Quantitative Assay for Autotaxin Using Monoclonal Antibodies Specific to Conformational Epitope

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Autotaxin (ATX), which is identical to lysophospholipase D (lysoPLD), is a tumor cell motility-stimulating factor originally isolated from melanoma cell supernatant and has been pointed out for its involvement in regulation of invasive and metastatic properties of cancer cells. It is also known as a multifunctional protein that is up-regulated in various malignancies including breast and lung cancer and potently stimulates cell proliferation, cell motility and angiogenesis, which is accounted for by its intrinsic lysoPLD activity that is capable of releasing lysophosphatidic acid (LPA) from lysophosphatidylcholine (LPC) or sphingosine 1-phosphate (S1P) from lysosphingomyelin. LPA is both a mitogen and a motogen that acts through G protein-associated Edg (endothelial differentiation gene) receptors and is well known to be associated with tumor aggressiveness and tumor cell-directed angiogenesis. Considering the importance of ATX as an enzyme that exerts lysoPLD activity and produces LPA, it was deemed that it might be important to measure the serum ATX. Until now ATX has been analyzed by its lysoPLD enzymatic activity or immunological methods, like a Western blotting. In this study we have produced monoclonal antibodies specific to conformational epitope on ATX and developed the ATX immunoenzymetric assay which is simple and can be performed in an automated immunoassay analyzer AIA-system without cumbersome procedure such a sample pretreatment.

1. Introduction

Autotaxin, also known as NPP2 (nucleotide pyrophosphatase/phosphodiesterase 2), is a 125kDa glycoprotein and has been shown in various malignant tumor tissues including non-small cell lung cancer [1], breast cancer [2], renal cell cancer [3], and hepatocellular carcinoma [4] Indeed, ATX not only stimulates the growth of cancer [5], but also acts as a tumor motility factor [6,7], augment the tumorigenecity of ras-transformed cells [8] and induces a strong angiogenic response [9]. All biological functions of ATX can be explained by its ability to act as an extracellular lysoPLD [10-13]. A major substrate of ATX is LPC, which is hydrolyzed into LPA and choline. The bioactive lysophospholipid LPA elicits a variety of biological responses, including cancer initiation, progression, and metastasis [14,15]. LPA is also reportedly elevated in plasma of ovarian cancer patients compared with the healthy control [16,17], while serum LPA level is significantly higher in multiple myeloma patients [18]. It is now established that these LPA actions are mediated through activation of its G protein-coupled receptors (GPCR), *i.e.*, LPA_{1-3} (as the Edg family receptors) [19,20,21] and LPA_4/p2y9/GPR23 [22] and LPA_5/GPR92, which are structurally distant from the Edg family [23]. Although the physiological functions of ATX are still unclear, ATX is widely expressed with the highest mRNA levels in brain, placenta, ovary, and small intestine [24] and furthermore its mRNA levels are overexpressed in various malignancies, such as thyroid, renal cell, breast cancer, and glioblastoma [16,25-28].

The measurement of ATX in serum has currently carried out by lysoPLD enzymatic activity assay or immunological methods, like Western blotting.

Unfortunately lysoPLD enzymatic activity assay is lack of versatility because of time-consuming and tedious method, while it is necessary to consider the interference of intrinsic factors, like choline, LPC, and other lysoPLDs. Likewise Western blot analysis is also lack of versatility, and furthermore it is just a qualitative method, not a quantitative method. In this study, we introduce a quantitative determination of ATX by an immunoenzymometric assay using monoclonal antibodies (mAbs) specific to a conformational epitope.

2. Materials and methods

2.1 Preparation of recombinant human ATX

Human cDNA for ATX was amplified by RT-PCR using a human liver cDNA library as template DNA base on the sequence information in the data base (GenBank accession no. L46720) and was introduced into the baculovirus transfer vector pFASTBac-1 (Invitrogen, CA, USA). In the same way, cDNA for ATX was introduced into pFASTBac HT (Invitrogen) to add a polyhistidine-tag at the NH_2-terminus of ATX. The preparation of recombinant baculovirus and the expression of recombinant ATXs were performed according to the manufacturer's protocol. The purification of recombinant polyhistidine-tagged ATX (hisATX) was performed using metal chelate column chromatography (BD TALON; BD Biosciences, CA, USA) according to the manufacturer's protocol. To analyze the epitope of anti-ATX mAbs, five different regions of ATX were expressed in *Escherichia coli (E.coli)* JM109 using the pCold TF vector system (TAKARA, Shiga, Japan) at 15℃. The five fragments, ATX F1 (amino acid residue; 1-310), ATX F2 (amino acid residue; 301-610), ATX F3 (amino acid residue; 601-863), ATX F4 (amino acid residue; 150-459), and ATX F5 (amino acid residue; 450-726), were expressed as fusion proteins with a trigger factor (48kDa) with polyhistidine-tag. To purify each ATX fragment, the sonicated supernatant of *E.coli* was applied onto the metal chelate column.

