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Immunochemical Measurement of Placental Tissue Protein 4 in Serum

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Using the avidin–biotin binding system, an enzyme immunoassay procedure was developed to measure the placental tissue protein 4 (PP4) in serum. The standard curve covered the range from 0.5 to 100 ng/ml of PP4. The intra- and inter-assay coefficients of variation were less than 5 and 10%, respectively. Recoveries of PP4 added to serum ranged between about 99 and 105%. The result obtained by this assay method correlated well with those obtained by the radioimmunoassay method. The PP4 serum level was under 10 ng/ml in non-pathological individuals. In pregnancies during 5—40 weeks, the level ranged from 0.9 to 15.0 ng/ml. In ovarian malignancies, the PP4 concentration in serum irrespectively scattered from 0.9 to 21 ng/ml. Significantly high PP4 levels were found in uterine cervical cancer in the early stage and also in endometrial cancer.

Keywords——placental tissue protein 4 (PP4); PP4 enzyme immunoassay; avidin–biotin binding system; PP4 serum level; uterine cervical cancer; endometrial cancer

The placental cells synthesize unique proteins to this organ under normal circumstances. Certain placental proteins are clinically significant as markers not only of placental function but also of cancer, since they are re-expressed in various types of cancer.[1–4]

Very recently, the placental tissue protein 4 (PP4) has been isolated from the solubilized protein fraction of human placentae, and it has been characterized as a glycoprotein with a molecular weight of about 35000.[5] PP4 has been immunocytochemically associated with the cell membrane of placentae and with certain other human tissues.[6] However, there has been no report on the concentrations in tissue and body fluids, and nothing is known of the biological function of this protein.

In the present study, a highly sensitive enzyme immunoassay for PP4 in serum was developed by using the avidin–biotin binding system, and this assay has been used to measure PP4 serum levels in non-pathological individuals, pregnant women and patients with some gynecologic malignancies.

Experimental

Antigens and Antisera——PP4 was highly purified from human placentae as described previously in detail.[5] Antisera against PP4 were raised in rabbits,[5] and the immunoglobulin fraction obtained by ion exchange chromatography[7] was used as anti-PP4 antibody. Anti-rabbit IgG from goat was purchased from Miles Laboratories, Inc., U.S.A.

Reagents——Avidin, biotin, and other chemicals were purchased from Sigma Chemical Co., St. Louis Mo., U.S.A. Inorganic salts were from Wako Pure Chemicals Co., Osaka, Japan. Horseradish peroxidase (HRP, EC 1.11.1.7) was supplied by Boehringer Mannheim, Mannheim, FRG, as a suspension in ammonium sulfate solution. Bovine serum albumin and gamma globulin were from Miles Laboratories.

Preparation of Biotinyl-PP4 and Avidin–HRP Conjugate——Biotin was bound to *N*-hydroxysuccinimide by means of the carbodiimide reaction.[8] The biotinyl-*N*-hydroxysuccinimide ester (1 mg/0.1 ml of dimethylformamide) was added to PP4 solution (1 mg in 1 ml of 0.1 M sodium phosphate buffer, pH 8.0), and the solution was stirred overnight at room temperature. The mixture was applied on Sephadex G-25 column (2.0 i.d. × 30 cm), and eluted with 0.02 M sodium phosphate buffer, pH 7.2. The biotinyl-PP4 fraction was stored at −70 °C prior to use. Procedures for enzyme labeling with avidin and for measurement of enzyme activity have been described elsewhere in detail.[9,10]

Enzyme Immunoassay of PP4——Non-treated polystyrene microtiter plates were coated with anti-PP4 antibody solution (5 μg/ml in 0.05 M sodium bicarbonate buffer, pH 9.5).[9] Serial dilutions of tracer, standards or samples were prepared in 0.01 M sodium phosphate-buffered saline (PBS, pH 7.2) containing 0.1% bovine albumin and gamma globulin, 0.1% gelatin, and 0.05% Tween-20. Biotinyl-PP4 tracer (2.5 ng/50 μl) and 100 μl of serum sample (or standard) were added to anti-PP4 antibody on each well of the micro-plate, and left to stand overnight at room temperature. After washing of the plate with 0.9% NaCl solution 3 times, enzyme labelled avidin was added, and the plate was allowed to stand for 15 min. After washing of the plate with 0.9% NaCl solution 3 times, the enzyme activity was measured by using H_2O_2 and *o*-phenylenediamine.[10] The calibration curves were plotted as the ratio of the enzyme activity at given standard PP4 to the activity at zero standard PP4, *i.e.*, B/B_0.

