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Analysis of the Urine Proteome of Human Contrast-Induced Kidney Injury Using Two-Dimensional Fluorescence Differential Gel Electrophoresis/ Matrix-Assisted Laser Desorption Time-of-Flight Mass Spectrometry/Liquid Chromatography Mass Spectrometry

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Key Words

Urinary biomarker · Mannose-binding lectin · Contrast-induced nephropathy · Acute kidney injury · Two-dimensional differential gel electrophoresis · Mass spectrometry

Abstract

Background: The pathogenesis of contrast-induced nephropathy (CIN) remains unclear and is defined by changes in serum creatinine which is not a sensitive biomarker for acute kidney injury. Search for differentially expressed urinary protein or peptide could contribute to further understanding of the disease and may provide new biomarkers. ***Methods:*** This is a small sample research. Urine samples were obtained from patients who underwent percutaneous coronary intervention and were labeled with 3 different fluorescent dyes. After 2-dimensional electrophoresis was run, the differentially regulated spots were picked out and identified by mass spectrometry. Another 31 patients were used as validation group. ***Results:*** Among 56 significantly changed spots, mannose-binding lectin (MBL) and MBL-associated serine protease 2 were both significantly upregulated. Compared to the baseline value, urinary MBL was significantly increased in the CIN group (2.08, 1.42–5.72, vs. 1.09, 0.516–1.411; $p < 0.01$). Postprocedure urinary MBL in the CIN group was also significantly higher than that in the non-CIN group (2.08, 1.42–5.72, vs. 1.057, 0.738–1.885; $p < 0.05$). ***Conclusion:*** The studies suggested that MBL may be associated with contrast-induced acute kidney injury. It leads to an attempt to define a new pathogenesis and a novel biomarker for CIN.

Introduction

The incidence of contrast-induced nephropathy (CIN) continues to increase with the expansion of the use of iodinated contrast medium. When CIN complicates percu-

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taneous coronary intervention (PCI), it is associated with significant in-hospital and long-term morbidity and mortality, as well as with a prolonged hospital stay [1–5]. CIN remains defined by an increase in serum creatinine (SCR) [6]. However, following a toxic insult SCR rises slowly, usually over days. Early detection of acute kidney injury would assist in facilitating early interventions.

The protein profiles of urine could reflect acute kidney injury directly and promptly. And by virtue of its noninvasiveness and availability, peptidome profiling of human urine is now becoming an important method for investigating kidney physiology and detecting novel disease-associated markers of renal diseases [7, 8]. Surface-enhanced laser desorption/ionization time-of-flight (TOF) mass spectrometry (MS) is useful for the clinical urinary protein patterns, but definitive identification of differentially regulated proteins is challenging. Two-dimensional (2D) polyacrylamide gel electrophoresis has been used in many types of protein expression profiling; however, quantitative analysis of the urinary protein from numerous 2D gels is difficult, and gel-to-gel variation will further complicate the analysis [9–11]. Two-dimensional differential gel electrophoresis (2D-DIGE) in which samples are labeled with 3 different fluorescence cyanine (Cy) dyes and are resolved simultaneously within the same 2D gel could reduce variability and enable a reliable and precise comparison of urinary protein profiles among different gels [12]. Following 2D-DIGE, identification of protein by matrix-assisted laser desorption/ionization (MALDI) TOF MS or liquid chromatography MS are feasible and reliable. In the present study, 2D-DIGE and MALDI-TOF MS were employed to investigate protein expression alterations between preprocedure and postprocedure urine from patients who underwent PCI.

Mannose-binding lectin (MBL) and MBL-associated serine protease 2 (MASP2) are two key components in the immune response in that they can initiate the lectin pathway of the complement cascade of the innate immune response [13, 14]. Recent studies have shown that complement plays a crucial role in the pathogenesis of renal injury. Zhou et al. [15] demonstrated that complement-deficient mice are protected against renal ischemia/reperfusion (I/R) injury, others showed that renal I/R injury could be abrogated by treatment with complement inhibitors [16–18]. We identified MBL and MASP2 as two significantly upregulated spots in the urine profile after PCI. Furthermore, validation of MBL in human urines between patients with and without CIN suggests that MBL may contribute to the pathogenesis of CIN and urine MBL may be a possible early biomarker.

