Production and Characterization of Monoclonal Antibodies against Human Pancreatic Phospholipase A₂

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Summary Five hybridoma cell lines producing monoclonal antibodies against human pancreatic phospholipase A₂ (PLA₂) were established after selection from prepared hybridomas on the basis of the affinity of the antibodies they produced. All the monoclonal antibodies (1008, 1015, 1045, 1085, and 2003) proved to have very high affinity toward human pancreatic PLA₂ ($K_a = 1.5 \times 10^{11} - 1.2 \times 10^{12} \text{ M}^{-1}$), but to have no cross reactivity with porcine pancreatic PLA₂, snake venom PLA₂, and membrane-associated PLA₂ from human spleen. Cross reactivities with human pancreatic prophospholipase A₂ (proPLA₂) ranged from 4 to 49%. Three of the monoclonal antibodies neutralized the enzyme activity of PLA₂, while the other two had no effect on it. The three neutralizing antibodies had smaller cross reactivities with proPLA₂ than the other two. On radioimmunoassay using these monoclonal antibodies, the PLA₂ contents of sera from patients with acute pancreatitis were above normal in every case. These monoclonal antibodies are expected to be useful tools in the diagnosis of acute pancreatitis.

Key Words: human pancreatic phospholipase A₂, monoclonal antibody, radioimmunoassay, cross reactivity, acute pancreatitis

Human pancreatic phospholipase A₂ (PLA₂; EC 3.1.1.4) is a digestive enzyme synthesized by pancreatic acinar cells as an enzymatically inactive prophospholipase A₂ (proPLA₂), which is secreted into the duodenum and converted to the active form by tryptic cleavage of a seven-amino acid peptide from the N-terminus. The importance of PLA₂ in the development and aggravation of acute pancreatitis has been discussed [1–3], and the elevation of PLA₂ activity in the sera from patients with acute pancreatitis has been reported [4–7].

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Serum PLA₂ can be measured enzymologically or immunologically. Although several authors have reported the measurement of the enzyme activity of PLA₂ in human sera [4-7], these methods require long incubation times because of the low PLA₂ activity in the sera, and tedious procedures are needed for the extraction of the product of the enzyme reaction. These drawbacks make activity measurements difficult to apply to clinical use. The immunological method is expected to be more sensitive, convenient and accurate than the enzymological method, and moreover, is able to detect the enzyme as the whole enzyme molecule, including those that have no enzyme activity. Radioimmunoassay (RIA) and time-resolved fluoroimmunoassay of human pancreatic PLA₂ using polyclonal antibodies (pAbs) have been reported [8, 9], and these methods show significant elevations of serum PLA₂ levels in patients with acute pancreatitis.

We describe here the development of monoclonal antibodies (mAbs) against human pancreatic PLA₂, which were found to have high affinity and specificity and which can be applied to clinical use.

MATERIALS AND METHODS

Materials. Porcine pancreatic PLA₂ and fetal calf serum were purchased from Boehringer Mannheim GmbH (Germany); and Naja naja venom PLA₂ came from Sigma Chemical Co. (St. Louis, MO). RPMI 1640 Medium (autoclavable) was purchased from Nissui Pharmaceutical Co., Ltd. (Tokyo). Immunobead (rabbit anti-mouse immunoglobulin) was a product of Bio-Rad Laboratories (Richmond, CA). Other chemicals were of the highest grade available. Assay buffer for RIA was 0.01 M PBS (pH 7.4) containing 1 mM EDTA, 0.01% NaN₃ and 0.2% BSA.

Human pancreatic PLA₂ and proPLA₂. Human pancreatic PLA₂ was purified from pancreatic juice as described by Nishijima *et al.* [8]. Human pancreatic proPLA₂ was purified from the same according to Grataroli *et al.* [10], except that chromatography on CM-Sepharose was done twice. Both of them were found to be pure by amino acid analysis and SDS-PAGE. Pancreatic juice was kindly donated by Dr. M. Ogawa (Osaka University Medical School).

