Immunochemical Measurement and Immunohistochemical Detection of Membrane-Associated Placental Tissue Protein 1

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Using the avidin–biotin binding system, an enzyme immunoassay procedure was developed to measure the membrane-associated placental tissue protein 1 (MP1) in serum. The standard curve covered the range from 10 to 1000 ng/ml of MP1. The intra- and inter-assay coefficient of variations (C.Vs) were less than 5 and 10%, respectively. Recoveries of MP1 added to serum ranged between about 96 and 101%. The MP1 serum level was over 10 and under 112 ng/ml in non-pathological men, and under 240 ng/ml in non-pathological women. The MP1 level in the ovulatory phase was higher than in other phases of the menstrual cycle. In pregnancies during 6—39 weeks, the MP1 level ranged from 10 to 540 ng/ml, and it increased during the third trimester of gestational age. In benign gynecologic diseases, the MP1 concentration in serum ranged from 10 to 215 ng/ml. The MP1 levels in benign diseases were compared with those in ovarian malignancies, in endometrial carcinoma, and in uterine cervical cancer. The immunohistochemical location of MP1 was detected in the cell membrane of ovarian cystadenocarcinoma.

Keywords membrane-associated placental tissue protein 1 (MP1); MP1 enzyme immunoassay; MP1 immunohistochemical location; MP1 serum level; menstrual cycle; gestational age; gynecological disease

The human placentae is a highly specialized organ and contains a wide variety of biologically active compounds, some of which are 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]

Recently, the membrane-associated placental tissue protein 1 (MP1) 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 180000.[5] MP1 has an electrophoretic mobility intermediate between those of the $alpha_2$- and $beta_1$-globulins, an isoelectric point of 4.75 and a sedimentation coefficient of 6.65 S. Immunocytochemically, MP1 has been associated with the cell membrane and cytoplasm of syncytiotrophoblast in human placentae, but not other human tissues.[6] On the other hand, there has been no report on the measurement of MP1 in tissues and body fluids.

In the present study, a highly sensitive enzyme immunoassay for MP1 was developed to examine the diagnostic significance of MP1, and this assay was used to measure MP1 serum levels in non-pathological individuals, in pregnant women, and in patients with some benign gynecologic diseases and malignancies. Also, the location of MP1 was studied immunocytochemically in benign and malignant gynecological diseases.

Experimental

Antigens and Antisera MP1 was highly purified from human placentae as described previously in detail.[5] Antisera against MP1 were raised in rabbits,[5] and the immunoglobulin fraction obtained by ion exchange chromatography[7] was used as anti-MP1 antibody.

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 from Boehringer Mannheim, Mannheim, FRG, as a suspension in ammonium sulfate solution. Bovine serum albumin and gamma globulin were from Miles Laboratories. Human choriogonadotropin (hCG), human lutropin (LH), and human follitropin (FSH) were from Boehringer Mannheim. Estradiol and progesterone were supplied by Teikoku Hormone Mfg. Co., Ltd.

Preparation of Biotinyl-MP1 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 MP1 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-MP1 fraction was stored at −70 °C prior to use. Procedures for enzyme labeling with avidin and for measurement of enzyme activity have been described in detail elsewhere.[9,10]

Enzyme Immunoassay of MP1 Non-treated polystyrene microtiter plates were coated with anti-MP1 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-MP1 tracer (10 ng/100 μl) and 100 μl of serum sample (or standard) were added to anti-MP1 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. Following 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 MP1 to the activity at zero standard MP1, *i.e.*, B/B_0.

Validation of Immunoassay The effect of serum on the assay was studied by adding known amounts of MP1 to normal sera and by determining the analytical recovery of MP1. The precision and reproducibility of the assay were assessed by repeatedly assaying several serum samples, to which standard MP1 had been added.

Serum Samples From apparently normal, non-pregnant individuals (41 female, 15 male), and 47 pregnant women at 6—39 weeks of gestation, blood samples were obtained to measure the MP1 serum concentration.

As benign gynecologic diseases, there were ovarian dermoid cyst (4 cases), ovarian serous cystadenoma (4 cases), ovarian mucinous cystadenoma (4 cases), ovarian Brenner's tumor (2 cases), ovarian thecoma (2 cases), endometriosis (10 cases), and uterine myoma (10 cases).

