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Effect of PA-MSHA vaccine on plasma phospholipids metabolic profiling and the ratio of Th2/Th1 cells within immune organ of mouse IgA nephropathy

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Abstract

Phospholipids as a class of important constituents in the biomembranes have been paid increasing attention in many fields. IgA nephropathy is now generally known to be the most common form of primary glomerulonephritis in the world. However, phospholipids metabolism in IgA nephropathy was not clear. Until recently, there was no effective treatment available for patients with IgA nephropathy. In this paper, effect of PA-MSHA vaccine on plasma phospholipid metabolic profile of mouse IgA nephropathy was investigated using high performance liquid chromatography/mass spectrometry (HPLC/MS) and principal components analysis (PCA). Female Balb/c mice were divided into four groups: model group, control group, PA-MSHA treatment group and medicine control group (dipyridamole + common threewingnut root). The experimental IgA nephropathy model was established by the immunity combination method of oral BSA and injection of SEB. It was found that combination of LC/MS technology with PCA can be successfully applied to phospholipids profile analysis, clearly classify the model group and normal group, and PA-MSHA treatment group is closer to the normal control group than medicine control group. The result showed that Th_2/Th_1 (=$CD_4^+CD_{30}^+/CD_4^+CD_{30}^-$) of the model group is 20.70 ± 3.57, which is significantly higher than that of the control group (1.34 ± 0.14) ($P < 0.001$). The Th_2/Th_1 ratio of the PA-MSHA treatment group and the medicine control group are lower than that of the model group ($P < 0.01$). It is suggested that mouse IgA nephropathy has the phospholipids metabolic abnormality, PA-MSHA vaccine cannot only regulate the abnormal phospholipids metabolism mouse with the IgA nephropathy, but also correct the over unbalance of Th_2/Th_1 proportion.
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1. Introduction

Phospholipid is an important part that forms the cell membrane. Changes of phospholipid combination affect the physical and chemical properties of two-layer membrane. Membrane phospholipids are a complex mixture of molecular species containing a variety of fatty acyl and head group compositions, such as phosphatidylserine (PS), phosphatidylethanolamine (PE), phosphatidylcholine (PC), phosphatidylinositol (PI), lysophosphatidylcholine (LysoPC) and sphingomyelin (SM). In addition to their structural role, some phospholipids also participate in biological processes in various pathways such as signal transduction [1], membrane trafficking and sorting [2], morphogenesis [3], and source of second messengers [4–7]. Phospholipid has been paid increasing attention in many fields, for example as biomarkers in some disease studies. Animal models will help in understanding the relationship between phospholipids and diseases.

Metabonomics is the identification and measurement of metabolic profile dynamics of living systems to toxin or drug, environmental changes and diseases [8]. Metabolic profiling methods are gaining widespread use for the investigation of disease and drug efficacy in both the clinical and non-clinical settings [9–13]. These techniques involve NMR [8,14–16], mass

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spectrometry [8,17–20] and optical spectroscopic techniques [21]. High-performance liquid chromatography coupled with mass spectrometry (HPLC/MS) is a powerful alternative that offers high selectivity and good sensitivity [12,22,23]. Because of its sensitivity, specificity and efficiency, recent advances in mass spectrometry have provided the foundation for the development of approaches to identify and quantify complex lipids from biological samples and alternations in lipid metabolism and lipid-mediated signaling process.

A single LC/MS metabolite profile can yield hundreds of components. This provides a wealth of information to be interpreted and leads to significant challenges while processing the data. Thus, it is necessary to utilize a wide range of statistical data reduction method [24], i.e. multivariate analysis including principal components analysis (PCA).

Immunoglobulin A (IgA) nephropathy is a relatively new recognized disease. It is now generally known to be the most common form of primary glomerulonephritis (GN) in the world. Primary IgA nephropathy (IgAN) is the most common form of glomerulonephritis in the developed world and it progresses to end-stage renal failure in about one third of the patients within 10 years [25]. IgAN is characterized by predominantly mesangial IgA deposition. Nephrotic-range proteinuria, microscopic hematuria and renal failure impairment are strong predictors of an adverse clinical outcome [26,27]. The function disorder of immune system is involved in the progression of kidney disease, especially the change of T lymphocyte subtypes proportion makes the proportion of various cytokines unbalance and abnormal immune inflammatory reaction occur. Finally, it results in the impairment of kidney. In glomerular disease, the disorder of lipid metabolism is suspected as factors exacerbating glomerular dysfunction. Although many reports regarding metabolism disorders of lipids in nephritis syndrome have been published [28,29], there have been few published reports describing metabolism disorders of phospholipids in chronic glomerular disease. In particular, there has been no report on the relationship between proteinuria, the ratio of T helper cell-2 versus T helper cell-1 (Th_2/Th_1) and phospholipids level. Until recently, there was no effective treatment available for patients with IgA nephropathy. Since IgA nephropathy may affect up to 1.3% of the population [25,30], there is a need for novel therapeutic agents capable of preserving renal function. Substantial progress has been made in the past several years in treating IgA nephropathy such as angiotensin-coverting-enzyme inhibitors [31], corticosteroids [32] and $n-3$ polyunsaturated fatty acids [33,34]. But no convincing evidence has been provided to support the use of theses treatments.