Serum samples. Sera from patients with acute pancreatitis were kindly donated by Dr. M. Ogawa (Osaka University Medical School). The diagnosis of acute pancreatitis had been made by him based on the following criteria: (a) the clinical condition and its evolution; (b) the elevated amylase activity in serum and/or urine; (c) the results of computed tomography and/or laparotomy.

Enzymatic assay of PLA₂. The enzyme activity of PLA₂ was measured according to the method described by Katsumata *et al.* [11], except that L- α -phosphatidylcholine, 1-palmitoyl-2-[1-¹⁴C]palmitoyl (Amersham Japan, Tokyo) was used instead of a ³H-labelled substrate, and the volume of the enzyme reaction was reduced to one tenth. The enzyme reaction was run for 1 h at 37°C and terminated by addition of 20 μ l of 5% Triton X-100 containing 0.2 M EDTA. The

product of the enzyme reaction was extracted with 750 μ l of hexane containing 0.1% acetic acid after addition of approximately 500 mg of anhydrous Na₂SO₄. The radioactivity in 500 μ l of the hexane layer was determined by liquid scintillation counting.

Preparation of mAbs. Human pancreatic PLA₂ dissolved in saline was emulsified with Freund's complete adjuvant and administered subcutaneously to female BALB/c mice three times at three-week intervals. The administered dose of PLA₂ each time was 25 μ g for fusion 1 and 5 μ g for fusion 2. PLA₂ solution in saline (50 μ g for fusion 1 and 10 μ g for fusion 2) was injected intraperitoneally into the mice with the highest anti-PLA₂ titer three days before the cell fusion.

The fusion procedure was based on the method of Galfr and Milstein [12]. Spleen cells from immunized mouse were fused with P3-NS1/1-Ag4 mouse myeloma cells in the presence of 50% polyethylene glycol (MW4,000, Merck). Cells were then suspended in HAT (hypoxanthine, aminopterin, and thymidine) medium and seeded into wells of 96-well culture plates.

All wells containing hybridomas were screened for antibodies to human pancreatic PLA₂ by the enzyme-linked immunosorbent assay (ELISA) described below. Hybridomas in positive wells were allowed to expand in about 10 ml of culture medium and then frozen. Culture supernatants collected at that time were subjected to the displacement test (see below). Hybridomas selected by this test were cloned by limiting dilution.

ELISA. In this assay, 96-well microtiter plates (Nunc-Immuno Plate, Nunc, Roskilde, Denmark) were coated with human pancreatic PLA₂ by addition of 50 μ l of PLA₂ solution (200 ng/ml in 0.05 M carbonate buffer, pH 8.5) to each well and incubation of the plates overnight at 4°C. The wells were blocked with 0.2% BSA-PBS and washed with 0.5% Tween 80-PBS. Next, 50 μ l of each hybridoma supernatant was added to a PLA₂-coated well, and the plates were incubated for 1 h at 37°C. Antibodies bound to the wells were detected by a commercial kit utilizing biotinylated anti-mouse IgG and avidin-biotinylated peroxidase complex (Vectastain ABC kit, Vector Laboratories, Burlingame, CA) with ABTS as the coloring agent.

Radioiodination of human pancreatic PLA₂. A 5- μ g sample of human pancreatic PLA₂ was radioiodinated with 18.5 MBq of Na¹²⁵I by the chloramine-T method [13]. ¹²⁵I-Human pancreatic PLA₂ was separated from free ¹²⁵I⁻ by gel filtration on a Sephadex G-25 column (1 $\times$ 25 cm). The specific activity of ¹²⁵I-human pancreatic PLA₂ was 2.6–3.0 MBq/ μ g.

Displacement test of culture supernatants. This test was done to estimate which of the frozen hybridomas produced antibody of high affinity. Serial dilutions of culture supernatants (100 μ l) were mixed with 100 μ l of ¹²⁵I-human pancreatic PLA₂ (40,000 cpm) and 100 μ l of 2% bovine γ -globulin dissolved in assay buffer (γ -globulin buffer) and incubated overnight at room temperature. B/F separation was performed by the polyethylene glycol method. The same procedure was done by addition of human pancreatic PLA₂ (50 ng/ml) in γ -globulin

buffer in place of γ -globulin buffer, and the decrease of radioactivity in the bound fraction was examined.