Table 1. Experimental design of DIGE gels

	Gel 1	Gel 2	Gel 3
Cy2	internal standard	internal standard	internal standard
Cy3	postproc. group A	preproc. group C	postproc. group C
Cy5	preproc. group B	postproc. group B	preproc. group A

Preproc. = Preprocedure CAG or PCI; postproc. = postprocedure CAG or PCI.

Patients and Methods

Patient Selection

Twelve patients admitted to Renji Hospital of Shanghai Jiaotong University for diagnostic or therapeutic cardiac catheterization were recruited as 2D-DIGE group. All of these patients were strictly selected by the following criteria: male, age over 60 but below 80 years, body mass index between 22 and 28, with a history of hypertension, history of diabetes mellitus and angina pectoris, preprocedure SCR below 90 μmol/l. Patients with a history of any invasive procedure 2 weeks before, signs of acute renal failure or acute myocardial infarction, genitourinary tract infection and anemia were excluded.

Salt intake of these patients was unified as lower than 3 g/day. The calorie intake for these patients was also calculated as 25 cal/day/kg. Protein, lipid and glucose account for 20, 20 and 60%, respectively, in the daily calorie composition.

Two freshly voided urine samples were studied; the first was obtained before cardiac catheterization, the second approximately 12–18 h after the procedure. In the course of routine monitoring, SCR and blood urea nitrogen levels were measured before, 24 and 48 h after the procedure. Low-osmolar contrast medium (Isovue 370, Bracco Diagnostics Inc.) had been administered to all patients as a radiocontrast agent.

Another 31 patients (13 CIN, 18 non-CIN) from our former study of urine biomarkers of CIN were used as validation group. The urine and serum samples were collected on site and were frozen at –80°C for later use. Whether MBL was upregulated 24–48 h after cardioangiography (CAG) or not was tested by enzyme-linked immunosorbent assay (ELISA).

CIN was defined as an increase in SCR of more than 25%, or 44.2 μmol/l over the baseline level 48–72 h after the procedure.

Ethical Considerations

This investigation was approved by the institutional review board of Renji Hospital, Medical School, Shanghai Jiaotong University. All patients were informed and consented before enrollment.

Sample Preparation for 2D-DIGE and Protein Isolation

For DIGE analysis, the urine samples were collected from 12 patients before and after PCI. Protease inhibitors (Cocktail Set I; Calbiochem, Merck) were added and samples cooled at 4°C at once. The samples were stored at 4°C for no more than 2 h until centrifugation to remove the insoluble materials. Ultrafiltrated by Centriprep 5-kDa cutoff YM-3 membrane concentrators (Milli-

Table 2. Clinical and demographic data for patients in whose urine proteomes were analyzed

Case	Age years	HPT or DM	FBG mmol/l	Basic SCR μmol/l	Postproc. SCR, μmol/l	CIN	ACEI or CCB	NSAIDs	CM ml	TC mmol/l	Urine protein mg/l
1	68	both	5.8	89.7	95.8	no	both	yes	150	4.16	negative
2	77	HPT	6.4	78.2	114.3	yes	both	yes	200	9.83	0.25
3	67	both	12.8	87.4	114.9	yes	both	yes	200	7.84	0.25
4	60	HPT	6.4	88.4	77.4	no	both	yes	100	6.70	negative
5	75	both	7.8	82.0	78.8	no	both	yes	150	5.66	negative
6	71	HPT	5.6	67.6	84.5	yes	both	yes	180	7.13	negative
7	71	both	8.0	90.0	89.7	no	both	yes	100	5.24	0.15
8	69	HPT	7.0	85.0	77.8	no	both	yes	100	2.60	negative
9	63	both	6.8	73.7	81.1	no	both	yes	150	3.48	negative
10	65	both	8.9	64.4	69.8	no	both	yes	100	6.38	negative
11	66	both	16.1	69.3	94.4	yes	both	yes	220	4.00	0.25
12	72	HPT	6.8	75.3	82.5	no	both	yes	180	6.38	0.15

All 12 patients were male, with age ranging from 60 to 77 years (68.7 ± 4.9), body mass index from 22 to 28 (25.9 ± 1.7), all took angiotensin-converting enzyme inhibitors (ACEI) and calcium channel blockers (CCB) and had hypertension; besides, most had type 2 diabetes at the same time; they all took nonsteroidal anti-inflammatory drugs (NSAIDs) such as acetylsalicylic acid. HPT = Hypertension; DM = diabetes mellitus; FBG = free blood glucose; postproc. SCR = SCR 48 h after the procedure; CM = contrast medium; TC = total cholesterol.

pore, Billerica, Mass., USA) at 4°C, the concentrated samples were immediately stored at –80°C until use.