Professor Xiya Mu, a Chinese microbiologist, has got a variant strain by applying bio-technology, and developed *Pseudomonas aeruginosa*-mannose sensitive hamemagglutination vaccine (PA-MSHA vaccine) [35]. This vaccine increases the antigen presenting function by activation of proliferation and differentiation of dendritic cells (DC cells) by body, in this way, it breaks down the complete and incomplete immunological tolerance state of the body, completely activates the polyclonal T cell and B cell, regulates the unbalance state of cell-mediated immunity and humoral immunity, increases the number and proportion of T cell and its subgroup, and stimulates the differentiation of intrinsic immunological active factor [36,37].In this paper, we used PA-MSHA vaccine to treat the IgA nephropathy mice, and utilized the plasma phospholipids metabolic profiling method combined to the ratio of Th2/Th1 cells to study how PA-MSHA vaccine affects the transformation of the T helper cell subtypes and phospholipids metabolism of IgA nephropathy. The result indicated that mouse IgA nephropathy has the phospholipids metabolic abnormality, PA-MSHA vaccine cannot only regulate the abnormal phospholipids metabolism mouse with the IgA nephropathy, but also correct the over unbalance of Th_2/Th_1 proportion.

2. Materials and methods

2.1. Animals husbandry and experimental protocol

Female Balb/c mice, 6–8-week old (weight 18 ± 2 g), were obtained from the Laboratory Animal Centre (Dalian Medical University, China). The Balb/c mice mentioned above were fed in clean-grade room at constant temperature (21 ± 2 °C) and humidity (45%) under a 12:12-h light/dark cycle. The routine urine test was checked after one week pre-feeding. Thirty-two mice whose urinary protein and urinary RBC were negative were selected, then were randomized to four groups: model group ($n = 10$), control group ($n = 6$), PA-MSHA treatment group ($n = 10$) and medicine control group (dipyridamole + common threewingnut root) ($n = 6$). All animal experiments were performed with the approval of the Institutional Animals Care and Animals Use Committee of the UA Greater Los Angeles Healthcare System.

Except the control group, mice in all other groups were orally given 0.1% bovine serum albumin (BSA) (Sigma Chemical Co., St. Louis, MO, USA) with acidified water (6 mM HCl) on alternate days [38,39]. After 6 weeks, each mouse was injected via tail veins with 0.4 ml 1% BSA buffer solution at fixed time, once a day, for 3 days. From 9th week on, the mice were injected with staphylococcal enterotoxin B (SEB), which was purchased from the Academy of Military Medical Sciences (Beijing, China), diluted by sterile saline with the dosage 0.8 mg/kg/week, once a week, for 3 weeks.

Mice in the control group were given oral acidified water on alternative days until death. After 6 weeks and on the 9th week, they were injected via tail veins with saline with the same quantity and time as for the model group.

PA-MSHA treatment group: at 11th week, after intravenous SEB, the subcutaneous injection of PA-MSHA vaccine (donated by Professor Xiya Mu, and each piece is 1 ml, containing inactivated PA-MSHA strain 1.8×10^9) was started. The first dose, 0.2 ml/mouse; the second dose, 0.3 ml/mouse. After the third time, the dose was controlled at 0.5 ml/ time, two times each week, lasted 4 weeks.

Medicine control group: at 11th week, after intravenous SEB, mice started to administrate by oral common threewingnut root (0.04 mg/g/day) and dipyridamole (0.2 mg/g/day), lasted 4 weeks.

Balb/c mice were fed with standard food and had free access to distilled water until 15 weeks. Urine was collected using metabolic cage. After completion of immunization, all mice were maintained for an additional week with free access to food and plain acidified water. On 16th week, all Balb/c mice were killed.

2.2. Urinary analyses and histochemistry technique

Protein quantitiation: 24-h-urine sample was collected and the protein quantitation per deciliter was examined by using Biuret colorimtry with automatic analyzer (Hiachi Co., Japan). The total urinary protein quantitation could be calculated according to the 24-h-urine volume.

RBC determination: random urine sample was collected and centrifuged (3000 rpm, 5 min). Red blood cell in urine precipitate was counted in ten high power fields (HPF) in every urine sample. Then the average RBC count per HPF was calculated.

Immediately after sacrifice, tissue from the left kidney was collected for immunofluorescence technique. The kidney frozen sections of 5 μm thickness on glass slides were air-dried. Sections were stained with fluorescein isothiocyanate (FITC)-labelled goat anti-mouse IgA (Sigma Chemical Co., St. Louis, MO, USA) (diluted 1:10).