As malignant samples, we obtained pretherapeutic sera from patients with ovarian serous cystadenocarcinoma (5 cases), ovarian mucinous cystadenocarcinoma (5 cases), ovarian papillary adenocarcinoma (5 cases), ovarian clear cell carcinoma (3 cases), other ovarian malignancies (3 cases), uterine cervical carcinoma (20 cases), and uterine endometrial carcinoma (15 cases).

Immunohistochemical Detection Tissue samples were obtained from the same patients at operation. These samples were immediately cut into small cubes and embedded in paraffin after fixation in a mixture of 90% ethanol and acetic acid.[11] Sections (3—4 μm) were then prepared. An

avidin–biotin immunoperoxidase complex technique (ABC methold)[12] was applied to detect MP1 in ovarian neoplasms. Control specimens were incubated with normal rabbit serum (non-immune : non-pregnant rabbit serum; Boehring Inst., Marburg, FRG).

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 10 ng/ml (5×10^{-11} mol) of MP1, which caused the 6% reduction in the enzyme activity obtained without MP1 standard. The quantitative range lied between 10 and 1000 ng/ml.

The effect of serum on the recovery of MP1 is summarized in Table I. The recoveries of MP1 added to each serum sample ranged between about 96 and 101% in the enzyme immunoassay. The precision and reproducibility were investigated; the inter-assay coefficient of variation (C.V.) was less 10%, and the intra-assay C.V. was less than 5% (Table II).

Serum Levels of MP1 Among non-pathological individuals, MP1 serum levels ranged from 10 to 112 ng/ml in men and from 10 to 240 ng/ml in non-pregnant women (Table III). The MP1 serum level in the ovulatory phase was higher than in other phases of the menstrual cycle. The MP1 level ranged from 12 to 122 ng/ml in postmenopausals. In pregnancy during 6—39 weeks of gestation, the MP1 serum level ranged from 10 to 540 ng/ml, and the MP1 concentration increased in the third trimester (Fig. 2).

In various kinds of benign ovarian tumors, the MP1 serum levels ranged from 10 to 160 ng/ml (Table IV). The mean and standard deviation values were 105 and 53 ng/ml, respectively. In endometriosis, the MP1 serum level ranged

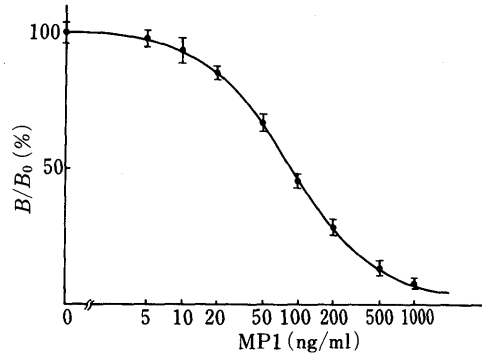

Fig. 1. Typical Standard Curve in Enzyme Immunoassay of MP1
Each point is the mean ± standard deviation of 5 measurements.

TABLE I. Recovery of MP1 Added to Serum Samples in Enzyme Immunoassay

Sample No.	MP1 concentration (ng/ml) Found in original serum	Added	Total found ($n=5$; mean ± S.D.)	Recovery of MP1 (%)
1	18.5	50	67.1 ± 2.9	98 ± 4
2	32.3	50	80.8 ± 2.3	98 ± 3
3	46.8	50	93.0 ± 3.7	96 ± 4
4	13.7	100	111 ± 3	98 ± 3
5	66.2	100	168 ± 5	101 ± 3

S.D., standard deviation.

TABLE II. Precision of MP1 Enzyme Immunoassay

Mean amount of MP1 (ng/ml)	C.V. (%)
Intra-assay ($n=10$)	
18.6	4.9
46.8	3.7
112	3.0
Inter-assay ($n=5$)	
13.7	9.7
32.3	9.0
66.2	7.5

C.V., coefficient of variation.

TABLE III. Serum MP1 Level in Normal Non-pathologic Individuals

	Number of case	Mean	S.D.	Range
Men	15	43	33	10—112
Non-pregnant women	41	48	53	10—240
Follicular phase	13	45	51	10—180
Ovulatory phase	6	105	86	10—240
Luteal phase	14	18	15	10— 47
Postmenopausals	8	61	34	12—122

MP1 concentration in ng/ml; S.D., standard deviation.