The precipitated urine protein pellet which was processed by adding –20°C acetone to the concentrated urine samples was resuspended, and the protein concentration was determined using Bradford's spectrophotometric method [19] measured by UV-1700 Pharmspec (Shimadzu).

Fluorescent Tagging

The 12 selected patients were randomly divided into 3 groups. Three DIGE gels were run; each gel compared the preprocedure to postprocedure urine proteomes of different groups of patients (table 1). The preprocedure or postprocedure protein was labeled on ice for 30 min with Cy3 or Cy5, respectively. An internal standard which comprised equal amounts of pre- and postprocedure protein was labeled with Cy2. For each gel, these 3 samples (internal standard, preprocedure and postprocedure) were pooled prior to 2D examination.

Isoelectric Focusing, Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis, Image Analysis

For analysis, 200 μl of the mixture was used to rehydrate 11-cm, pH 4–7 immobilized pH gradient strips (GE Healthcare) in an immobilized pH gradientphor (GE Healthcare) unit. Once rehydration was completed, samples were focused according to the isoelectric point.

Immediately after isoelectric focusing, immobilized pH gradient strips were incubated in equilibration buffers I and II, second-dimension separations (SDS-PAGE) were performed on an SE600 vertical electrophoresis system (GE Healthcare) and gels were scanned using the DIGE-enabled Typhoon Scanner (GE Healthcare).

The gel image was processed by the differential in-gel analysis module of Decyder software version 5.01 to detect differentially expressed spots. The estimated number of spots for each codetection procedure was set to 1,500.

MS and Peptide Mass Fingerprinting

Spots of interest were cut from a preparative 2D gel which was loaded with 800 μg urine protein mixture of pooled internal samples. After they had been destained and in-gel digested, the spots were processed by a Shimadzu-Biotech Axima CFRPlus MALDI-TOF instrument (Shimadzu-Biotech, Manchester, UK) to get the peptide mass fingerprinting (PMF). PMF spectra were then interpreted with the Mascot (http://www.matrixscience.com/) against National Center for Biotechnology Information nonredundant protein database.

Linear trap quadrupole (LTQ) Bioworks 3.2 (Finnigan LTQ Mass Spectrometer LTQ; Thermo Electron Corporation) was also used for liquid chromatography MS identification of spots which could not be identified by MALDI-TOF MS analysis.

Enzyme-Linked Immunosorbent Assay

ELISA kits designed for human MBL (USCN Life, Missouri City, Tex., USA) were used in samples collected from another 13 CIN and 18 non-CIN patients. The minimum detectable dose of human MBL of the kit is 0.24 μg/l, and the detection range is 0.94–60 μg/l. Absorption was measured at 450 nm wavelength with an ELX800 Universal Microplate reader (Bio-Tek instrument Inc.).

SCR was measured by a modified Jaffe method with protein precipitation using an alkaline picrate reaction.

The biochemical assays were performed blinded to the sources and identities of all the specimens ordered at random.