2.3. Flow cytometry

After the execution of animals, under sterile condition the spleen cells were separated, and cultured in 1640 culture medium (1×10^6 cells/plate) (Gibco/BRL Co., USA), after 24 h PHA-P (2 μg/plate) (Sigma, USA) was added to the culture fluid, after 4 h FITC anti-mouse CD_4 (Clone NO RM 4–5, eBioscience, Co., USA), and R-PE anti-mouse CD_{30} (Clone NO $mCD_{30.1}$, eBioscience, Co., USA) were added. Then, RBC lysis buffer of mouse (BD Co., USA) was added, the collected cells were incubated at room temperature for 30 min for the analysis with FACScan Flow Cytometer (BD Co., USA).

2.4. Plasma preparation and chemicals

The phospholipid standards (1,2-dimyristoyl-*sn*-glycero-3-phosphoethanolamine (C14:0/C14:0 PE phosphatidyl ethanolamine), 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (C14:0/C14:0 PC phosphatidylcholine), 1,2-dimyristoyl-*sn*-glycero-3-[phospho-L-serine] (sodium salt) (C14:0/C14:0 PS phosphatidylserine) and 1-lauroyl-2-hydroxy-*sn*-glycero-3-phosphocholine (C12:0 lysoPC lysophosphatidylcholine) were from Avanti Polar Lipids, Alabaster, AL, USA) were dissolved (approximately 1 mg/ml) in chloroform/methanol (2:1, v/v) (HPLC grade, TEDIA, USA), and further diluted in hexane/1-propanol (3:2, v/v) (HPLC grade, TEDIA, USA).

To prevent platelet activation and phospholipase activity, blood samples were collected in EDTA-containing tubes. Mouse plasma was stored at −80 °C before lipid extraction. The lipids in the samples were extracted essentially as described earlier [40]. Prior to analysis, the extracted samples were redissolved in 250 μl of chloroform/methanol (2:1, v/v) (HPLC grade, TEDIA, USA) and then was diluted 10 times by hexane/1-propanol (3:2, v/v).

Table 1
Linear gradient composition

Time (min)	A (%)	B (%)
0	68	32
20	20	80
33	20	80
38	68	32
60	68	32

Note—Solvent mixture A: hexane/1-propanol/formic acid/ammonia (25%) (79/20/0.6/0.06, v/v/v/v); solvent mixture B: 1-propanol/water/formic acid/ammonia (88/10/0.6/0.06, v/v/v/v).

2.5. High-performance liquid chromatography and mass spectrometry

An HP 1100 series HPLC system (Agilent Technologies, Palo Alto, CA, USA) was used. The LC separation was performed on a diol column (Nucleosil, 100-5 OH, Germany), 250 mm × 3.9 mm (i.d.) × 5.0 μm. The total flow rate was 0.4 ml/min. The flow from the LC was split using a Micro-Splitter Valve such that the flow to the electrospray was approximately 0.28 ml/min. The column temperature was kept at 35 °C. The linear solvent gradient was shown in Table 1.

The mass spectrometric detection was conducted on a QTRAP LC/MS/MS system from Applied Biosystems/MDS Sciex (USA) equipped with a turbo ion spray source. The combination of highly selective triple quadrupole MS–MS scans and high sensitivity ion trap product scans on the same instrument platform provides rapid identification of phospholipids of extracted mice blood sample. The detection of phospholipids eluted from the chromatographic column was performed in a survey scan named as “enhanced MS” (EMS). The split HPLC effluent entered the MS through a steel ES ionization needle was set at −4500 V (in negative ion mode) and the ion source temperature was set to 250 °C. The ion source and ion optic parameters were optimized with respect to the positive or negative molecular related ions of the phospholipids standards. The nebulizing gas and turbo gas were both set at 40 psi, the curtain gas was set at 30 psi. The declustering potential was set at 80 V. The other parameters were as follows: EMS as survey scan (mass range *m/z* 450–950, scan speed 1000 Da s^{-1}, fill time 20 ms) and EPI as dependent scan (scan speed 1000 Da s^{-1}, fill time 150 ms, collision energy set from −50 to −35 eV in the negative-ion mode.)

The structures of phospholipids were elucidated according to the tandem mass spectra acquired under the “enhanced product ion scan” (EPI) mode [42–44].

2.6. Data analysis

Negative-ion LC/MS chromatograms were inspected for profiling the phospholipid species in plasma. Negative-ion mode ESI–MS was chosen because it gave more information-rich data than positive-ion mode. Masses corresponding to the

Table 2
Quantitative urinary proteins and counting of urinary RBC

	Mice	Urinary protein (mg/24 h)	Urinary RBC (HP)
Control group	6	91.8 ± 19.6	1.1 ± 1.3
Model group	10	1472.7 ± 329.5[a]	8.4 ± 3.1[a]
PA-MSHA treatment group	10	441.8 ± 95.08[b]	3.7 ± 1.4[b]
Medicine control group	6	1141.6 ± 105.8[c]	5.8 ± 3.4[d]

[a] Compared with control group, $P < 0.001$.
[b] Compared with model group, $P < 0.01$.
[c] Compared with model group, $P > 0.05$.
[d] Compared with model group, $P < 0.05$.

quasi-molecular anions $[M-\mathrm{H}]^-$ (for PE, PS, and PI species) or $[M-15]^-$ (for PC, SM and lyso-PC species) for each phospholipid species were plotted against elution time. From the LC/MS profile of a plasma sample, 64 phospholipid species were discovered and the peaks that constantly occurred in each IgA nephropathy mice were collected to form a database. The peak intensities in the extracted ion chromatography were normalized (i.e. the peak intensities were divided by their corresponding internal standards' intensities) by that of internal standard using the home-made software.

