Clinical Proteomics Identifies Urinary CD14 as a Potential Biomarker for Diagnosis of Stable Coronary Artery Disease

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Abstract

Inflammation plays a key role in coronary artery disease (CAD) and other manifestations of atherosclerosis. Recently, urinary proteins were found to be useful markers for reflecting inflammation status of different organs. To identify potential biomarker for diagnosis of CAD, we performed one-dimensional SDS-gel electrophoresis followed by liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS). Among the proteins differentially expressed in urine samples, monocyte antigen CD14 was found to be consistently expressed in higher amounts in the CAD patients as compared to normal controls. Using enzyme-linked immunosorbent assays to analyze the concentrations of CD14 in urine and serum, we confirmed that urinary CD14 levels were significantly higher in patients (n = 73) with multi-vessel and single vessel CAD than in normal control (n = 35) (P < 0.001). Logistic regression analysis further showed that urinary CD14 concentration level is associated with severity or number of diseased vessels and SYNTAX score after adjustment for potential confounders. Concomitantly, the proportion of CD14+ monocytes was significantly increased in CAD patients (59.7 ± 3.6%) as compared with healthy controls (14.9 ± 2.1%) (P < 0.001), implicating that a high level of urinary CD14 may be potentially involved in mechanism(s) leading to CAD pathogenesis. By performing shotgun proteomics, we further revealed that CD14-associated inflammatory response networks may play an essential role in CAD. In conclusion, the current study has demonstrated that release of CD14 in urine coupled with more CD14+ monocytes in CAD patients is significantly correlated with severity of CAD, pointing to the potential application of urinary CD14 as a novel noninvasive biomarker for large-scale diagnostic screening of susceptible CAD patients.
Introduction

Coronary artery disease (CAD) is the most common type of heart disease and cause of heart attacks [1]. Recent research has shown that inflammation plays a key role in CAD and other manifestations of atherosclerosis [1]. Cytokine or growth factors produced in the inflamed intima induces monocytes entering the plaque to differentiate into macrophages, leading to the development of atherosclerosis [2]. Effector molecules from immune cells that dominate early stage of atherosclerotic lesions accelerate progression of the lesions and further elicit acute coronary syndromes. These inflammatory factors, such as C-reactive protein (CRP) or monocyte chemoattractant protein-1 (MCP-1), represent attractive biomarkers for the prediction of risk for developing CAD [3]. Although these biomarkers hold promises, the invasive procedures for their clinical applications still emphasize an urgent need for non-invasive biomarkers that can correlate with severity of CAD.

Urine has especially become one of the most attractive body fluids in biomarker discovery as it can be obtained non-invasively in large quantities and is stable as compared with other body fluids. Recently, urinary proteins were also found to be useful markers for reflecting inflammation status of different organs. However, applying approaches for biomarker discovery to urinary samples have been encumbered by concerns for reproducibility and poor standardization of protocols until lately the development of state-of-the-art proteomic methodology [4–6].

Proteomic approaches in healthy and pathological samples are especially helpful to facilitate discerning differential protein expression patterns associated with normal and diseased states. Mainly attributable to the advent of emerging proteomics, the analysis and identification of complex protein mixtures in biological tissues have recently become amendable to routine analysis [7–9]. The study of proteins at the level of cellular systems derived from various tissues by proteomics methods has provided a firm basis for understanding the complex proteome profiles of total protein mixtures from complex whole tissues or cells at various disease stages [10]. As a result, proteomics has successfully been used to examine dynamic changes in protein expression of various tissues or body fluids, resulting in the identification of clinically useful biomarkers for disease diagnosis and prognosis [11–13]. Therefore, comparative proteomics analysis of normal and urinary samples from patients with a defined disease stage is particularly valuable for studying the differences of proteins expressions between diseased and normal subjects [7].