Radioimmunoassay——Quantitation of PP4 was carried out by a conventional radioimmunoassay[11] using the competitive double antibody technique. The diluent contained 0.1% bovine albumin and 0.05% Tween-20 in PBS. Tracer (^{125}I-PP4: 2 ng/100 μl), standard (or sample: 100 μl), and the anti-PP4 (antisera diluted 10000 times: 100 μl) solutions were mixed, then incubated overnight at room temperature. The anti-rabbit IgG antibody was added to the mixture, and allowed to stand overnight at room temperature. The suspensions were centrifuged, then the precipitates were counted.

Validation of Immunoassay——The effect of serum on the assay was studied by adding known amounts of PP4 to normal sera and by determining the analytical recovery of PP4. The precision and reproducibility of the assay were assessed by repeatedly assaying several serum samples, to which standard PP4 had been added. The PP4 values obtained by enzyme immunoassay were compared with those obtained by radioimmunoassay.

Serum Samples——From apparently normal, non-pregnant individuals (10 cases/female; 10/male), and 59 pregnant women at 5—40 weeks of gestation, blood samples were obtained to measure the PP4 serum concentration.

As malignant samples, we obtained pretherapeutic sera from patients with ovarian serous cystadenocarcinoma (6 cases), ovarian mucinous cystadenocarcinoma (4 cases), ovarian papillary adenocarcinoma (3 cases), ovarian clear cell carcinoma (3 cases), other ovarian malignancies (5 cases), uterine cervical carcinoma (20 cases), and uterine endometrial carcinoma (20 cases).

Results

Analytical Considerations

The standard-dose response curve obtained by enzyme immunoassay is shown in Fig. 1. The sensitivity of the assay was determined to be 0.5 ng/ml of PP4, which caused a 10% reduction in the enzyme activity obtained without PP4 standard. The quantitative range lied between 0.5 and 100 ng/ml, as in the conventional radioimmunoassay.

The effect of serum on the recovery of PP4 is summarized in Tables I and II. The recoveries of PP4 added to each serum sample ranged between about 99 and 105% in the enzyme immunoassay. The precision and reproducibility were investigated; the inter-assay coefficient of variation (CV) was less than 10%, and the intra-assay CV was less than 5% (Table III).

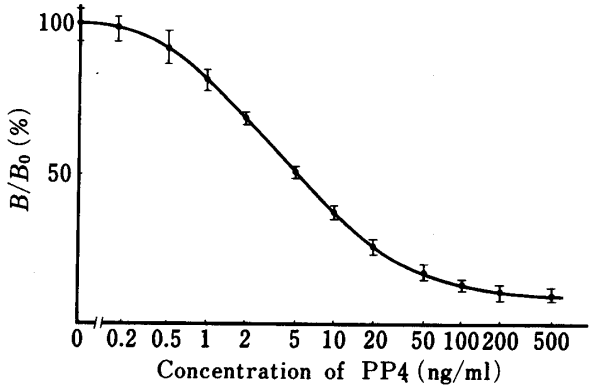

Fig. 1. Typical Standard Enzyme Immunoassay Curve for PP4

Each sample was run in triplicate.

TABLE I. Recovery of PP4 Added to Serum Samples in Enzyme Immunoassay

Sample No.	PP4 concentration (ng/ml)			Recovery of PP4 (%)
	Found in original serum	Added	Total found ($n=5$; mean ± SD)	
1	0.5	10	10.5 ± 0.2	100 ± 2
2	2.1	10	12.6 ± 0.3	105 ± 3
3	9.8	10	20.0 ± 0.5	102 ± 5
4	3.4	50	55.3 ± 2.2	104 ± 4
5	5.6	50	55.0 ± 1.0	99 ± 2
6	7.3	50	57.0 ± 1.4	99 ± 3