Then the data were fed to principal component analysis (PCA) and partial least squares-discriminant analysis (PLS-DA) with the SIMCA-P software (Version 10.0 DEMO, Umea, Sweden). Partial least squares (PLS) was a powerful method of data analysis for the minimal demands on measurement scales, sample size, and residual distributions. Although PLS can be used for theory confirmation, it can also be used to suggest where relationships might or might not exist and to suggest propositions for later testing. PLS regression is a recent technique that generalizes and combines features from principal component analysis and multiple regression. It is particularly useful when we need to predict a set of dependent variables from a large set of independent variables. In our study, PLS regression method (using SIMCA-P software) was employed to find the correlation between phospholipids and urinary protein or Th2/Th1. Phospholipids species were served as independent variables (X), and urinary protein, Th2/Th1 were served as dependent variables (Y), respectively.

3. Results and discussion

3.1. Treatment of PA-MSHA vaccine on mouce IgA nephropathy

IgA nephropathy has been considered to be a kind of immune complex nephritis, and accumulation of IgA immune complex is the important mechanism of IgA nephropathy origination. The experimental IgA nephropathy model was established by using the immunity combination method of giving oral BSA and injection of SEB. The results show that the proteinuria concentration is 1472.7 ± 329.5 mg/24 h in the model group, which is much higher than that in the control group (91.8 ± 19.6 mg/24 h) ($P < 0.001$). The counting of urinary RBC in model group is much higher than that in the control group (8.4 ± 3.1 versus 1.1 ± 1.3) ($P < 0.001$) (Table 2). In the immunofluorescent staining, immunoglobulins A distributed in clumps on the mesangical areas or walls of capillary in all of ten Balb/c mice in the experimental model group, whereas sections from all controls contained negligible IgA. It accords with the clinical appearances of IgA nephropathy. The model used here affords a basis for further study of the nephropathy mechanism.

At the same time, Table 2 also indicates that the urinary protein concentration in the PA-MSHA treatment group is 441.8 ± 95.1 mg/24 h, which is much lower than that of model group ($P < 0.01$), whereas the urinary protein concentration in the medicine control group (1141.6 ± 105.8 mg/24 h) has no obvious difference from those in the model group ($P > 0.05$); the urinary RBC counting (3.7 ± 1.4) in the PA-MSHA treatment group is significantly lower than that in the model group ($P < 0.01$), whereas the urinary RBC counting in the medicine treatment group is also decreased. Immunofluorescence also shows that the IgA immune complex accumulation in mesangial area or capillary of glomeruli in the PA-MSHA treatment group is significantly less than that in the model group. Whereas, the IgA accumulation in the glomeruli of medicine control group is similar to that of model group (Fig. 1). It suggests that PA-MSHA vaccine significantly decreased the urinary protein and hematuria, and released the pathological change of kidney. It could be seen that the PA-MSHA vaccine has obvious treatment effect on mice with the IgA nephropathy.

3.2. PA-MSHA vaccine might modulate the imbalance of Th_2/Th_1 within spleen of mouse IgAN

Differentiation mature degree of T cells from low to high are $CD_4^-CD_8^-$ double-negative T cell, CD_4^+ or CD_8^+ single-positive cell, and $CD_4^+CD_{30}^+$ (Th_2) or $CD_4^+CD_{30}^-$ (Th_1) T cell. It has been well known that Th_1 is mainly involved in cell-mediated immunity and Th_2 is mainly involved in humoral immunity. By using two-color fluorescence method, the percentages of $CD_4^+CD_{30}^+$ and $CD_4^+CD_{30}^-$ cells of spleen in each group were detected by flow cytometry, and the result is given in Fig. 2. Th_2/Th_1 (=$CD_4^+CD_{30}^+/CD_4^+CD_{30}^-$) of the model group is 20.70 ± 3.57, which is significantly higher than that of the control group (1.34 ± 0.14) ($P < 0.001$), which indicated Th_2 hyperfunction had the relationship with onset of IgA nephropathy [41]. We suggest that hyperfunction of Th2 cell is a signification pathogenic factor in mouse IgA nephropathy; the balance between Th1 and Th2 might play a role in the development of immunopathologic lesions in mouse IgA nephropathy.