RIA. A 100- μ l sample of human pancreatic PLA₂ standard solution or sample, 100 μ l of ¹²⁵I-human pancreatic PLA₂ (40,000 cpm), 100 μ l of diluted ascites, and 200 μ l of assay buffer were mixed in disposable polystyrene tubes and incubated overnight at room temperature. One hundred microliters of Immunobead (rabbit anti-mouse immunoglobulin, 1 mg/ml assay buffer) was then added; and after 1 h at room temperature, the tubes were centrifuged (2,000 $\times$ g, 5 min). The supernatant was removed, and the radioactivity of the pellet was measured.

RESULTS

Preparation of mAbs

Two fusions were performed. Hybridoma cell colonies appeared in 508 wells for fusion 1 and in 471 wells for fusion 2. In fusion 1, more than 200 wells were initially positive by ELISA. Although some of them became negative during successive culture, we froze hybridomas from 85 wells while they were positive. In fusion 2, the number of initially positive wells was 52, and the cells from 24 of them were frozen.

Fig. 1. Displacement test of the hybridoma culture supernatants. Dilution curves of the culture supernatants collected at the time of hybridoma freezing were prepared by RIA using ¹²⁵I-human pancreatic PLA₂ in the absence (●) or presence (○) of 5 ng/tube of human pancreatic PLA₂. Out of the total of 109 samples, results for three culture supernatants which showed (a) large, (b) moderate, or (c) little displacement are shown as examples.

Culture supernatants collected at the time of hybridoma freezing were subjected to the displacement test. We found that there were great differences in the degree of displacement among the supernatants. Figure 1 shows, for example, the results of the displacement test for three culture supernatants: they showed large, moderate, or little displacement.

Our main aim in this study was to develop an immunoassay for human pancreatic PLA₂. The sensitivity of the immunoassay is greatly influenced by the affinity of the antibody used in the assay system. We considered that hybridomas showing large displacement in the displacement test produced antibodies of high affinity; and from this point of view, we selected five hybridomas, which were then established by cloning.

Thus, we obtained five hybridoma cell lines: 1008, 1015, 1045, and 1085 from fusion 1, and 2003 from fusion 2. Ascitic fluids were prepared for each of these hybridomas, and the properties of the mAbs in these ascites were studied. IgG was purified from the ascites by use of a protein A column (Bio-Rad MAPS kits), if necessary.

Immunoglobulin class and affinity constants

All five mAbs were IgG₁ and had κ light chains as determined by ELISA using a Mouse Mono Ab-ID EIA kit (ZYMED Laboratories, San Francisco, CA).

The affinity constants calculated from the Scatchard plots [14] are shown in Table 1. They ranged from $1.5 \times 10^{11} \text{ M}^{-1}$ for 1045 to $1.2 \times 10^{12} \text{ M}^{-1}$ for 1085. Thus, all of these hybridoma cell lines produced mAbs of very high affinity for human pancreatic PLA₂.

Standard curves of RIA

Standard curves for human pancreatic PLA₂ were prepared for RIA using five mAbs. Dilutions of ascites were from 1 : 360,000 for 1008 to 1 : 800,000 for 1085, which made the B_0/T approximately 40%. The 90% intercept values of the standard curves were as follows: with 1008, 0.4 ng/ml; with 1015, 0.4 ng/ml; with 1045, 0.5 ng/ml; with 1085, 0.3 ng/ml; and with 2003, 0.4 ng/ml. Figure 2 shows the standard curve obtained with mAb 1085. Human pancreatic PLA₂ could be measured with good sensitivity by RIA using any of the five mAbs.

Table 1. Properties of mAbs.

mAb	$K_a \text{ (M}^{-1}\text{)}$	Cross reactivity (%) with human pancreatic proPLA ₂
1008	4.1×10^{11}	49
1015	1.0×10^{12}	4
1045	1.5×10^{11}	39
1085	1.2×10^{12}	19
2003	3.9×10^{11}	7

Fig. 2. Standard curve of the RIA for human pancreatic PLA₂ using mAb 1085. Similar curves were obtained for the other four mAbs with some differences in the sensitivity. The conditions of the assay were as described in Materials and Methods.