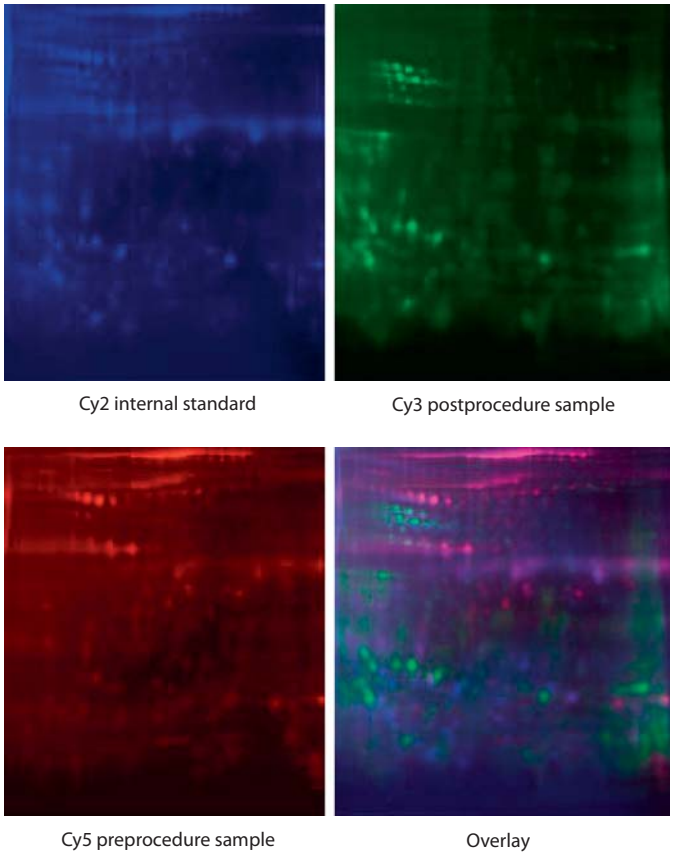

Fig. 1. Overlaid 2D-DIGE gel images of preprocedure (red in the online version), postprocedure (green in the online version) and internal standard (blue in the online version) protein spot patterns. The internal standard is Cy2 labeled, Cy3 labels the postprocedure urine sample and Cy5 the preprocedure urine sample.

Statistical Analysis

A Decyder biological variation analysis (GE Healthcare) software module was used for statistical analysis of protein abundance changes between samples by matching spots displayed in internal standard in each of the gels. Analysis of variance (ANOVA) was applied, and the data were filtered to retain spots with ANOVA statistical significance, $p < 0.05$, and spots that appeared in >75% of the spot maps.

The distribution of the urine and serum MBL ELISA data was skewed by the 1-sample Kolmogorov-Smirnov test and was found not normally distributed. For univariate analysis, a comparison between CIN and non-CIN patients was performed using the Student t test or Kruskal-Wallis H test for continuous variables as appropriate, and categorical data were analyzed with the Pearson χ^2 test. The Wilcoxon rank sum test was used in paired samples before and after the procedure. Results were expressed as means ± SD or medians and interquartile ranges where appropriate.

All tests of significance were 2-sided, and differences were considered statistically significant at $p < 0.05$. The software used for data analysis was SPSS version 13.0.

Results

DIGE and PMF

The clinical data of the patients whose urine had been used for DIGE analysis is presented in table 2. In figure 1, protein variation is visualized on gels of different time points and the internal standard of urine samples labeled by different Cy dyes.

From the comparison result analyzed by Decyder software through differential in-gel analysis and biological variation analysis modules, there were at last found 56 confirmed spots ($p < 0.01$ by ANOVA) including 39 increased and 17 decreased as compared between preprocedure and postprocedure samples. All of these 56 spots were cut from a preparative gel, and their peptide fragments of the proteins were identified. Some representative spots of the MALDI and PMF analysis results are presented in table 3. Twenty-one of 56 spots were identified as albumin originated and showed as albumin precursor or albumin proteins, and some of the spots were identified as the same protein (data not shown). However, among these proteins, MBL and MASP2 which were within the same pathway to activate the complement were both significantly upregulated. The MS analysis of the spots which were identified as MBL and MASP2 is shown in figure 2. MALDI-TOF MS analysis of MBL was not so satisfactory, but the same spot was identified as MBL with the possibility over 80% by liquid chromatography MS analysis.

Enzyme-Linked Immunosorbent Assay

To validate the DIGE result and to confirm whether urine MBL was increased in CIN patients, the supernatants of preprocedure and postprocedure urine and serum samples in 13 CIN patients and 18 non-CIN patients were tested by ELISA. Among 13 CIN patients, 1 patient experienced a short episode of oliguria and needed treatment with diuretics. None of the patients needed renal replacement therapy after CAG.

The clinical data of these 31 patients are presented in table 4. Twenty-four hours after the procedure, the urinary MBL level was significantly increased (fig. 3). The same studies of serum MBL did not show any significant differences between CIN and non-CIN groups.