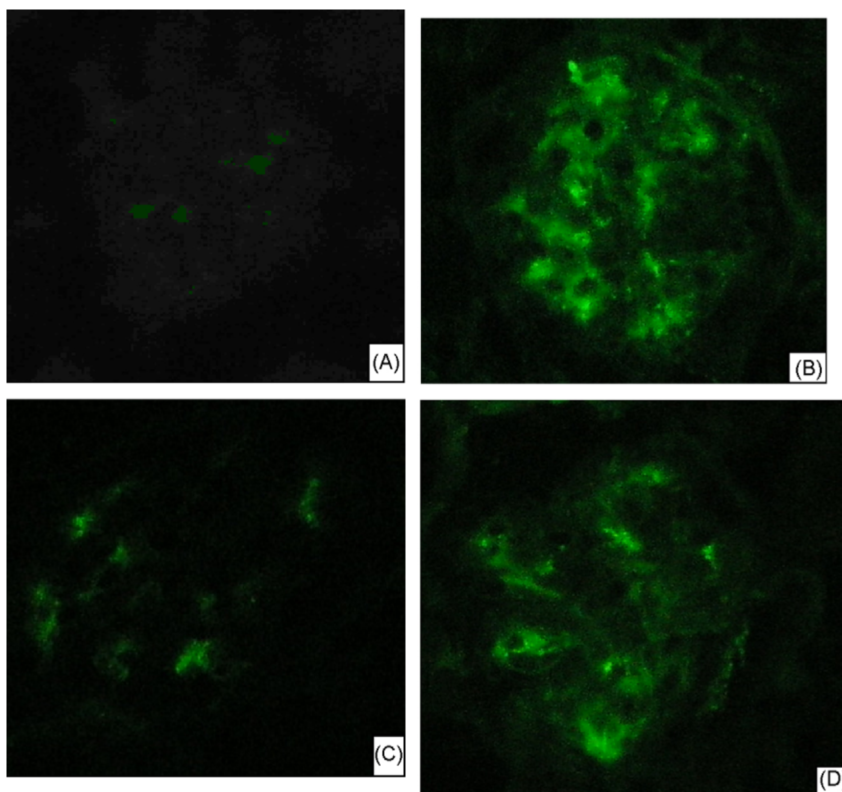

Fig. 1. IgA accumulation to glomeruli stained with anti-IgA fluorescein isothiocyanate (FITC). (A) Control glomerulus. (B) Glomerulus from the model mouse. (C) Glomerulus from the PA-MSHA treatment mouse. (D) Medicine control glomerulus.

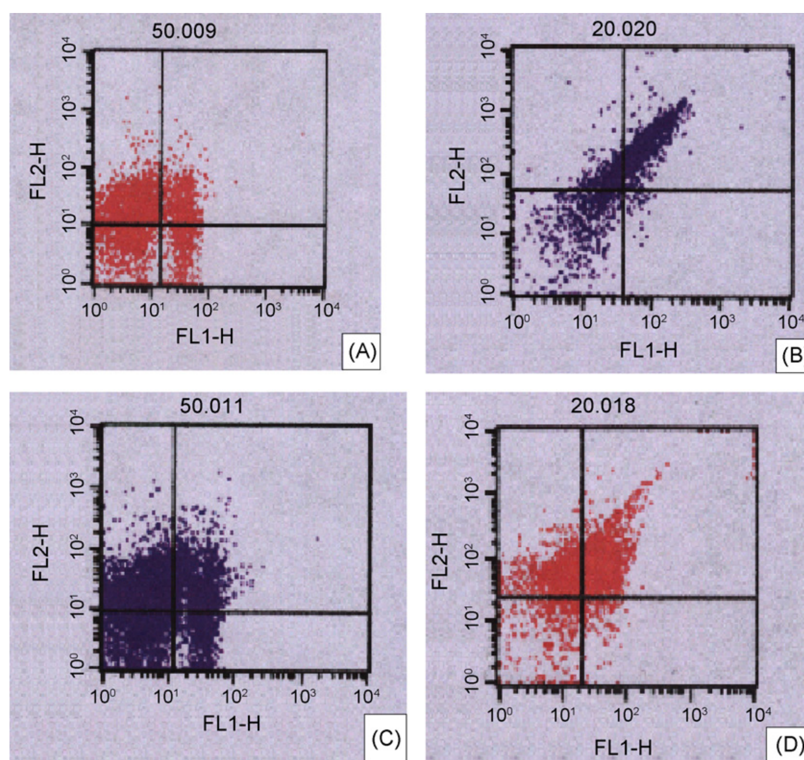

Fig. 2. Percentages of $CD_4^+CD_{30}^+$ and $CD_4^+CD_{30}^-$ cells of spleen in each group by flow cytometry. (A) Control; (B) IgA nephropathy model; (C) PA-MSHA treatment; (D) Medicine control.

The experimental result further showed that the Th_2/Th_1 ratio in the PA-MSHA treatment group (1.86 ± 0.07) are lower than that in the model group ($P < 0.01$), which indicated PA-MSHA vaccine made Th2 differentiation index decreased, and shift from Th2 cell to Th1 cell increased. The tendency CD_4^+ T cells in spleen of mouse with IgA nephropathy over differentiation to Th_2 cells was corrected to a degree after the treatment of PA-MSHA vaccine. Therefore, PA-MSHA vaccine regulating T cell differentiation direction helps improving the imbalance of Th_2/Th_1 within immune organs—spleen.