TABLE II. Recovery of PP4 Added to Serum Samples in Radioimmunoassay

Sample No.	PP4 concentration (ng/ml)			Recovery of PP4 (%)
	Found in original serum	Added	Total found ($n=5$; mean ± SD)	
1	1.5	10	11.3 ± 0.3	98 ± 3
2	4.6	10	14.7 ± 0.5	101 ± 5
3	7.3	10	17.2 ± 0.5	99 ± 5
4	3.7	50	56.2 ± 2.2	105 ± 4
5	6.6	50	58.1 ± 1.5	103 ± 3
6	7.0	50	57.5 ± 2.1	101 ± 4

TABLE III. Precision of PP4 Enzyme Immunoassay

Mean amount of PP4 (ng/ml)	CV (%)
Intra-assay ($n=10$)	
5.2	4.7
18.1	3.0
30.0	4.0
Inter-assay ($n=5$)	
4.7	8.0
15.3	7.5
24.6	9.4

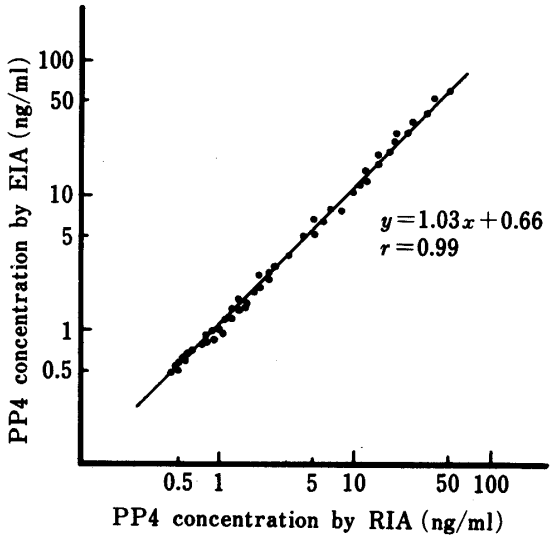

Fig. 2. Correlation between PP4 Values Obtained by Enzyme Immunoassay and Radioimmunoassay

The enzyme immunoassay method was compared with the conventional radioimmunoassay, with the use of serum samples from 50 patients (Fig. 2). The results obtained by the two methods showed a good correlation. The correlation coefficient (r) was 0.99, with a confidence limit of 99% ($p>0.0001$). The regression line was $y=1.03x+0.66$, where y represents the results of enzyme immunoassay.

