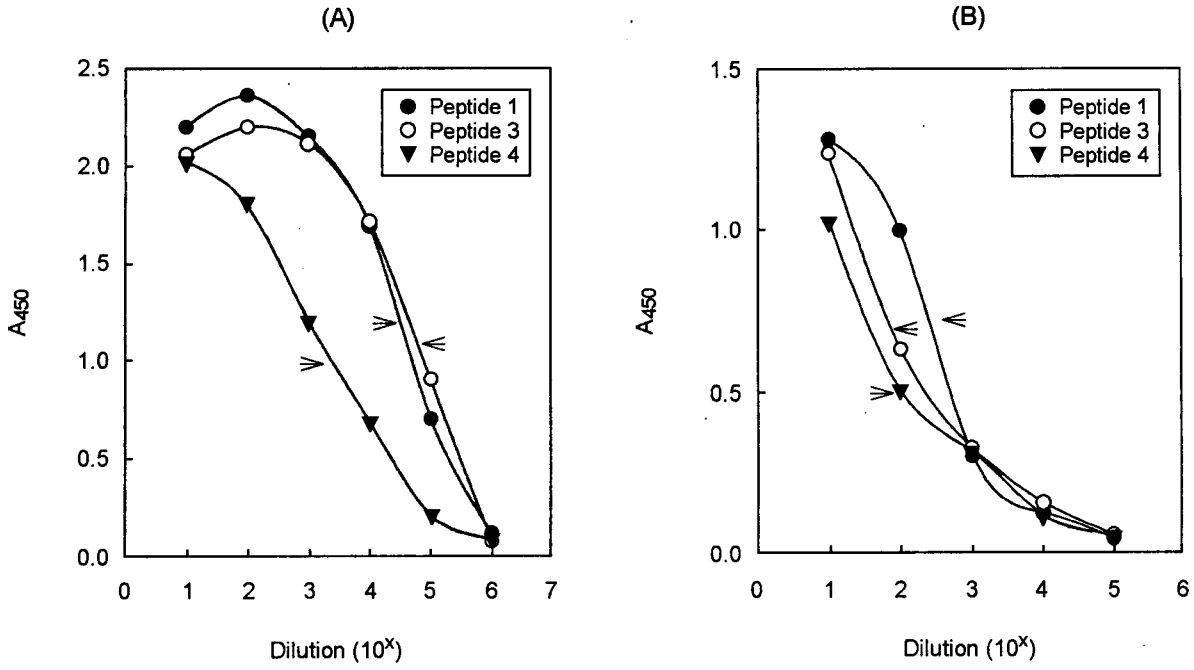

Fig. 2. Titration curves of anti-synthetic peptide antisera, using synthetic peptide-KLH conjugate **(A)** or AFP **(B)** as antigen. The antisera were diluted as indicated on the abscissa and used in the ELISA. The arrows indicate optimal dilution points where 50% of maximum binding was obtained.

Table 2. Result of the specificity analysis of whole antiserum raised against synthetic peptide 1-KLH conjugate by ELISA

Antigens	Specificity (%)
Peptide 1-KLH Conjugate	100*
Human AFP	46
Human serum albumin	1

* Relative specificity of the antiserum to each coated antigen is represented when 100% specificity was determined by binding of peptide 1-KLH conjugate to antiserum

tibodies are that they can be prepared immediately after determining the amino acid sequence of a protein (either from protein sequencing or from DNA sequencing) and that particular regions of a protein can be targeted specifically for antibody production[10,19]. Rapid conversion from DNA sequence information to antibodies has enormous potential for application in molecular biology. Similarly, the production of site-specific antibodies has immediate implications for functional and clinical studies. Synthetic peptides corresponding to the amino acid sequences of AFP can be thus used to produce antibodies which are highly specific to AFP, in the event that it is really difficult to produce the antibody specific to AFP due to the similarity with serum albumin.

The antibody directed against synthetic peptide-conjugated KLH was raised in rats. The titer was analyzed to determine the optimal dilution of the antibody used in the ELISA. All five kinds of antibodies showed good bindings to peptide-KLH conjugate in the experiment of antibody titer. In particular, the antibodies against peptide 1, peptide 3, and peptide 4 had high titers and antibody titers were 2×10^{-5}, 1×10^{-5}, and 2×10^{-4}, respectively (Fig. 2A). In the reactivity with AFP, antibodies against peptide 1, peptide 3, and peptide 4 also represented higher titers than those of anti-peptide 2 and 5 antibodies, and antibody titers were 2×10^{-3}, 1×10^{-2}, and 1×10^{-2}, respectively (Fig. 2B).