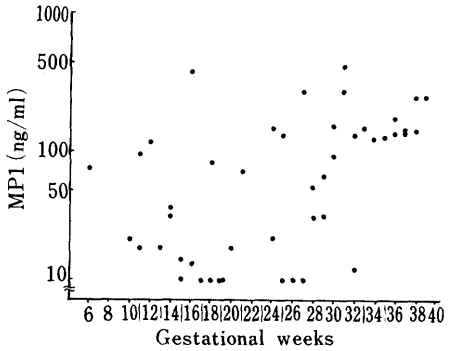

Fig. 2. The MP1 Serum Level in Pregnancy during 6—39 Weeks

TABLE IV. Serum MP1 Level in Benign Gynecological Diseases

	Number of case	Mean	S.D.	Range
Benign ovarian tumors	16	105	53	10—160
Serous cystadenoma	4	129	28	95—160
Mucinous cystadenoma	4	89	61	34—153
Dermoid cystadenoma	4	142	10	130—143
Brenner's tumor	2	78		10—145
Thecoma	2	41		38— 43
Endometriosis	10	75	64	10—210
Uterine myoma	10	56	76	10—215

MP1 concentration in ng/ml; S.D., standard deviation.

TABLE V. Serum MP1 Level in Gynecological Malignancies

	Number of case	Mean	S.D.	Range
Ovarian malignancies	21	54	43	20—165
Serous cystadenocarcinoma	5	64	65	20—165
Mucinous cystadenocarcinoma	5	38	34	20— 97
Papillary cystadenocarcinoma	5	57	33	20—103
Clear cell carcinoma	3	65		20—145
Metastatic adenocarcinoma	3	49		20—107
Endometrial carcinoma	15	81	47	20—145
Uterine cervical carcinoma	20	110	52	20—240

MP1 concentration in ng/ml. S.D., standard deviation.

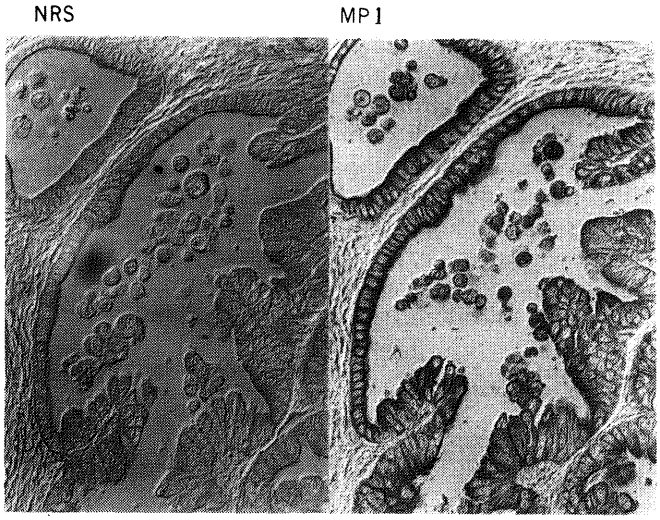

Fig. 3. Immunohistochemical Location of MP1 in Ovarian Serous Cystadenocarcinoma, Interference Phase Contrast (10×25)

NRS, normal rabbit serum as a control to anti-MP1 antibody. MP1 is mainly in the membrane of malignant cells.

from 10 to 210 ng/ml, and the mean value was 75 ng/ml. In uterine myoma, the MP1 level ranged from 10 to 215 ng/ml, and the average value was 56 ng/ml (Table IV).

In the case of ovarian malignancies, the MP1 serum levels ranged between 20 to 165 ng/ml, and the mean and standard deviation values were 54 and 43 ng/ml, respectively (Table V). In uterine endometrial carcinoma, the MP1 serum level ranged from 20 to 145 ng/ml, and the mean value was 81 ng/ml. In the case of uterine cervical cancer, the MP1 concentration in serum ranged from 20 to 240 ng/ml, and the mean and standard deviation values were 110 and 52 ng/ml, respectively (Table V).