2.2 Preparation of anti-ATX mAbs

Eight-wk-old Wister Kyoto rat was immunized by the footpad injection with 250μg of purified hisATX antigen which was emulsified with the complete Freund's adjuvant (Difco Laboratories, MI, USA). One month after first immunization, the fusion of B cells obtained from rat iliac and inguinal lymph nodes with PAI myeloma cells was performed. Hybridoma cells were cultured in a synthetic medium (GIT medium; NIHON PHARMACEUTICAL CO., LTD, Tokyo, Japan) without serum supplementation. The hybridoma supernatants were screened for the antibodies that bind to the native form ATX in solution by ELISA (Enzyme Linked Immunosorbent Assay), not to ATX antigen directly coated on solid phase. Briefly, 50 ng/well of the anti-rat IgG in TBS (10 mmol/L Tris-HCl, 15 mmol/L NaCl, pH 7.4) was coated on a 96-well Maxisorp immunoassay plate (NUNC; Nalge Nunc International, NY, USA) for 16 h at 4℃. The wells were blocked with 200 μL of TBS containing 3% bovine serum albumin (BSA). After washing the wells 3 times with TBS, the hybridoma culture supernatant was added to each well and incubated for 2h at room temperature. After washing with TBS containing 0.05% Tween-20 (TBST) 4 times to remove unbound antibody, 50 ng of hisATX in 50 μL of TBST containing 1% BSA (1% BSA-TBST) was added to each well. The bound hisATX was detected by HisProbe-HRP (Pierce Chemical Company, IL, USA). Absorbance at 450nm by the addition of TMB substrate(3,3',5,5'-Tetramethylbenzidine, Kirkegaard & Perry Laboratories, Inc., MD, USA) was measured. All procedures were performed without hisATX for background of nonspecific bindung. The hybridoma cells selected were established by a limiting dilution method. In addition, a synthetic peptide (DSPWTNISGSC, amino acid residue; 49-59) was conjugated with keyhole limpet hemocyanin (KLH), and an anti-peptide mAb, named P101, was established as a tracer antibody for detecting ATX by the immunization of the peptide-KLH conjugate.

2.3 Reactivity of mAbs with ATX directly coated on immunoassay plate

To analyze the reactivity of anti-ATX mAbs with ATX antigen directly coated on the immunoassay plate, 50 μL of 1mg/L purified recombinant ATX was coated on a microtiter well for 16h at 4℃. After blocking with 3% BSA-TBS, the anti-ATX mAbs were added to each well. The antibody bound was detected by peroxidase-labeled anti-rat IgG (American Qualex, CA, USA),

followed by addition of TMB substrate as described above. All procedures were performed without ATX coating for background of nonspecific binding.

2.4 Reactivity profile of mAbs with ATX by Western blotting

One microgram of the purified recombinant ATX and the ATX fragments were subjected to SDS-PAGE. After electrophoresis, the proteins on SDS-gel were transferred to a PVDF membrane using the Semidry-Blot apparatus (BioRad Laboratories, CA, USA) at 20 A/m^2-gel for 2h. After transferring the protein, the PVDF membrane was blocked with TBS containing 1% skim milk for 2h at room temperature. The anti-ATX mAb (1mg/L) was added to the membrane and the antibody bound was detected by alkaline phosphatase-labeled anti-rat IgG (American Qualex), followed by addition of the CDP-Star chemiluminescense substrate (PerkinElmer, MA,USA).

2.5 Adsorption of ATX from human serum by anti-ATX mAbs

To analyze an ability of absorption of ATX from human serum by anti-ATX mAbs, human serum was mixed with the anti-ATX mAbs bound magnetic particle and the remaining lysoPLD activity in treated human serum was determined. Briefly, 10 μg of anti-ATX mAbs were added to 25 μL of the magnetic particle suspension immobilized anti-rat IgG (BioMag goat anti-rat IgG Fc; QIAGEN, Hilden, Germany). After washing the particles with TBST to remove unbound anti-ATX mAb, 50 μL of human serum and 150 μL of TBST were added and the mixture was incubated for 2h at room temperature. The remaining LysoPLD activity in the supernatant of the mixture was determined.