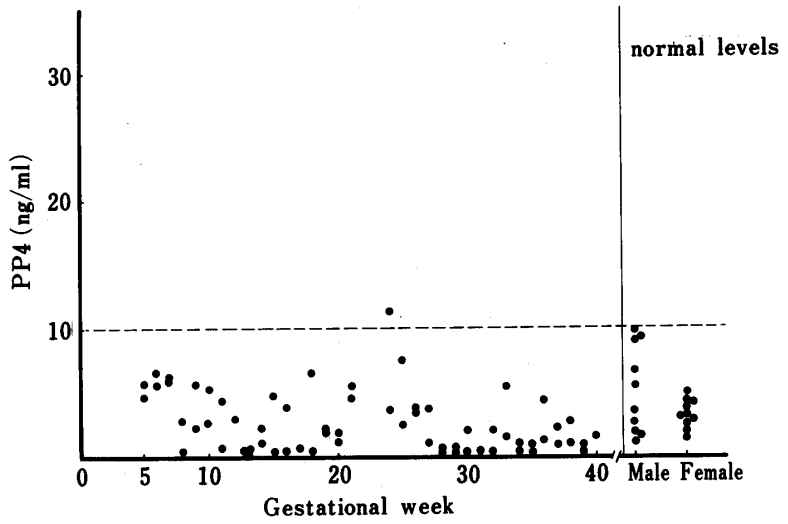

Fig. 3. The PP4 Serum Levels in Pregnant and Non-pathological Individuals

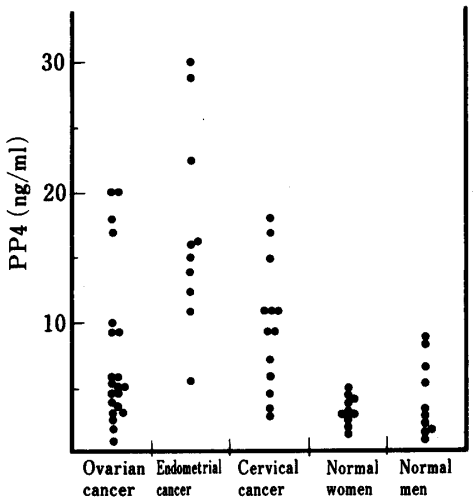

Fig. 4. The PP4 Serum Levels in Patients with Malignancies

Serum Levels of PP4

In non-pathological individuals, PP4 serum levels ranged from 1.6 to 5.0 ng/ml in women and from 1.1 to 9.8 ng/ml in men (Fig. 3). There was no case showing a concentration over 10 ng/ml in these 20 individuals. In pregnancy during 5-40 weeks of gestation, the PP4 serum level ranged from 0.9 to 15.0 ng/ml, and 55 pregnant women among 59 cases showed levels under 10 ng/ml (Fig. 3). The PP4 serum level seemed to be stable throughout pregnancy.

In various kinds of ovarian malignancies, the PP4 concentration in serum ranged from 0.9 to 21.0 ng/ml, and the mean value was 7.8 ng/ml (Fig. 4). Four out of 21 cases showed a PP4 serum level over 10 ng/ml. The frequency of elevation of the PP4 concentration over 10 ng/ml was 67% in ovarian clear cell carcinoma, while it was low in other ovarian malignancies. In the case of uterine cervical cancer, the PP4 serum level ranged between 3.0 and 18.0 ng/ml, and the mean value was 9.7 ng/ml. The frequencies of levels over 10 ng/ml were 43% and 50% in stage-I_a and -I_b, respectively. In uterine endometrial carcinoma, the PP4 concentration in serum was between 3.9 and 30.0 ng/ml (mean: 17.2 ng/ml). The frequency of occurrence of PP4 concentration over 10 ng/ml reached 87.5% in endometrial carcinoma.

Discussion

To measure the PP4 concentration in serum, an enzyme immunoassay was developed

using horseradish peroxidase and the avidin–biotin binding system. The enzyme immunoassay described above allows quantitation of PP4 in serum in the range of 0.5 to 100 ng/ml. When less than 2.5 ng of the biotinyl-PP4 was used as a tracer, less than 0.25 ng/ml of PP4 in serum could be determined. The recoveries of PP4 added to serum ranged between 99 and 105% (Table I). The enzyme immunoassay has a precision that is within acceptable immunoassay standards,[11] with an intra-assay CV of less than 5% and an inter-assay CV of less than 10% (Table III). The PP4 serum concentrations determined by this assay agreed with those obtained by the conventional radioimmunoassay. Storage of the biotinyl-PP4 tracer and enzyme-labeled avidin in solution at 4 °C for six months did not affect the performance of the enzyme immunoassay.

PP4 is immunocytochemically associated with the cell membrane of syncytio- and cytotrophoblasts as well as with the villous stroma of human placentae,[6] and it has been detected in stomach, kidney and bladder by the use of the immunodiffusion technique.[5] By using the present enzyme immunoassay, PP4 was also detected in sera of healthy men and non-pregnant women. The PP4 serum level was less than 10 ng/ml. Even in pregnant women, the mean value was 3.8 ng/ml, and only 6.8% of pregnant women developed PP4 serum levels over 10 ng/ml. The pregnant cases with elevated PP4 serum level showed no specific range of gestational age (Fig. 3).

On the other hand, the PP4 concentration in serum frequently increased in the patients with uterine carcinoma, especially uterine endometrial cancer, while the frequency of the elevation was not so high (19%) in ovarian malignancies (Fig. 4). In uterine endometrial carcinoma, the frequency of the PP4 elevation reached 85.7% even in stage I cases. The frequency of PP4 elevation in uterine cervical cancer (stage I) was approximately 46%, which is as high as that of the tissue antigen 4. TA-4,[12] as a tumor marker for uterine cervical cancer.

PP4 is not always specific or highly related to the uterus. In the present study, however, elevation of the PP4 serum concentration was very frequently observed in the early stages of uterine cervical and endometrial carcinomas. Such a high frequency may imply usefulness of PP4 as a marker of endometrial carcinoma.

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