Cross reactivity with other PLA₂s

No detectable decrease in the radioactivity of the bound fraction was observed in the RIA using any of the five mAbs by addition of porcine pancreatic PLA₂ (up to 5 μ g/ml), *Naja naja* venom PLA₂ (up to 5 μ g/ml), or membrane-associated PLA₂ from human spleen (up to 2 μ g/ml) that had been purified in our laboratory [15]. These concentrations were more than or nearly 1,000 times the concentration of human pancreatic PLA₂ exhibiting a 50% intercept on the standard curve. Therefore, our mAbs do not cross-react with these three kinds of PLA₂.

Cross reactivity with human pancreatic proPLA₂

All the mAbs gave standard curves for human pancreatic proPLA₂ parallel to the curve for human pancreatic PLA₂ on RIA. Cross reactivities with human pancreatic proPLA₂ calculated from the 50% intercept of both standard curves are shown in Table 1. They varied from 4% for 1015 to 49% for 1008, suggesting differences in binding sites among the mAbs.

Effect on PLA₂ enzyme activity

As shown in Fig. 3, the mAbs could be divided into two groups: 1015, 1085, and 2003, which completely neutralized the enzyme activity of human pancreatic PLA₂, and 1008 and 1045, which had little effect on it. The amount of the neutralized enzyme agreed fairly well with that of the enzyme to which bivalent IgG would bind, which assures that this neutralizing effect is a specific one. Also, the former group of mAbs (neutralizing mAbs) was found to have smaller cross

Fig. 3. Effect of the mAbs on the enzyme activity of PLA₂. Constant amounts (3.6×10^{-13} mol/tube) of human pancreatic PLA₂ and serial dilutions of the purified IgG solution of mAb 1008 (■), 1015 (□), 1045 (○), 1085 (●), or 2003 (▲) were mixed and incubation was done for 1 h at 37°C. After that, the enzyme activity of PLA₂ in the mixture was measured as described in Materials and Methods. The values are presented as a percent of the enzyme activity found in the absence of antibody.

Table 2. Within- and between-assay variations of RIA using mAb 1008.

Assay	Number of determinations	Mean $\pm$ SD (ng/ml)	Coefficient of variation (%)
Within-assay			
Serum 1	6	2.45 ± 0.08	3.3
Serum 2	6	8.60 ± 0.16	1.9
Serum 3	6	22.87 ± 1.11	4.9
Between-assay			
Serum 1	5	2.43 ± 0.08	3.2
Serum 2	5	8.46 ± 0.27	3.2
Serum 3	5	23.21 ± 0.96	4.1

reactivities with human pancreatic proPLA₂ while the latter (non-neutralizing mAbs) had larger cross reactivities (see Table 1).

Reproducibility

Reproducibility of the RIA was estimated with human sera having different PLA₂ levels. On RIA using mAb 1008, the coefficients of variation for within- and between-assay series were 1.9–4.9% ($n=6$) and 3.2–4.1% ($n=5$), respectively, as shown in Table 2. Other four mAbs also gave values below 10%.

Fig. 4. Dilution test of human sera. Three serum samples having different PLA₂ levels were diluted serially with assay buffer, then measured by the RIA using mAb 1008.

Fig. 5. Serum levels of PLA₂ in healthy individuals (H) and patients with acute pancreatitis (P). Human pancreatic PLA₂ contents were measured by the RIA using mAb 1008 and 1085, and PLA₂ enzyme activities were measured by the enzyme assay described by Isaji [16], for the same serum groups.

Dilution test of serum

Serial-dilution experiments of human sera gave linear lines that passed through the origin when any of the five mAbs was used for the RIA. Figure 4 shows the result for an RIA using mAb 1008.