Immunohistochemical Detection The location of MP1 was examined immunohistochemically in ovarian neoplasms. No positive staining was detected in normal ovary, ovarian dermoid cyst, or mucinous cystadenoma as a benign ovarian tumor. On the other hand, MP1 was detected immunohistochemically in 5 of 9 patients (56%) with ovarian serous cystadenocarcinoma, and in 2 of 9 patients (22%) with ovarian mucinous cystadenocarcinoma (Fig. 3).

Discussion

To measure the MP1 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 MP1 in serum in the range from 10 to 1000 ng/ml. When less than 5 ng of the biotinyl-MP1 was used as a tracer, less than 5 ng/ml of MP1 in serum could be determined. The recoveries of MP1 added to serum ranged between 96 and 101% (Table I). The enzyme immunoassay is precise within acceptable immunoassay standards,[13] with an intra-assay C.V. less than 5% and an inter-assay C.V. of less than 10% (Table II). Storage of the biotinyl-MP1 tracer and enzyme-labeled avidin in solution at 4 °C for six months did not affect the performance of the enzyme immunoassay.

MP1 was immunocytochemically associated with the cell membrane and cytoplasm of syncytio-trophoblast in human placentae,[6] while it could not be immunochemically detected (in Ouchterlony's gel diffusion test) in concentrated extracts of stomach, kidney, uterus, liver, spleen, adrenal, colon, rectum, bladder, and erythrocytes.[5] Because of this, MP1 appeared to be a protein more "specific" to the placentae.[5] By using the present enzyme immunoassay, however, MP1 was detected also in the sera of healthy men and women. The MP1 serum level was less than 112 ng/ml in men. In non-pregnant women, the MP1 serum level was less than 240 ng/ml, and the data showed a periodic change reflecting the menstrual cycle (Table III). The MP1 serum level in the ovulatory phase was higher than in other phases, while the MP1 level in the luteal phase was lower (t test: $p<0.1$). In postmenopausals, the MP1 serum level bore a very close to that in men (Table IV). In the present enzyme immunoassay, there was no cross-reaction (less than 0.001%) with FSH, LH, HCG, progesterone, and estradiol. These findings may imply a relationship between MP1 and the menstrual cycle, although nothing is known of the biological function of MP1.

In pregnancy during 6—39 weeks of gestation, the MP1 serum level ranged from 10 to 540 ng/ml, and the MP1 concentration increased in the third trimester (Fig. 2). Such a fluctuation is different from that[14] of placental tissue protein 4 (PP4), suggesting possible clinical significance as a marker of placental function.

The MP1 serum levels in some gynecological diseases are summarized in Tables IV and V. The MP1 concentrations in serum lied between 10 and 215 ng/ml in benign gynecologic diseases (Table IV), and the MP1 level ranged from 20 to 240 ng/ml in patients with pretherapeutic gynecologic malignancies (Table V). In benign ovarian tumors, the MP1 serum levels ranged from 10 to 160 ng/ml, and the MP1 level lied between 20 and 165 ng/ml in ovarian malignancies. In the case of endometriosis, the MP1 serum level ranged from 10 to 210 ng/ml, while it ranged from 20 to 145 ng/ml in endometrial carcinoma. In uterine myoma, the MP1 serum level ranged between 10 and 215 ng/ml, and it ranged from 20 to 240 ng/ml in uterine cervical carcinoma. In these malignancies, there is no relationship between the MP1 serum concentration and the clinical stage by FIGO. As shown in Tables IV and V, the MP1 serum level in benign gynecologic diseases is comparable with that in the gynecological malignancies. The MP1 in serum may not be a suitable tumor marker.

On the other hand, the location of MP1 was studied immunohistochemically in ovarian neoplasm. No positive staining was detected in normal ovary, ovarian mucinous cystadenoma or ovarian dermoid cyst. In ovarian cystadenocarcinomas, however, MP1 was identified in 56% (5/9 cases) of ovarian serous cystadenocarcinomas and in 22% (2/9) of ovarian mucinous cystadenocarcinomas. The MP1 was associated mainly with the membrane in ovarian cystadenocarcinoma cells, as shown in Fig. 3. Although the MP1 serum level in the ovarian cystadenocarcinoma was comparable with that in benign ovarian tumor, the frequency of MP1 immunohistochemical detection in the former was obviously higher than that in the latter. These finding may suggest a localization of MP1 in ovarian cystadenocarcinoma tissue.

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