LysoPLD activity was assessed by measuring choline liberation from the substrate LPC [5]. Briefly, the reactions were performed in 100 μL aliquots; the serum samples (20 μL) were incubated with 2 mmol/L 1-myristoyl (14:0)-LPC (Avanti Polar Lipids Inc., AL, USA) in the presence of 100 mmol/L Tris-HCl, pH 9.0, 500 mmol/L NaCl, 5 mmol/L $MgCl_2$, 5 mmol/L $CaCl_2$, and 0.05% Triton X-100 for 3 h at 37℃. The liberated choline was detected by an enzymatic photometric method using choline oxidase (Wako Pure Chemical, Osaka, Japan), horseradish peroxidase (TOYOBO, Osaka, Japan), and TOOS reagent (N-ethyl-N-(2-hydoroxy-3-sulfoproryl)-3-methylaniline; DOJINDO, Kumamoto, Japan) as a hydrogen donor. Absorbance was read at 550nm and converted to the amount of choline by comparison with a standard curve by choline chloride.

2.6 Cross reactivity of anti-ATX mAbs with various animal ATXs

To analyze the cross-reactivity of anti-ATX mAbs to ATX of various animal species, an adsorption assay by anti-ATX mAbs were performed using mouse, rat, rabbit, horse and bovine serum. Briefly, various animal sera were incubated with anti-ATX mAbs and the remaining lysoPLD activity of each sample was determined describe above.

2.7 Combination of mAbs for two-site immunoenzymetric assay

Anti-AXT mAbs were purified from the hybridoma culture supernatant using HiTrap Protein G (GE Healthcare, Buckinghamshire, UK). The purified mAbs were then biotinylated with sulfo-NHS-LC-LC-biotin reagent (Pierce) according to the manufacturer's protocol. Microtiter wells were coated with 100 ng of anti-ATX mAbs and blocked with 3% BSA-TBS. After blocking, 50 ng of purified recombinant ATX was added to the wells. ATX bound to primary anti-ATX mAbs was detected by the biotinylated secondary anti-ATX mAbs, followed by peroxidase-labeled streptavidin (Zymed Laboratories, CA, USA) and TMB substrate described above. All procedures were performed without ATX for background of nonspecific binding. Base on the analysis of this screening for two-site immunoenzymometric assay, we selected R10.23 as solid phase primary antibody and R10.21 as enzyme-labeled secondary antibody.

2.8 Automated immunoenzymetric assay for quantitative determination of ATX

Antigen concentration of ATX in serum was determined using a specific two-site immunoenzymometric assay. To prepare the ATX immunoenzymetric assay reagent, R10.23 was digested with pepsin and the purified $F(ab)_2$ form using phenyl-5PW (TOSOH, Tokyo, Japan)

hydrophobic column chromatography in order to avoid nonspecific binding of human antibodies to various animal IgG in human specimens, like HAMA (human anti-mouse antibodies). R10.23 $F(ab)_2$ was coated by physical absorption on a *in house* magnetic bead made from ethylene-vinyl alcohol and ferrite. Coating efficiency of antibody on a bead was approximately 100 ng. ATX immunoenzymetric assay reagent was prepared as follows. Twelve R10.23-coated beads were placed in the reaction cup and then 35 μg of alkaline phosphatase-labeled R10.21 in assay buffer (5% BSA, 5% sucrose, 10 mmol/L Tris-HCl, 10 mmol/L $MgCl_2$, pH 7.4) was added to the cup. After addition of all materials, ATX immunoenzymetric assay reagent was prepared by immediate freeze-dry procedure of the reaction cup. The ATX immunoassay reagent can be used with an automated immunoassay analyzer AIA-system (TOSOH). The AIA-system includes automated specimen dispensation, incubation of the reaction cup, bound/free washing procedure, 4MUP (4-methylumbelliferyl phosphate) substrate dispensation, fluorometric detection, and a result report. The antigen-antibody reaction time is only 10 min., and the first result is reported within 22 min; the throughput of the system is 60 and 180 samples per hour using the AIA-600Ⅱ system and the AIA-1800 system, respectively.

2.9 Preparation of standard ATX calibrator

To purify the non-tagged ATX from the culture supernatant of Sf9 insect cells which expressed recombinant human ATX, R10.23-immobilized column was prepared using NHS-activated HiTrap (GE Healthcare), according to the manufacturer's protocol. One liter culture supernatant of the Sf9 cells was applied to the 5 mL bed volume of R10.23 column immobilized 20 mg of antibody. The concentration of purified ATX was determined by BCA Protein Assay Reagent (Pierce) with BSA as standard. Subsequently the serum was applied to the R10.23-immobilized column to deplete ATX in normal human serum. The pass-through fraction was collected and the remaining lysoPLD activity was measured to confirm the complete depletion of activity. ATX calibration fluids having six different concentrations (0, 0.34, 0.675, 1.35, 2.70, and 5.40mg/L) were prepared by spiking the purified recombinant ATX into the ATX depleted serum.

