Enzyme immunoassay of pancreatic oncofetal antigen (POA) as a marker of pancreatic cancer

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SUMMARY For the quantitative measurement of pancreatic oncofetal antigen (POA), an enzyme immunoassay for POA has been developed, and is based on the sandwich method using antibody-coupled glass beads and enzyme (peroxidase)-labelled antibody. Serum POA concentrations were increased significantly in patients with pancreatic cancer, but not in those with chronic pancreatitis or other miscellaneous diseases, or in normal subjects. It is concluded that the enzyme immunoassay could be used for the assay of POA and our results show that the determination of serum POA would be useful in the diagnosis of pancreatic cancer.

Presence of oncofetal antigen relatively specific to human pancreatic cancer, designated as pancreatic oncofetal antigen (POA), was first described by Banwo *et al* in 1974,¹ and we have reported high positivity of POA in pancreatic juice of patients with pancreatic cancer by a qualitative assay method of double immunodiffusion or counter electro-immunophoresis.²

Attention has been focused on the detection of POA for the serological diagnosis of pancreatic cancer. Until now, however, assay of POA has not been used widely because a quantitative assay procedure for POA has not been established.

In this paper we describe a quantitative assay of POA using enzyme immunoassay, and its application as a clinical test for pancreatic cancer.³

Methods

SUBJECTS

Serum was collected from 21 patients with pancreatic cancer, in whom the diagnosis was confirmed by operation or necropsy, 28 patients with chronic pancreatitis, 74 patients with miscellaneous diseases including 37 patients with malignancy of the oesophagus, stomach, colon, biliary tract, or liver, and 46 healthy persons as normal controls.

PREPARATION OF ANTI-POA SERUM

Human fetal pancreatic tissue was obtained at about

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23 weeks of fetal life and homogenised in a buffered solution containing protease inhibitors; 200 KIE/ml of aprotinin and 60 mg/ml of epsilon aminocaproic acid (EACA). Homogenates were centrifuged at $100\,000 \times g$ for 50 minutes, and the pellet was discarded. After alpha-fetoprotein (AFP) was excluded by negative affinity chromatography with CNBr activated Sepharose 4B coupled with anti-AFP, the supernatant was emulsified with an equal volume of complete Freund's adjuvant to immunise rabbits by subcutaneous injection. The rabbits were bled when a sufficiently high antibody titre was achieved. The rabbit serum was absorbed with insolubilised normal human plasma, and adult and fetal human liver.⁴

IGG FRACTION OF ANTISERUM

The IgG fraction of the absorbed antiserum was isolated by ammonium sulphate precipitation,⁵ and by DEAE cellulose chromatography.⁶

ANTIBODY COUPLED GLASS BEADS

The rabbit (anti-POA) IgG-coupled glass beads were prepared by the method of Hamaguchi *et al*.⁷ Glass beads (6.8 mm in diameter) were heated at 500°C for five hours, and then immersed in 2% solution of 3-aminopropyltriethoxysilane (Nakarai Ltd) in acetone at 40°C for 24 hours. The beads were washed with acetone and dried, and were then immersed in 1% aqueous solution of glutaraldehyde at room temperature for one hour. After washing with 0.25 M sodium phosphate buffer, pH 7.5, 320 beads were immersed in 50 ml of 0.25 M sodium phosphate buffer, pH 7.5, containing 2 mg of rabbit

IgG fraction isolated from anti-POA serum at 4°C overnight. After washing with 0.25 M sodium phosphate buffer, pH 7.5, they were further immersed in 0.02 M phosphate buffered saline (PBS), pH 7.3, containing 0.1% NaN₃ and 1% bovine serum albumin (BSA) (Fraction V, Nakarai Ltd), before storing at 4°C.

PEROXIDASE-LABELLED ANTIBODY

The IgG fraction of anti-POA serum was coupled to horseradish peroxidase by the method of Nakane and Kawaoi.⁸ Five milligram of horseradish peroxidase (Grade II, RZ 3.4, 290 PU/mg, Toyobo Ltd) was dissolved in 1.0 ml of 0.3 M NaHCO₃, pH 8.1, and 0.1 ml of 1% fluorodinitrobenzene (FDNB) in 99.5% ethanol was added and mixed gently at room temperature for one hour. Then 1.0 ml of 0.06 M NaIO₄ in distilled water was added and mixed for 30 minutes at room temperature and 0.1 ml 1.6 M ethylene glycol in distilled water was further added and mixed gently for one hour at room temperature. The solution was dialysed against 0.01 M sodium carbonate buffer, pH 9.5, at 4°C overnight. Five milligram anti-POA IgG was added to 3 ml of the solution and mixed gently for three hours at room temperature and 5 mg NaBH₄ was then added and mixed at 4°C for three hours. The solution was dialysed against 0.02 M phosphate buffer, pH 7.3, at 4°C overnight. The peroxidase conjugate was purified by Sephadex G-150 column chromatography, and fractions of peroxidase-labelled IgG were collected to which equal volumes of PBS containing 2% BSA were added before storing at -40°C.