Discussion

For studying a large number of proteins simultaneously, 2D-DIGE, which combined a 2D gel with 3 different kinds of fluorescent Cy dyes, has great potential in

Table 3. Identified spots

Spot No.	Protein	Up- or down-regulated	GI No.	Top mowse score	MW/PI	Sequence coverage %
1	leucine-rich α_2-glycoprotein 1	up	114590928	116	43.2/5.76	34
3	kininogen 1 isoform 2	up	4504893	93	48.94/6.29	20
12	CGI-70 protein	down	4929609	66	36.42/5.13	26
13	chain B, human zinc α_2-glycoprotein	down	4699583	133	31.854/5.70	34
14	α_2-glycoprotein 1, zinc, isoform CRA-b	down	119597029	138	23.1/5.98	46
26	prolyl 4-hydroxylase, β-subunit	up	23510448	108	57.12/4.69	33
29	semenogelin I *Homo sapiens* (human)	up	134426	102	52.16/9.3	38
30	α_1-microglobulin/bikunin precursor	up	4502067	108	39.89/5.95	30
31	heparan sulfate proteoglycan 2 (perlecan), isoform CRA-a	up	119615400	66	38.86/6.44	14
32	MASP2	up	21264361	108	74.19/5.42	47
37	chain, human complement regulatory protein Cd59	up	149242594	68	9.33/5.67	36
40	serine proteinase inhibitor clade A	up	50363219	70	46.74/5.43	51
44	lectin, mannose-binding 2	up	5803023	77	26.23/5.39	45
45	prostaglandin H_2 D-isomerase	up	32171249	80	21.03/5.23	45
47	immunoglobulin λ light chain variable region	up	3091210	93	12.33/4.85	30

GI = Genoinfo identifier; MW = molecular weight; PI = isoelectric point.

biomarker discovery and may provide clues regarding the pathogenesis of kidney diseases [20–22]. Therefore, we used 2D-DIGE in analyzing human urine proteomes before and after contrast medium had been injected.

Urine sample preparation presents several unique challenges to proteomic analysis. Protein degradation may be minimized with the combination of protease inhibitors and storage at –80°C. It has been recognized that urine protein levels were altered with timing of the collection as well as with the diet. To obviate these changes, we collect urine samples at the bedside by quickly adding protease inhibitors and cooling to 4°C at once; the samples were kept at 4°C for less than 2 h for later centrifugation. Right after centrifugation at 4°C, the supernatants were frozen to –80°C for long-term conservation. The diets of the enrolled patients were controlled 3 days before urine collection until 2 days after the procedure. We also controlled the patients on some basic status element such as age, gender, BMI, smoking history, basic disease and medicine intake. The use of angiotensin-converting enzyme inhibitors, calcium channel blockers and nonsteroidal anti-inflammatory drugs was unified in these 12 patients to minimize the medication influences on urine proteomes. The basic SCR, free blood glucose and total cholesterol levels were similar among the patients enrolled. All of the patients in the 2D-DIGE analysis had normal basic renal function and negative to trace amounts of urine proteins. Patients with any other invasive procedure, acute renal failure or acute myocardial infarction, genitourinary tract infection and anemia were excluded; thus, the variability of individual patients was minimized and the result of DIGE would be more reliable.

The abundant protein such as albumin and IgG in the urine could influence the result of the proteomic experiment. Removal of the abundant protein will eliminate some of the small-molecule proteins at the same time. To minimize this kind of influence, only patients with negative to trace amounts of basic urine protein were enrolled.

Because of the lower incidence of CIN and delayed diagnosing, the urine samples for proteomic research were not all from CIN patients, the samples were from patients at high risk of coronary artery disease with basic hypertension and type 2 diabetes mellitus. For these patients, the procedures of CAG and PCI were usually more complicated, and the volume of contrast medium used was larger. There were 4 CIN cases among the 12 patients enrolled in the 2D-DIGE group and the incidence was 33.3%. Furthermore, the results were tested by ELISA in another group of CIN patients afterwards.

It is interesting that we found, in the postprocedure urine proteome profiles, MBL and MASP2 both significantly upregulated. MBL and MASP2 are two important