3. Results and Discussion

3.1 Recombinant human ATX and ATX fragments

For the production of anti-ATX mAbs, a recombinant human hisATX expressed in baculovirus system was purified using BD TALON metal chelate column. Finally, approximately 1 mg of recombinant human ATX exhibiting lysoPLD activity was obtained. The purified ATX stained by CBB (Coomassie brilliant blue) was detected as single band, whereas the doublet bands were detected by Western blot analysis (**Fig. 1**). This result could be attributed to a different glycosylation of hisATX or a degradation of hisATX in the course of purification procedure. To determine the epitope of anti-ATX mAbs, five ATX fragments were expressed in *E.coli* using the pCold TF vector system. All fragments expressed at 15℃ were well soluble in TBS and it was easy to purify them by metal chelate column chromatography (**Fig. 2** (a)). Although epitope determination of each mAb was tried by Western blotting using these ATX fragments, all 23 mAbs except R10.48 and P101, which is anti-peptide mAb, did not show any reaction with these ATX fragments (**Fig. 2** (b) , 2(c) and 2(d)). In addition, no inhibition was observed by various ATX fragments to the binding of anti-ATX mAbs to soluble ATX antigen (data not shown). These results strongly suggested that the anti-

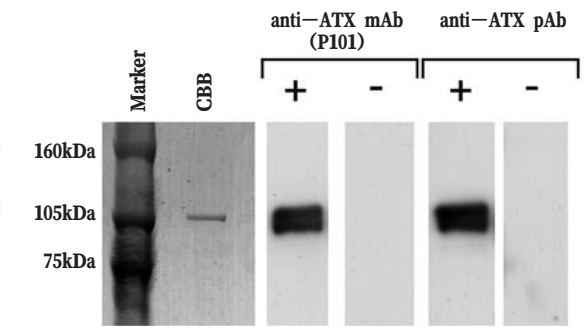

Fig. 1 Purified hisATX and identification as ATX
The purified hisATX recombinant protein (1 μg/lane) expressed in baculovirus system was subjected to SDS-PAGE. The proteins were detected by CBB staining (CBB). The purified hisATX was analyzed by Western blotting using anti-ATX mAb (P101) and anti-ATX polyclonal antibody (pAb) against the synthetic peptide (amino acid residue 652-666; CVRPDVRVSPSFSQN). The results of Western blot analysis with anti-ATX Abs (+) and with only secondary antibodies (-) were shown.

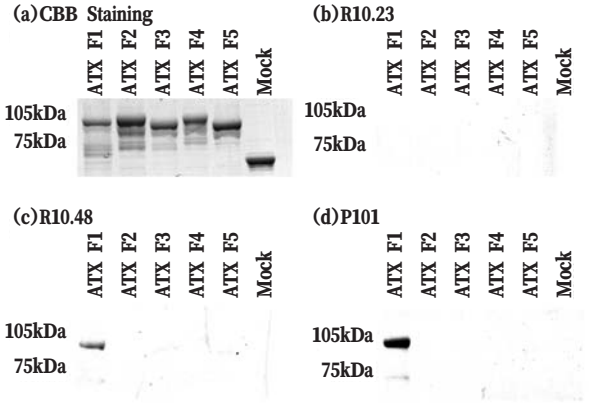

Fig. 2　Epitope analysis of anti-ATX mAbs by Western blotting
Five ATX fragments expressed in *E.coli* were subjected to SDS-PAGE and the proteins were detected by CBB staining (a). Reactivity of R10.23 (b), R10.48 (c) and P101 (d) as a positive control with each ATX fragment was analyzed by Western blotting. Among 23 anti-ATX mAbs, only R10.48 reacted with ATX F1 fragment.

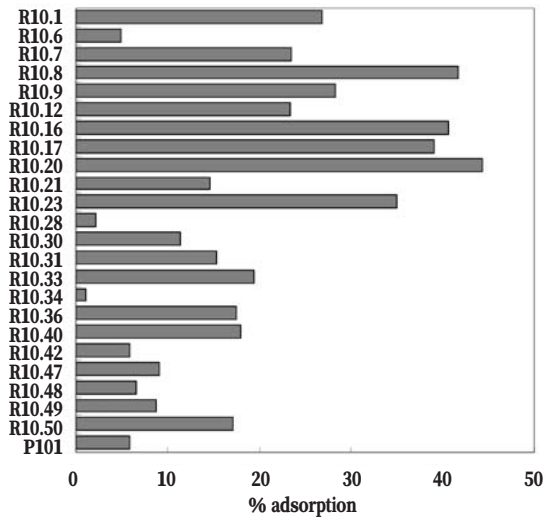

Fig. 3　Binding ability of anti-ATX mAbs to soluble ATX in human serum
The purified anti-ATX mAbs were incubated with the anti-rat IgG immobilized particle. Normal human serum was added to the particle and the lysoPLD activity in the supernatant of the mixture was determined. The percent adsorption ［(initial activity - residual activity in supernatant) / (initial activity) x 100］ was calculated. The initial activity was determined by the sample which was treated by the particle without anti-ATX mAb.