3.3. Separation of phospholipid classes

The full scan of phospholipid species in plasma was in the negative-ion mode because in this mode most of phospholipid species have relatively high sensitivity. PE was eluted firstly, followed by PS (PI), PC, SM and lysoPC in a successive manner for phospholipids containing a given fatty acyl composition. Fig. 3(top) shows the total-ion current (TIC) of phospholipids species in IgA nephropathy Balb/c mouse plasma based on the negative-ion HPLC-ESI/MS. Since different molecular species within a given class have the same polar head, their retention times in normal-phase HPLC have minor difference. The retention time difference of compounds within one same class is less than that in two different classes, which can be used to align the retention time of phospholipid species in the extraction ion chromatography in order to avoid the retention time fluctuation between different injections. Hence, the chromatographic retention times can be used to identify each class and most importantly the system separated isobaric species from different phospholipid classes. On the other hand, after normal-phase HPLC separation, the ions with different *m*/*z* was separated by MS. Finally we can obtain the two-dimensional phospholipid profiling (Fig. 3, down).

The internal standards (C14:0/C14:0 PE, C14:0/C14:0 PS, C14:0/C14:0 PC and C12:0 lysoPC) were selected based on their solubility and the lack of any demonstrated endogenous molecular ions in that region, which was verified by acquiring a mass spectrum without internal standards. These phospholipid species are quasi-quantified by the comparison of the individual

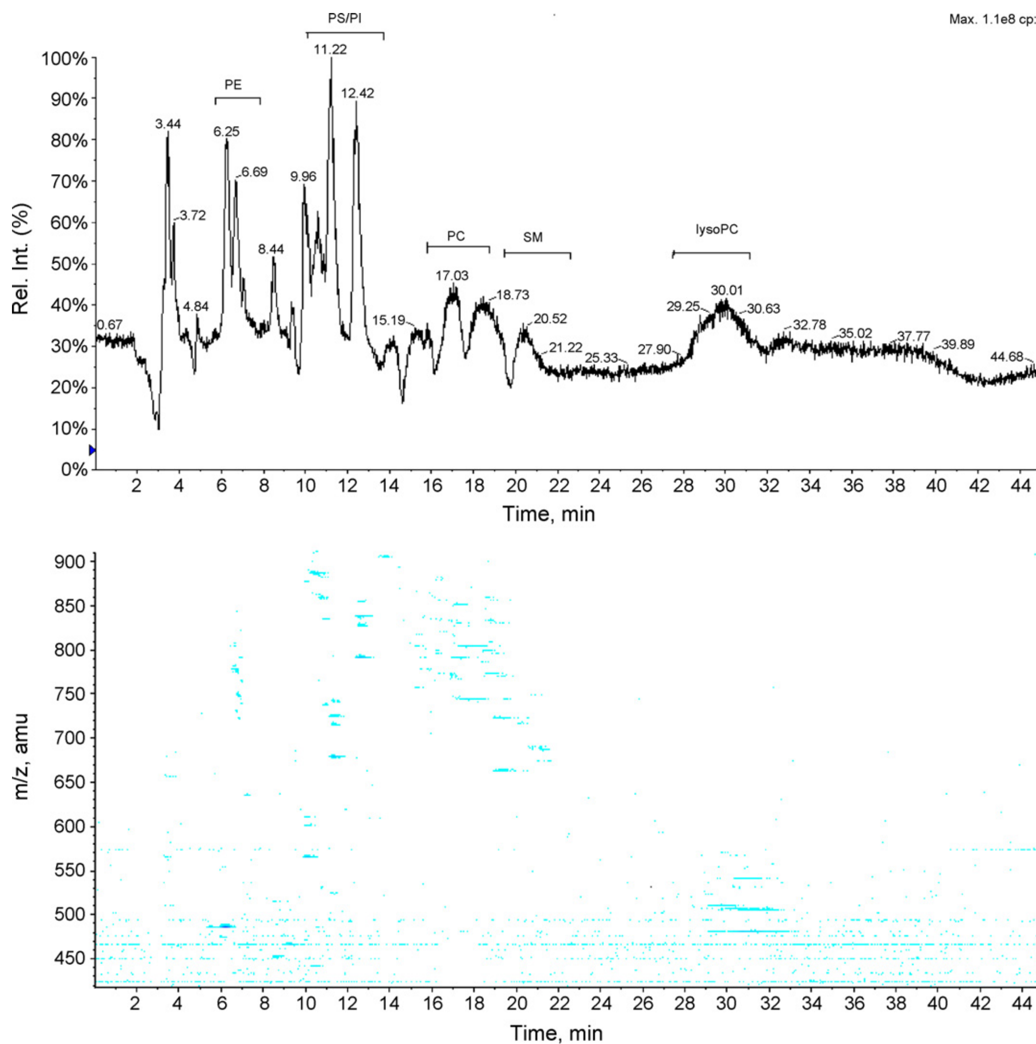

Fig. 3. Total ion chromatogram of LC/ESI–MS analysis of phospholipid mixture obtained from blood sample on IgA nephropathy Balb/c mouse showing both total ion chromatogram (top) and a two-dimensional plot including retention times and *m*/*z* values (bottom). PS, phosphatidylserine; PE, phosphatidylethanolamine; PC, phosphatidylcholine; PI, phosphatidylinositol; LysoPC, lysophosphatidylcholine; SM, sphingomyelin.

ion peak intensity with that of corresponding internal standard. For PI and SM species, due to the lack of commercial internal standards, their retention times were close to those of PS and PC species, and their quasi-quantification was based on PS and PC internal standards (i.e. C14:0/C14:0 PS and C14:0/C14:0 PC, respectively).