PRINCIPLES OF THE METHOD

The POA enzyme immunoassay that we have developed is a solid phase enzyme immunoassay based on the sandwich principle, as illustrated in Figure 1. Glass beads coated with rabbit anti-POA were incubated with the samples. Pancreatic oncofetal antigen present in the samples was bound to the glass beads and unbound materials were removed by washing. Subsequently, anti-POA conjugated with peroxidase was incubated with the beads to form anti-POA-peroxidase conjugates on the beads which were then incubated with enzyme substrate to develop a colour which measured the amount of bound anti-POA-peroxidase conjugate. The enzyme reaction was stopped by the addition of sulphuric acid and the intensity of the colour developed was read using a spectrophotometer at 492 nm. A standard curve was obtained by plotting against different concentrations of standard POA.

ASSAY PROCEDURE

Figure 2 shows an outline of the steps in the POA-enzyme immunoassay. One-tenth millilitre of the serum samples were incubated with antibody-coated glass beads in 0.2 ml of buffer consisting of PBS containing 1% BSA at 37°C for 12 hours. The beads were washed with 0.85% saline solution, and then incubated with 0.3 ml of peroxidase-conjugated anti-POA at 37°C for three hours. After washing, the beads were transferred to 2 ml of a substrate solution containing 0.8 mg of o-phenylenediamine (Nakarai Ltd) and 0.2 µl 30% H₂O₂ in 0.1 M citric acid with 0.2 M dibasic sodium phosphate, pH 4.8, before incubation at 37°C for 30 minutes. The

Fig. 1 Principle of the enzyme immunoassay for POA.

Fig. 2 The assay procedure of POA enzyme immunoassay.

enzymatic reaction was stopped by adding 0.5 ml 6 M H₂SO₄, and the absorbance of each solution was read at 492 nm.

STANDARD CURVE

A 10⁻⁴ dilution of homogenate of fetal pancreas was used as the standard POA, and defined as containing 1 unit/ml of POA. The standard curve for each sample was made with this standard POA.

Results

SPECIFICITY OF ANTI-POA SERUM

Absorbed rabbit anti-POA serum was tested by double immunodiffusion. As shown in Figure 3, there was no reaction with normal human serum, alpha-fetoprotein (Behringwerke Ltd), carcino-embryonic antigen (Abbott Ltd), ferritin (Behringwerke Ltd) and beta-2-microglobulin (Pharmacia Ltd), while there was a clear precipitation line with human fetal pancreatic extract used as the control. The antiserum did not react with extracts of human adult liver, fetal liver, adult pancreas, and porcine fetal pancreas.

PEROXIDASE-LABELLED ANTIBODY

Figure 4 shows the elution pattern of peroxidase-conjugated antibody on Sephadex G-150 column

Fig. 3. Double immunodiffusion; reactions of absorbed anti-POA (centre well) to the wells of AFP, ferritin, CEA, beta-2-microglobulin, normal human serum and POA (fetal pancreas) (arrow: positive precipitation).

chromatography. The absorbance of the fractions was read spectrophotometrically at 280 nm for protein concentration and 403 nm for peroxidase concentration. The peroxidase-labelled antibody was obtained by collecting the fractions of the first peak. Approximately 78% of the horseradish peroxidase was found to be coupled with anti-POA IgG.

STANDARD CURVE

Figure 5 shows the standard curve for the enzyme immunoassay of POA, and covers a range from 1 unit/ml to 10 000 unit/ml.

REPRODUCIBILITY

Tables 1 and 2 show the reproducibility of the

Fig. 4 An elution pattern of anti-POA conjugated with horseradish peroxidase on Sephadex G-150 column chromatography.

Fig. 5 Standard curve for POA enzyme immunoassay.

enzyme immunoassay which was obtained by carrying out repeated assays using 11 samples (sample A-K). The intra-assay coefficients of variation (CV) ranged from 7.7–17.3%, and the inter-assay CV ranged from 2.7–23.1%.

RECOVERY

Recovery of added POA was assessed by assaying samples to which serum with a known POA concentration had been added. The recovery ranged from 90.7–101.1% with an average of 96.2%.