Serum level

The average PLA₂ levels (mean $\pm$ SD, ng/ml) of sera from five healthy individuals measured by RIA using each of the five mAbs were the following: with 1008, 3.1 ± 0.8 ; with 1015, 0.6 ± 0.1 ; with 1045, 2.4 ± 0.5 ; with 1085, 1.3 ± 0.2 ; and with 2003, 0.7 ± 0.2 . Significant differences in the measured values among the mAbs were observed, and those that had larger cross reactivities with proPLA₂ gave higher serum levels (see Table 1).

PLA₂ contents in sera from five healthy individuals and eight patients with acute pancreatitis measured by RIA using mAb 1008 and 1085 are shown in Fig. 5. PLA₂ enzyme activities in these sera measured by the enzyme assay described by Isaji [16] are also shown in this figure. In all cases of patients with acute pancreatitis, serum levels were above the normal range with both RIAs. Enzyme activities of PLA₂ in sera from patients with acute pancreatitis were also above normal. However, the difference in the measured values between the two serum groups was more prominent on RIA than on enzyme assay.

DISCUSSION

In this study, we established five hybridoma cell lines producing mAbs against human pancreatic PLA₂. All the mAbs were found to have very high affinity for human pancreatic PLA₂, allowing highly sensitive radioimmunoassays to be developed. These mAbs were also highly specific for human pancreatic PLA₂, having no cross reactivity with porcine pancreatic PLA₂, snake venom PLA₂, and membrane-associated PLA₂ from human spleen. These properties of the mAbs make them favorable as reagents in immunoassay systems for diagnostic purposes.

Hybridomas should be cloned as soon as possible after screening, but in this study, there were too many positive hybridomas to clone at one time. We therefore froze these positive hybridomas and selected by means of the displacement test those producing antibodies of high affinity. The selected hybridomas were then thawed and cloned. This method is considered to be effective when many wells are found to be positive in the first screening.

The mAbs were divided into two groups depending on their effect on the enzyme activity of human pancreatic PLA₂: those that completely neutralized the enzyme activity (neutralizing mAbs) and those that had little effect on it (non-neutralizing mAbs). The N-terminal region of PLA₂ has been suggested to have an important role in the catalytic activity of the enzyme [17]. We found that the neutralizing mAbs had smaller cross reactivities with human pancreatic proPLA₂,

which has an extra polypeptide sequence (seven amino acids) at the N-terminal of PLA₂, while the non-neutralizing mAbs had larger cross reactivities. Although the conformational changes between proPLA₂ and PLA₂ should be taken into consideration, we assume that the binding sites of the mAbs having smaller cross reactivities with proPLA₂ are near the N-terminal of PLA₂ and those of mAbs having larger cross reactivities with it are apart from the N-terminal. Thus, in this respect, our results are in accord with the suggested relationship of the N-terminal region of PLA₂ with the catalytic activity.

Our present RIA using the mAbs can determine the PLA₂ levels in human sera with good precision and accuracy. They clearly distinguished sera from healthy individuals and sera from patients with acute pancreatitis, with more prominent differences in the measured values between the two serum groups than found with the enzyme assay. This indicates that these mAbs can be useful tools in the diagnosis of acute pancreatitis.

The high specificity of the mAbs for human pancreatic PLA₂ allows the RIA using them to be unaffected by other PLA₂s. Especially, in the case of measurement of pancreatic PLA₂ levels in human sera, interference by the membrane-associated PLA₂ (type II PLA₂) is thought to be an important concern. It may be detected by the enzyme assay along with pancreatic PLA₂, whereas our present RIAs indicated no cross reactivities with it. This feature is considered to make our RIAs suitable for the diagnosis of acute pancreatitis. Moreover, the high affinity of the mAbs may make it possible to develop a more rapid immunoassay system that would be favorable for clinical use.

Five mAbs gave different values for the same serum group from healthy individuals, and the relationship between the measured values and their cross reactivities with proPLA₂ has been pointed out. Supposing that there exist on the average about 5 ng/ml of proPLA₂ and about 0.5 ng/ml of PLA₂ in normal human sera, these serum levels given by the five mAbs are reasonable. This suggests that pancreatic PLA₂ mostly exists as the inactive proPLA₂ molecule in normal sera. Further investigations are described in the following report.

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