The purity and specificity of antibodies were checked by ELISA and Western blotting methods and the fine results were shown for the antibody against peptide 1 that had the highest reactivity with AFP among the five kinds of peptides. In Western blotting analysis, positive bands were obtained when AFP or synthetic peptide 1-KLH conjugate was loaded on the gel and analyzed, but no band was obtained when serum albumin was used for analysis (Fig. 3). In the ELISA, anti-peptide 1 antibody showed specific bindings with peptide 1-KLH conjugate or AFP but no signal was obtained when human serum albumin was used for the analysis (Table 2). Therefore, these results clearly indicate that the

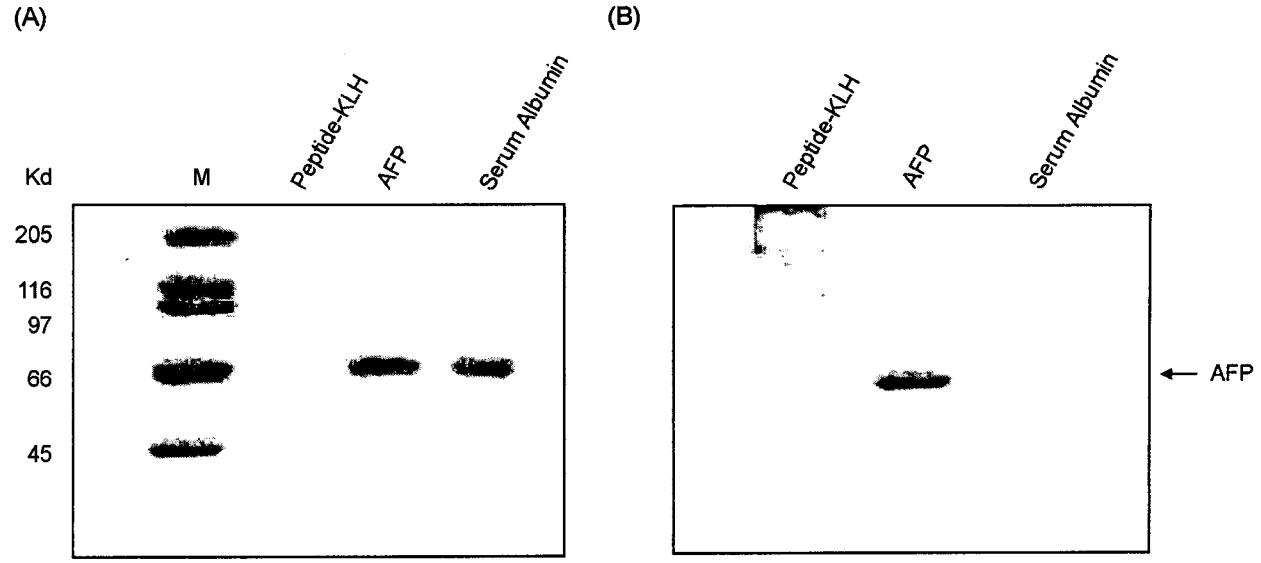

Fig. 3. Analysis of the specificity of anti-synthetic peptide 1 antibody by Western blotting. Proteins were loaded onto 10% SDS-polyacrylamide gel **(A)** and transferred to the nitrocellulose membrane. Membrane was incubated in diluted antiserum against peptide 1 and then visualized by peroxidase and its substrates **(B)**. Positive bands were obtained when AFP or synthetic peptide 1-KLH conjugate was loaded on the gel and analyzed, but no band was detected when serum albumin used for analysis.

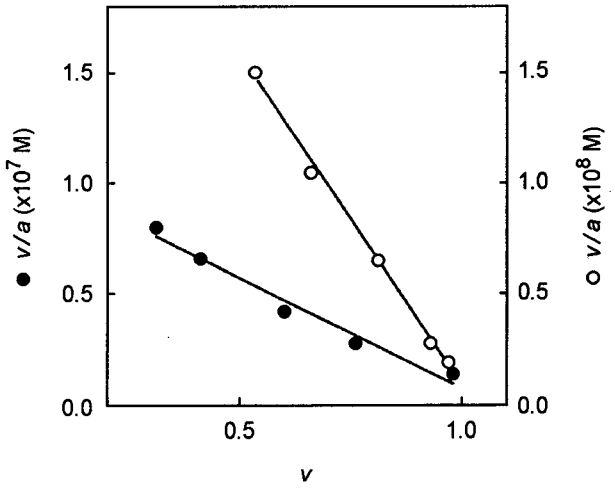

Fig. 4. Scatchard plots of the binding of anti-synthetic peptide 1 antibody to synthetic peptide 1 (○) or native AFP (●) measured by the ELISA. v is the fraction of the bound antibody and a the concentration of free antigen at equilibrium. v corresponds to $(A_0-A_0)/(A_0)$. A_0 is the absorbance measured in the absence of AFP.