SERUM POA CONCENTRATIONS IN PANCREATIC CANCER AND OTHER DISEASES

Figure 6 shows the serum POA concentrations in the 169 cases examined. The mean serum POA concentration in the normal controls was 763 ± 51 unit/ml (mean $\pm$ SEM), and 755 ± 91 unit/ml in patients with benign miscellaneous diseases, 749 ± 87 unit/ml in patients with malignancy other than pancreas, and 784 ± 116 unit/ml in chronic pancreatitis. The serum POA concentration in patients with pancreatic cancer varied from 940–2930 unit/ml with a mean value ($\pm$ SEM) of 2006 (± 122) unit/ml, and was significantly higher than that of other groups ($p < 0.001$, two sample rank test).

Abnormal POA concentrations above 1500 unit/ml accounted for 81% (17/21) of pancreatic cancer,

Table 1 Intra-assay reproducibility of POA enzyme immunoassay ($n=3$, sample A-C)

No	POA concentration (unit/ml)		
	A	B	C
1	285	2020	7070
2	265	1700	7040
3	248	2300	5120
4	304	1700	5120
5	285	1625	5000
6	248	1625	5140
7	285	1880	6800
8	255	1860	7260
SD	20.9	232.2	1049.1
m	271.6	1838.8	6068.8
CV	7.7	12.6	17.3

2% (1/46) of normal controls, 16% (6/37) of benign miscellaneous diseases, 8% (3/37) of malignant miscellaneous diseases and 14% (4/28) of chronic pancreatitis.

CHANGES OF SERUM POA CONCENTRATIONS IN PANCREATIC CANCER

In one resectable pancreatic cancer without metastasis, the serum POA concentration increased from 1610 to 2480 unit/ml with the progress of cancer during one month before operation. After removal of the tumour from the head of the pancreas ($35 \times 25 \times 20$ mm), the POA level fell rapidly to 900 and then to 112 unit/ml after one and two months (Fig. 7).

Discussion

Oncofetal antigens^{9 10} such as alpha-fetoprotein¹¹ and carcinoembryonic antigen¹² are now used for the immunological diagnosis of malignancy, and immunological approaches have been investigated^{13–16} to help in the early detection of pancreatic cancer.¹⁷

Pancreatic oncofetal antigen¹ has been reported

Table 2 Inter-assay reproducibility of POA enzyme immunoassay ($n=8$, sample D-K)

No	POA concentration (unit/ml)							
	D	E	F	G	H	I	J	K
1	760	860	1033	2220	2720	2930	3280	3830
2	600	746	824	2350	2860	3570	2670	3280
3	620	810	1100	1485	2750	2750	3780	3200
SD	87.2	57.1	144.0	466.4	73.7	431.0	555.9	343.0
m	660.0	805.3	985.7	2018.3	2776.7	3083.3	3243.3	3436.7
CV	13.2	7.1	14.6	23.1	2.7	14.0	17.1	10.0

Fig. 6 Serum POA concentrations in pancreatic cancer and other diseases (mean and standard error).

to be a valuable tumour marker for pancreatic cancer.²⁻¹⁸ Clinical trials carried out to detect POA in the pancreatic juice² or in the serum¹⁹⁻²⁰ have been made by double immunodiffusion and counter-immunoelectrophoresis. Gelder *et al*²¹ have reported that serum POA concentrations were

Fig. 7 Changes of serum POA concentrations in a resectable pancreatic cancer.

raised in 48% of patients with pancreatic cancer using rocket immunoelectrophoresis assay. A more sensitive assay method is required, however, to detect minute amounts of POA for early diagnosis.

We have developed an enzyme immunoassay²² for POA, which is a safe and sensitive quantitative assaying method with several advantages, as the procedure is simple and radioisotopes are not used.

Reproducibility studies of the established assay gave coefficients of variation of an average of 12.5% for intra-assay, and 12.7% for inter-assay, and the recovery test of the assay showed satisfactory results. These results indicate that the enzyme immunoassay used could be applicable in the preliminary measurement of serum POA concentrations.

Using the enzyme immunoassay, significantly raised serum POA concentrations were observed with a relatively high specificity in patients with pancreatic cancer, compared with those in chronic pancreatitis, other various diseases, or normal controls.

Although there was about 20% false negative rate and 10% false positive, the enzyme immunoassay of POA would be one of the useful aids in detecting the patients with cancer of the pancreas, and the estimation of serum POA could be combined with other morphological examinations such as ultrasonic tomography, computed tomography, endoscopic retrograde cholangio-pancreatography (ERCP), or angiography.

We conclude that POA would be the useful marker of pancreatic cancer, and the enzyme immunoassay of POA would be valuable in the diagnosis of pancreatic cancer.

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