To evaluate the reproducibility of HPLC–MS, the phospholipids standards were analyzed for six times in the selected conditions. R.S.D.s (%) of retention time and peak intensity ratio were <0.5% and <5%, respectively. Replicated analyses on the real plasma samples of mice were performed for five times. Reproducibility of HPLC/MS of the plasma phospholipid analyses was evaluated. The R.S.D.s (%) of peak height ratio were from 3.68 to 17.25% depending on the concentrations of phospholipids.

3.4. Effect of PA-MSHA vaccine on plasma phospholipids metabolic profiling by multivariate analysis

Phospholipids are the main constituents of biological membranes and have an important role in signal processing and as precursors for many other biologically active molecules [45–48]. We coupled HPLC with MS and investigated phospholipid species in Balb/c mice plasma. Sixty-four variables (phospholipid species) from the LC/MS spectrum (Fig. 3, down) established the row of matrix *X*, and 32 plasma samples (10 in the model group, 6 in the control group, 10 in the PA-MSHA treatment group and 6 in the medicine control group) established the column of matrix *X*, then matrix *X* was used in the PCA analysis. From Fig. 4(top), it is clear that the PCA analytic method achieves a good separation effect based on the plasma phospholipids, namely the model group is well separated from the control group. It hinted that mouse with the IgA nephropathy had an abnormal phospholipids metabolism.

From the result of the PCA analytic method, we further found that the PA-MSHA treatment group and the medicine control group distribute between the model group and the control group, and the PA-MSHA treatment group is closer to the control group. It could be seen that compared with the medicine control group, in the PA-MSHA treatment group PA-MSHA vaccine can regulate abnormal phospholipids metabolism. It suggests that PA-MSHA vaccine has a good treatment effect on the mouse with IgA nephropathy.

The loadings plot (Fig. 4, down) indicates the most influential ions that are responsible for separation between sample classes: the ions having the greatest influence in the PCA scores plot are those furthest away from the main cluster of ions. In turn, these compounds might be likely candidates for biomarkers. From this loadings plot, it shows that the possible biomarkers are those with *m/z* 790, 836 and 885 in the negative-ion mode. Moreover, the structures of these phospholipids molecules were identified as C18:0/C18:0 PS (phosphatidylserine) (*m/z* 790), C18:0/C22:5 PS (phosphatidylserine) (*m/z* 836) and C18:0/C20:4 PI (phosphatidylinositol) (*m/z* 885) by enhanced product ion (EPI) spectrum experiments under the negative mode. At the same time, the three phospholipid species in PA-MSHA treatment group were obviously different from those in control group. *T*-test in each of two groups was employed. Statistic significance was found (all $P < 0.01$). It suggests that C18:0/C18:0 PS, C18:0/C22:5 PS and C18:0/C20:4 PI were the most important phospholipid species for classifying IgA nephropathy from the normal controls and observing the therapy of PA-MSHA vaccine on IgA nephropathy.