ATX mAbs established recognize the only conformational epitope of native ATX molecule.

3.2 Characterization of anti-ATX mAbs

We have established 23 anti-ATX mAbs by a screening method based on an ability of capturing soluble ATX antigen. Sixteen of 23 mAbs were efficiently (more than 10% adsorption) able to deplete ATX from human serum (**Fig. 3**). Before using the screening method by an ability of capturing soluble ATX, we also tried to obtain anti-ATX mAbs by a screening method based on binding ability to ATX antigen directly coated on an immunoassay plate. The anti-ATX mAbs obtained by this method successfully work only for immunoassay methods, like Western blotting and cell staining under denatured condition, not for ELISA. In contrast, almost of all anti-ATX mAbs obtained by the screening method based on the ability

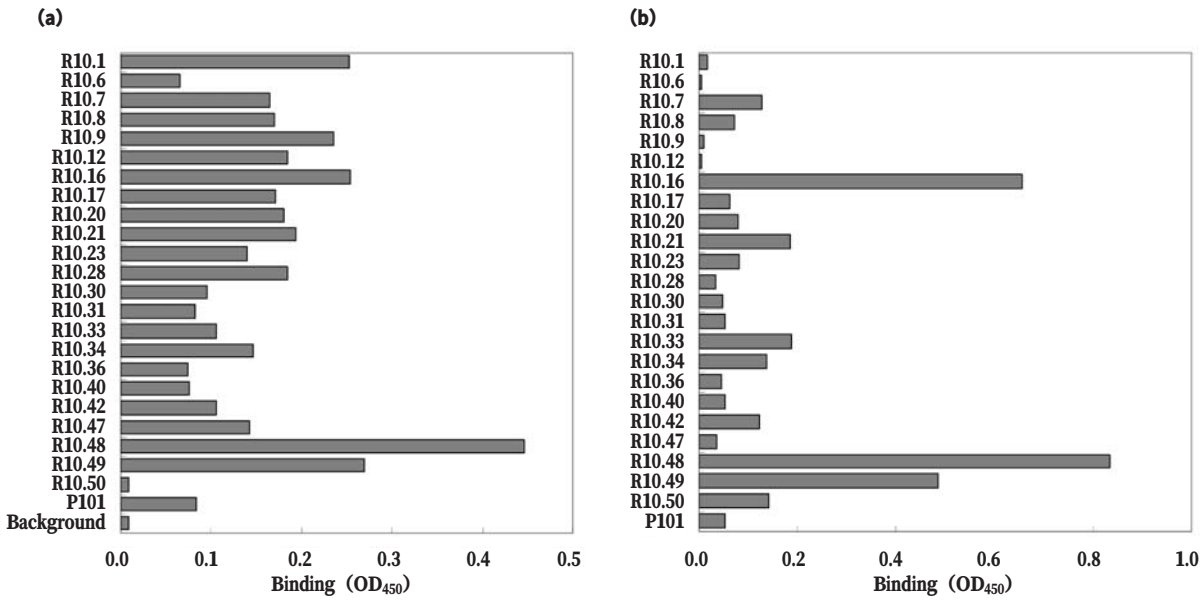

Fig. 4　Difference of anti-ATX mAbs binding to ATX antigen on the solid phase and soluble ATX antigen
(a) The purified anti-ATX mAbs were coated on the microtiter wells. HisATX recombinant protein was added to the wells and hisATX captured by anti-ATX mAb was detected by HisProbe-HRP. (b) The purified ATX recombinant protein was coated on the microtiter wells. The anti-ATX mAbs were added to the wells and the antibody bound was detected by peroxidase-labeled anti-rat IgG.

of capturing soluble ATX antigen could specifically bind to only soluble ATX antigen, neither to ATX antigen directly coated on an immunoassay plate (**Fig. 4**) nor to ATX antigen on a Western blot membrane (data not shown). As shown in **Fig. 3** and **4**, R10.28 and R10.34 could deplete only a recombinant ATX, not an ATX in human serum. This result suggested that these antibodies recognize the particular epitope on the only recombinant ATX. Anti-ATX mAbs showed the different depletion profile of the lysoPLD activity from various animal sera (**Fig. 5**) and an epitope of anti-ATX mAbs could not be determined using ATX fragments. These results demonstrated that anti-ATX mAbs established here could recognize the only conformational epitope on native ATX molecule and the epitope recognized by them was abundantly diverse.