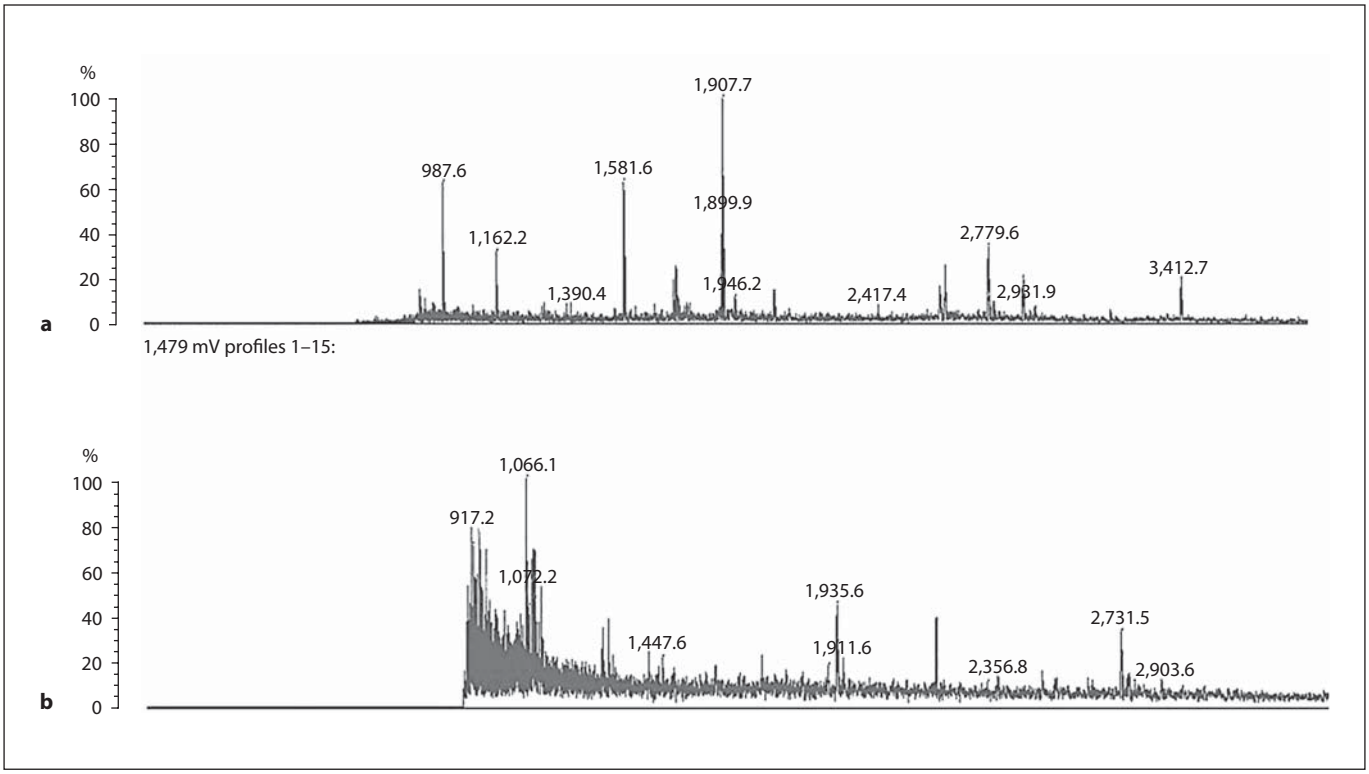

Fig. 2. MS analysis of the spots which were identified as 'MASP2' (**a**) and MBL (**b**) via PMF or via LTQ MS.

Table 4. Clinical data of patients tested by ELISA

Variable	CIN (n = 13)	Non-CIN (n = 18)	p
Age, years	66.3 ± 9.9	70.8 ± 11.9	0.166
Male gender, %	53.8	66.7	0.294
BMI	24.1 ± 2.8	23.2 ± 3.3	0.442
Current smoking, %	23.1	27.8	1.000
DM, %	30.8	5.6	0.134
Baseline SCR, μmol/l	63.3 ± 25.4	78.1 ± 21.8	0.091
CAG + PCI, %	61.5	44.4	0.078
CM amount, ml	155.4 ± 45.2	115.6 ± 54.1	0.039
Severe obstruction, %	92.3	72.7	0.359
ACEI, %	53.8	27.8	0.123
NSAIDs, %	76.9	66.7	0.256
TG, mmol/l	1.52 ± 0.65	1.6 ± 0.69	0.608
FBG, mmol/l	7.81 ± 2.56	6.50 ± 1.94	0.129
RBCs, n × 10^7/l	123.50 ± 12.76	123.8 ± 52.7	0.988

DM = Diabetes mellitus; CM = contrast medium; severe obstruction = ≥2 vessels of coronary injury were detected; ACEI = angiotensin-converting enzyme inhibitor; NSAIDs = nonsteroidal anti-inflammatory drugs; TG = triglyceride; FBG = free blood glucose; RBCs = red blood cells.