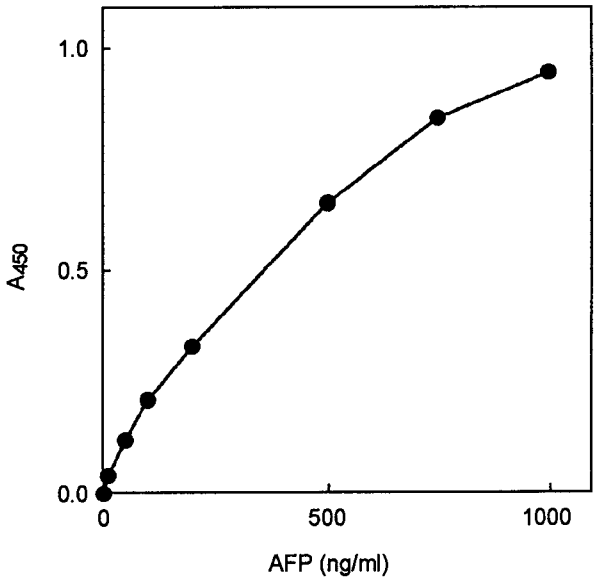

Fig. 5. Standard curve of the ELISA for human AFP. For detailed description of the assay conditions, refer to MATERIALS AND METHODS.

antibodies prepared in this study react with AFP with high purity and specificity.

Affinity constant is of particular interest, although Kd values can vary from 10^{-5} M to 10^{-11} M, since it influences the functional efficiencies of antibodies. High affinity antibodies, for example, may be very useful for diagnostic, therapeutic, or analytical applications. Affinity constant of antibody was measured in solution by ELISA and Scatchard analysis. The Kd value of anti-synthetic peptide 1 antibody showed 4.7×10^{-9} M for synthetic peptide and 6.8×10^{-8} M for AFP (Fig. 4). The affinity constant of AFP-antibody complex is lower than that of AFP-monoclonal antibody in our previous report[32]. However, it is thought that antibody prepared in this study has good binding affinity because the antibody against peptide from the primary

Table 3. The precision of ELISA developed for AFP assay

AFP (ng/ml)	CV (%)	
	Within-assay	Between-assay
10	4.1	9.5
50	3.9	10.2
100	4.5	8.8
200	4.8	11.5
500	5.3	11.8
750	5.7	12.4
1,000	5.5	12.6
mean±SD	4.83±0.70	10.97±1.48

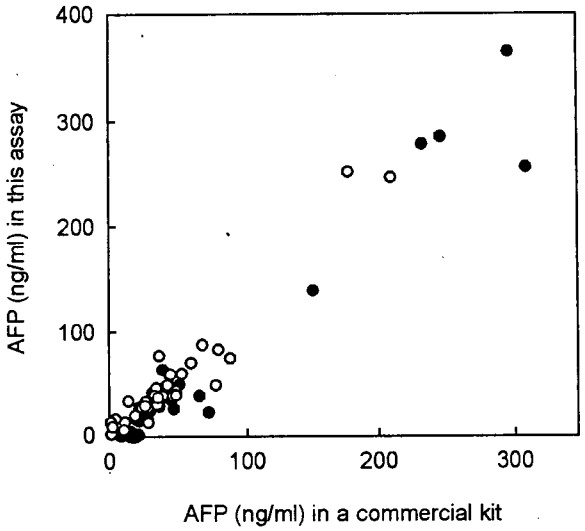

Fig. 6. A correlation study between AFP levels measured by both this assay and a commercial kit. A sample of 31 sera (●) and 33 amniotic fluids (○) were measured by synthetic peptide-based ELISA developed in the present work and the values were then compared with AFP levels by a commercial kit.

structure of protein may have low affinity for or not bind to three dimensional structure of native protein[18].

In order to determine if the antibody is desirable to measure the AFP, standard curve was drawn from ELISA (Fig. 5). In the standard curve, the working range was 10~1,000 ng/ml and the assay sensitivity was 10 ng/ml, indicating that this assay has a wider working range than that of 2~600 ng/ml in a commercial kit (RADIM, Italy) and permits less dilution of samples although there is a slightly low assay sensitivity. About the precision, the within-assay and between-assay coefficients of variance (CV) were 4.83% and 10.97%, respectively (Table 3). All samples were always run in the same assay since interassay CV was a little high while intraassay CV was pretty similar to that of other immunological techniques for human AFP[31,32].

To determine whether this assay correlated with the other immunoassay, we examined AFP levels by this assay and a commercial kit (Fig. 6). A sample of 31 sera and 33 amniotic fluids were measured and the values were then compared with AFP levels by a commercial kit. There was a good correlation between AFP concentrations determined in this study and AFP values in a commercial kit. However, there was found one limitation that a better correlation with AFP in amniotic fluid (γ=0.91) is shown than that of AFP in serum (γ=0.85), suggesting the possibility that serum may have any inhibitor of antigen-antibody binding.

In conclusion, this assay would be useful for diagnosis and prevention of pregnancy-associated pathology, tumor and non-tumor pathologies. Further study will focus to improve the assay sensitivity and interassay CV, to determine the cutoff value of AFP in several AFP-specific diseases, and to develop combined assays using AFP and other markers such as p53, human chorionic ganadotropin, and carcinoembryonic antigen[13,15,22,23].

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