3.3 Preparation of ATX immunoenzymetric assay reagent

To select the pairs of mAbs for two-site immunoenzy-mometric assay, all combinations of anti-ATX mAbs were examined. Among examined 529 combinations, 46 pairs of antibodies could form an immunocomplex, primary antibody - ATX - secondary antibody, and showed an enough binding signal for two-site immunoenzymetric assay to detect serum ATX. Although R10.16, R10.48 and R10.49 showed the binding to both ATX antigen on solid phase and soluble ATX antigen as shown in **Fig. 4**, no applicable partner was found for two-site immunoenzymetric assay. Finally we selected the combination of R10.23 as the primary antibody for solid phase and R10.21 as the enzyme-labeled secondary antibody for two-site

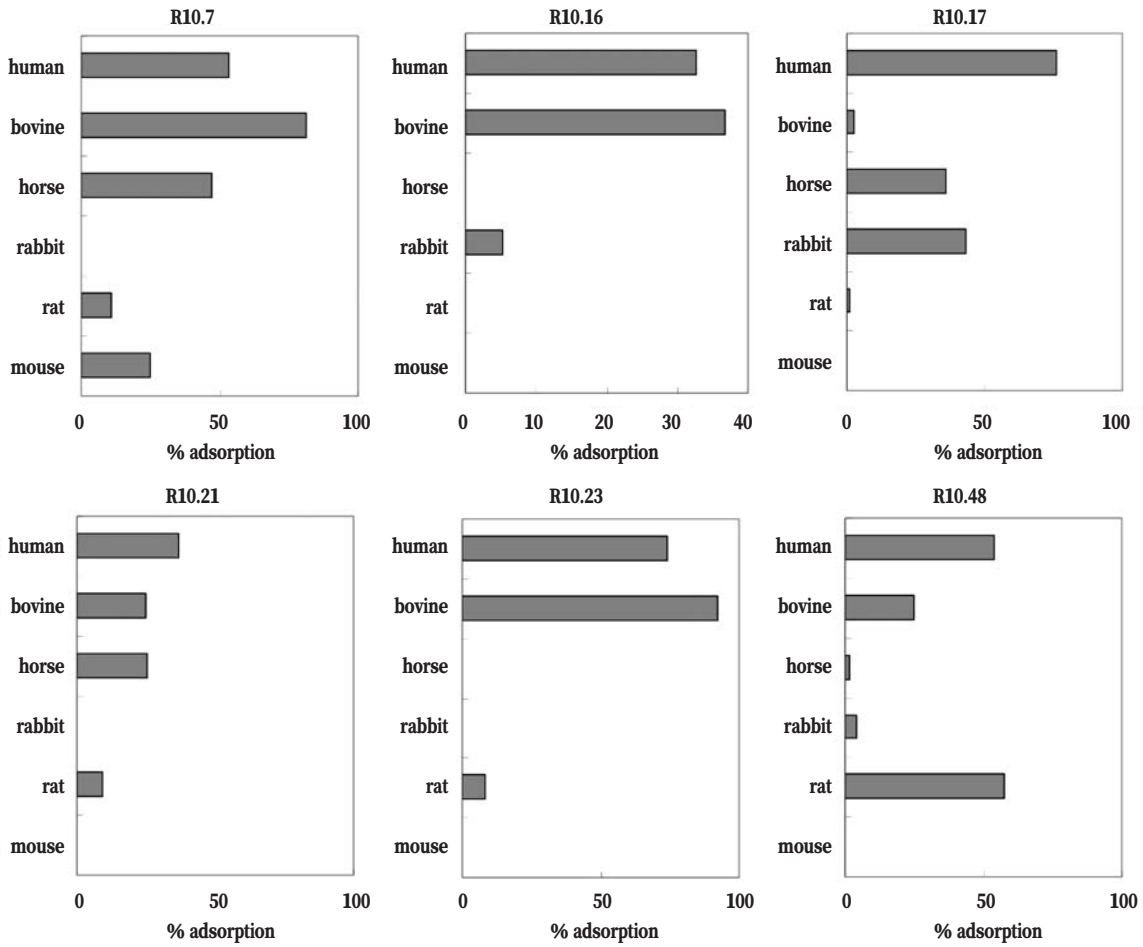

Fig. 5 Cross-reactivity of anti-ATX mAbs with various animal ATXs
Anti-ATX mAbs bound on magnetic particle were incubated with various animal sera and the remaining lysoPLD activity in serum was determined. The percent adsorption was calculated as described in **Fig. 3**. Typical cross-reactivity profiles of anti-ATX mAbs were shown.

immunoenzymometric assay because the combination of these antibodies showed a good correlation with lysoPLD activity in human serum. When we constructed two-site immunoenzymetric assay using these antibodies, we initially observed false ATX values frequently (20-30%) and the relationship between ATX concentration determined by the two-site immunoenzymometric assay and lysoPLD activity was poorly-correlated (data not shown). To solve this problem, both rat IgG and HBR-1 (Scantibodies, CA, USA), which is well known as heterophilic antibodies blocker, were added to the buffer of ATX immunoenzymetric assay reagent, these false values were completely reduced and the correlation was surprisingly better.