factors in initiating the lectin pathway which subsequently activates the complement cascade [23]. De Vries et al. [24] have demonstrated that the lectin pathway is activated followed by activation of the complement system in experimental renal I/R injury and clinical posttransplantation acute renal failure. As reported previously, in the healthy mouse kidney only glomerular mesangial cells stained MBL positive, after renal I/R injury with MBL deposited to injured tubular epithelial cells [25]. In the present study, we found that 24 h after the procedure, urine levels of MBL in CIN patients were significantly higher than those of non-CIN patients. In CIN patients, the level of postprocedure urinary MBL was significantly upregulated compared to that before the procedure. However, the serum MBL had no such changes. So it is reasonable for us to hypothesize that MBL was secreted by renal cells after having been injured by contrast medium. Further in vitro and in vivo researches are needed to precisely describe the mechanism. According to our former researches, urinary IL-18, urinary neutrophil gelatinase-associated lipocalin and also urinary N-acetyl-β-D-glucosaminidase were significantly upregulated 24 h after

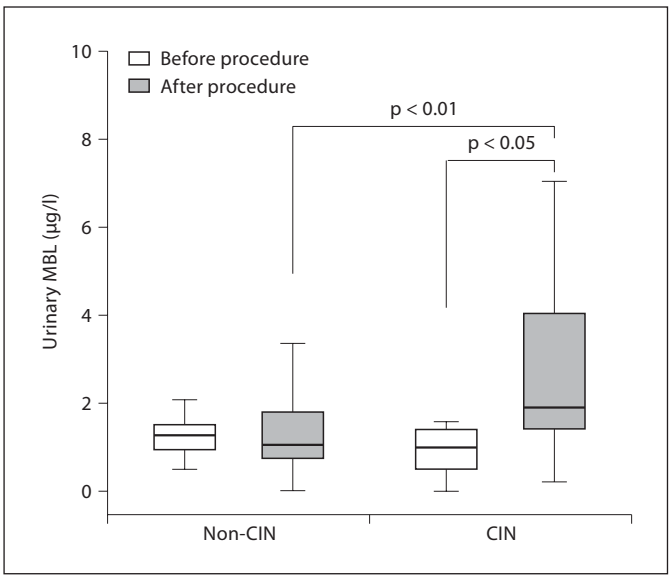

Fig. 3. Urinary MBL before and 24–48 h after CAG or PCI in CIN and non-CIN groups. Urinary MBL (µg/l) in the CIN group after the procedure (2.08; 1.42–5.72) was significantly increased compared to that before the procedure (1.09; 0.516–1.411; $p < 0.01$). It is also significantly increased in the CIN group (2.08; 1.42–5.72) compared to the non-CIN group (1.06; 0.738–1.885; $p < 0.05$) after the procedure. There is no significant difference of baseline values between the CIN and non-CIN groups.

the procedure in the current validation group of patients [26]. The level of urinary MBL elevated with these possible biomarkers also implicated that it was associated with tubular injury.

Although the nature of renal dysfunction following the use of iodinated radiocontrast agents has long been a matter of dispute, most previous studies demonstrated that regional ischemia might be the main pathogenesis of CIN [27–29]. No previous studies have focused on the lectin pathway and contrast-induced kidney injury, and high througput technologies such as proteomic solution were not adopted in the previous studies. Whether MBL and MASP2 secretion is induced by regional I/R injury or by other causes is still unknown. The proteomic technologies suggested that they might be associated with contrast-medium-induced kidney injury and indicated that the lectin pathway might be involved in CIN. To clarify the mechanisms of the lectin pathway in CIN, more advanced studies will be needed.

There are some other differentially expressed proteins found in our study. The data in table 2 show some representative spots, and some of them are frequent urinary proteins. Besides these spots, there are some others identified as albumin precursor and albumin proteins. Whether these albumin proteins are meaningful or not is still far from being understood.

The limitation of our study includes a lack of histochemistry evidence of the patients and the small population of the ELISA validation cases. Kidney biopsy is not usually carried out since CIN is a mild form of acute kidney injury. Seldom patients experienced oliguria or needed blood purification therapy. Multicenter investigations and longitudinal studies will have to be performed to establish a role for MBL as a biomarker.

Acknowledgments

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