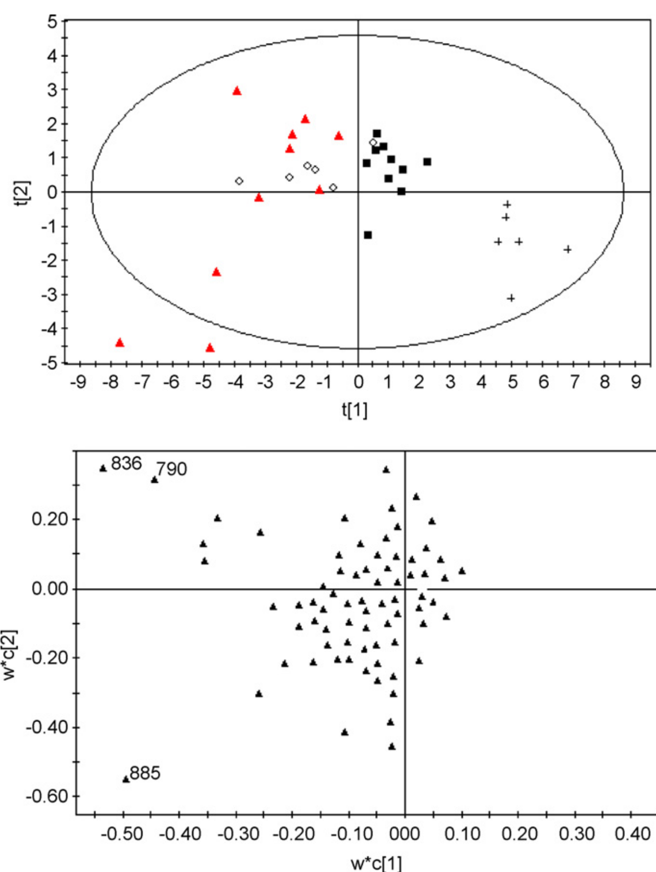

Fig. 4. Scores (top) and loadings (down) plots from PCA classifying plasma phospholipids from Balb/c mice in each group. (■) PA-MSHA treatment group; (▲) model group; (+) control group; (◊) medicine control group.

To study the relationship between phospholipids and proteinuria, the partial least squares-regression (PLS-R) model was used (Fig. 5). The horizontal axis shows the result of PLS performed according to the content of phospholipids species, and the vertical axis shows urinary protein. It can be seen from the result of Fig. 5 that during the development of IgA nephropathy and observation of the PA-MSHA treatment, a good relationship was seen between PLS-regression result using phospholipids data and urinary protein. Similarly, Fig. 6 gives the relationship between phospholipids and Th2/Th1 by PLS-R, the result points out that their relationship is not good. The possible mechanism is that mediated by PI [49], the intracellular protein kinase C signal transfer system is activated by the participation of PS, Ca^{+} and diacylglycerol, therefore the permeability of epithelial cells is increased [50] and the glomerular capillary barriers are injured, which results in proteinuria. However, PA-MSHA vaccine might restrain this signal transfer system, decrease the glomerular capillary barriers injury, which diminish the production of urinary protein. It is well known that proteinuria is an adverse predictor

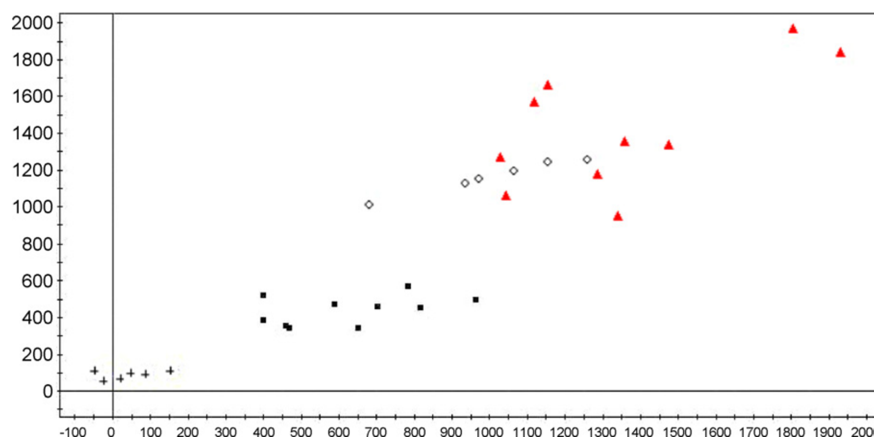

Fig. 5. Relationship between phospholipids and urinary protein by PLS-R. X: the result of PLS regression performed according to the content of phospholipids. Y: urinary protein. (■) PA-MSHA treatment group; (▲) model group; (+) control group; (◊) medicine control group.

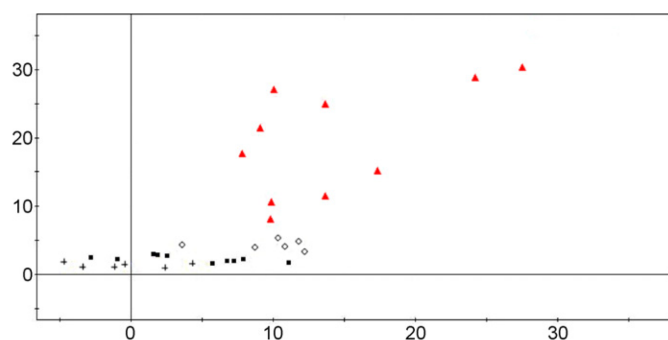

Fig. 6. Correlation between phospholipids and Th2/Th1 by PLS-R. X: the result of PLS performed according to the content of phospholipids. Y: Th2/Th1. (■) PA-MSHA treatment group; (▲) model group; (+) control group; (◊) medicine control group.

of this disease progress. We can assuredly presume that phospholipids could be an important biomarker during the study of mice with IgA nephropathy.

4. Conclusions

Combined the LC/MS technique with multivariate statistical analysis, it was found that mouse IgA nephropathy had the phospholipids metabolic abnormality. PA-MSHA vaccine not only decreases the urinary protein and microscopic hematuria of mouse with the IgA nephropathy, but also can correct the over unbalance of Th_2/Th_1 proportion, regulate the abnormal phospholipids metabolism. Thus, the purpose of treatment IgA nephropathy is achieved. The study of phospholipids metabolism change can be considered as proper criteria for diagnosis of nephropathy and observation of medicine treatment.

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