ATX expressed in baculovirus system for calibrator standard was effectively purified by a R10.23-immobilized column chromatography (**Fig. 6**). ATX bound to the R10.23-immobilized column was eluted by 100 mmol/L glycine (pH 3.5). Whereas both the antigenic activity and the lysoPLD enzymatic activity of ATX significantly decreased in the case of the elution with 100 mmol/L glycine (pH 3.0) or more acidic pH. To prepare the ATX deficient serum for calibrator base, normal human serum was applied to R10.23-immobilized column and the lysoPLD activity was completely depleted from 500 mL serum by once path-through procedure. Then, the six points of calibrator fluids were prepared using the purified ATX and the ATX depleted serum. The calibration curve of the AIA-system was well fitted by four-parameter logistic regression (**Fig. 7**).

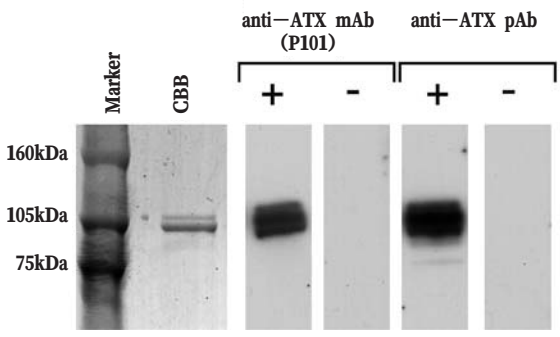

Fig. 6　Purified non-tagged ATX and identification as ATX
The purified ATX recombinant protein (1 μg/lane) expressed in baculovirus system was subjected to SDS-PAGE. The proteins were detected by CBB staining (CBB). The purified hisATX was analyzed by Western blotting using anti-ATX mAb (P101) and anti-ATX polyclonal antibody (pAb) against the synthetic peptide (amino acid residue 652-666; CVRPDVRVSPSFSQN). The result of Western blot analysis with anti-ATX Abs (+) and with only secondary antibodies (-) was shown.

3.4 Performance of ATX immunoenzymetric assay

We examined the within-run and the between-run reproducibility of our new serum ATX immunoenzymetric assay on AIA-system. Two pooled serum samples from normal healthy subjects were used to examine the within-run and the between-run coefficients of variation (CVs). A serum spiked recombinant ATX was additionally used for the within-run assay. In the within-run study, the measurement in the three samples was replicated 20 times. On the other hand, two samples were measured 20 times, in the between-run study. The mean ± standard deviation (SD) values for the three different samples in the within-run study were 0.59 ± 0.02, 1.25 ± 0.03 and 4.12 ± 0.10 mg/L, while the mean ± SD values for the two samples in the between-run study were 0.76 ± 0.03 and 1.48 ± 0.04 mg/L. The within-run and between-run CVs were 2.5 - 2.9% and 1.6 - 4.6%, respectively.

Various serum interferable substances that might interfere with the measurement were added to pooled serum samples (1:4 volume), followed by the ATX assay. Addition of up to 174 mg/L of conjugated bilirubin, 174 mg/L of free bilirubin, 4.4 g/L of hemoglobin, 50 mg/mL of human albumin, 20 g/L of triglycerides 200 mg/L of ascorbic acid, 100 mg/L of citric acid, 1 g/L of EDTA, 100 U/L of heparin and 5000 IU/L of rheumatoid factor did not affect on the recovery rate less than 10% in the present ATX

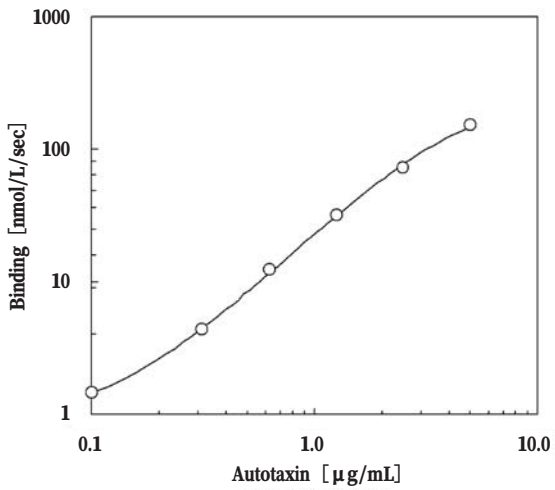

Fig. 7　Standard curve for the ATX immunoenzymetric assay on AIA-system
The calibration fluids consisting of six different concentrations (0, 0.34, 0.675, 1.35, 2.70, and 5.40 mg/L) were prepared. Binding rate (nmol/L/sec) of fluorescence intensity changes per second were measured and on AIA-system. Regression curve was drown by the equation; log (y) = a $\log(x)^{3}$ + b $\log(x)^{2}$ + c log(x) + d. The constant coefficient was as follows; a

immunoenzymetric assay.

The detection limit of the ATX antigen assay was defined as mean + 2 SD for the 5 replicates of zero calibrator. The minimum detection limit of this assay was estimated to be 0.110 mg/L.

3.5 Correlation study between ATX concentration and lysoPLD activity

It has been reported that ATX accounts for serum lysoPLD activity [29-30]. In fact, when human serum was applied to the R10.23-immobilized column, both ATX antigen and lysoPLD activity were completely undetectable. Therefore ATX concentration in human sera was determined on AIA-system and compared to their lysoPLD activity. The correlation between these two indices was analyzed (**Fig. 8**). The serum ATX antigen was very well-correlated to the serum lysoPLD activity (r=0.924). This result demonstrated that ATX exhibited almost all lysoPLD activity in human serum. Moreover this result indicated that the serum lysoPLD activity can be estimated by the measurement of the serum ATX antigen.

4. Conclusion

In this study, we found that the antibodies selected based on their capture ability of soluble ATX could hardly bind to ATX directly coated on microtiter wells. This result strongly suggested that we could not efficiently find out the antibody for two-site immunoenzymometric assay by the ordinary screening method base on antigen directly coated on immunoassay plate. Thus our results also suggested that the screening method based on an ability of capturing native antigen is indispensable for obtaining the antibody for immunoassay, like ELISA.

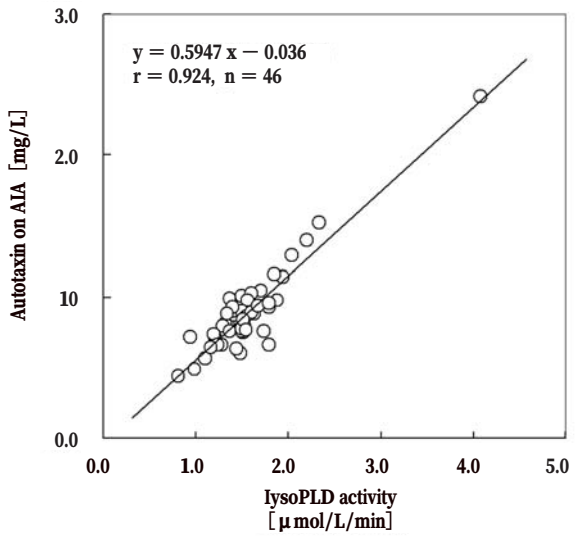

Fig. 8 Comparison study between the ATX concentration and the lysoPLD activity

ATX concentration in sera was determined by the AIA immunoenzymetric assay reagent on the AIA-system. The ATX concentration in serum samples was compared its lysoPLD activity by the linear regression method.

Ever since ATX has been reported to be identical to lysoPLD in 2002[5,31], which is producing enzyme of LPA, many scientists has been interested not only in physiological functions but also as a diagnostic marker. Although LPA has been suggested to be a serum biomarker for cancer metastasis by its physiological functions, it is not easy to determine the concentration of LPA in serum because LPA is not only unstable but also spontaneously generated in serum. By contrast, ATX is very stable in serum in comparison to the LPA instability. In fact, ATX remains its antigenic activity after several freeze-thaw cycles (data not shown). ATX concentration was determined by lysoPLD enzymatic activity assay until now but this method is time-consuming and really tedious. Whereas our new immunoassay is easy, quantitative, and high-throughput method in comparison with the lysoPLD enzymatic activity assay. In addition, our present result showed that the concentration of ATX antigen was well correlated with lysoPLD activity in serum. Therefore the ATX immunoenzymetric assay can be utilized for the determination of serum ATX concentration instead of the lysoPLD activity assay. Moreover regarding lysoPLD activity human specimens expect serum, like a seminal fluid, spinal fluid and urine, it is difficult to determine the lysoPLD activity by their intrinsic interfering substances. Especially lysoPLD activity in seminal fluid could not be determined because it contains high concentration, approximately 40 mmol/L, of choline which interferes with the lysoPLD enzymatic choline generation assay using 2 mmol/L LPC as a substrate (data not shown). Our preliminary result showed the ATX immunoenzymetric assay could determine ATX concentration in these human specimens (data not shown).

We performed the measurement of the ATX antigen in the serum using an automated analyzer and aimed to

adopt for clinical laboratory testing. Based on the results of the present study on within-run and between-run precision, interference, detection limit, we speculate that this ATX immunoenzymetric assay can be applied to clinical laboratory testing. The ATX immunoenzymetric assay, which is simple and can be performed in an automated analyzer, will contribute to the investigation of clinical signification of ATX as a novel diagnostics marker.

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