In this study, we employ an optimized gel-based coupled with gel-free shotgun proteomics strategy for urine biomarker discovery and a mechanistic investigation. Among the proteins differentially expressed in urine samples, monocyte antigen CD14 was found to be consistently expressed in higher amounts in the CAD patients as compared to normal controls. We have further made an endeavor to examine the differential manifestations of CD14 by proteomics and enzyme-linked immunosorbent assays in urine and flow cytometry on monocytes of normal control and CAD patients to provide some insight into the functional role of CD14 underlining the diagnostic or prognostic value for large-scale screening of CAD patients.

Materials and Methods

Study population

Seventy-three patients with angiographically proven CAD and 35 subjects with normal coronary arteries (mean age 65.5 ± 12.5 years), were enrolled in this successive study from 1 January to 30 June, 2012. The study was approved by the medical ethics committee of Kaohsiung Municipal Hospital and written informed consents were obtained from all participants. None of the patients had a history of active infectious disease, urinary tract infection, prior stroke, acute...
coronary syndrome or malignancies. Smoking status was recorded as three types, i.e. current smokers, ex-smokers or non-smokers. Body mass index (BMI) was calculated as weight (in kilograms) divided by height squared (in meters). A venous blood sample was collected for each patient on the day of examination after overnight fasting for at least 8 h. The following parameters were obtained by standard biochemical analysis techniques, including total cholesterol, low-density lipoprotein cholesterol, high-density lipoprotein cholesterol, triglycerides, serum creatinine, high sensitivity C-reactive protein (hs-CRP) and fibrinogen. Urine and plasma samples were collected before coronary angiography and stored at −80°C until analysis.

**Measurements of urinary protein, microalbumin and creatinine**

Urine protein and microalbumin were measured using commercial kits according to the manufacturer’s instructions (BioSystems, Barcelona, Spain). Serum and urinary creatinine levels were analyzed by a UV-Visible spectrophotometer at 505 nm by a standard spectrophotometric protocol. The ratio of urinary microalbumin to creatinine (UACR) was then calculated.

**Urine proteomics based on one-dimensional (1-D) gel**

Specimens of the first morning urine of about 10 ml each (collected between 8 and 10 A.M.) were collected in a sterile container for every test subject. This urine collection protocol is in accord with the “standard protocol for urine collection” currently under development by the Human Urine and Kidney Proteome Project and European Kidney and Urine Proteomics Action (EuroKUP) networks (www.eurokup.org; www.hukpp.org). The specimens were subsequently centrifuged at 1000 x g for 5 min to remove cellular debris. Precipitation of proteins was carried out in 10% trichloroacetic acid (TCA) containing 6 mM dithiothreitol (DTT) on ice for 30 min, and protein harvested by centrifugation at 14,000 rpm (18,078 x g) [14]. The washed protein pellet was washed twice with ice-cold 100% acetone/6 mM DTT. The pellet samples were always stored at −80°C until further analysis. For analysis, pellets were re-suspended in 0.4 ml SDS-containing loading buffer (10% SDS, 0.3125 M Tris-HCl, pH 6.8, 10% glycerol, 0.5 M DTT, 0.01% bromphenol blue) heating for 5 min at 95°C. About 10 μg of proteins (estimated based on a BCA protein-assay reagent kit (Pierce, Rockford, IL)) were loaded on 12.5% 1-D SDS-PAGE for protein separation with a 5% stacking gel. The gels were stained with Coomassie brilliant blue R-250 and destained in 10% methanol/7% acetic acid.

**In-gel digestion and nanoLC-ESI-MS/MS**

Based on the 1-D SDS-gel analysis of samples collected from 8 patients with different severity of CAD, the protein bands separated on the gels were sliced and destained three times with 25 mM ammonium bicarbonate buffer (pH 8.0) in 50% acetonitrile (ACN) for 1 h. The gel pieces were dehydrated in 100% ACN for 5 min and then dried for 30 min in a vacuum centrifuge. Enzyme digestion with trypsin and the peptide fragments analyzed by nanoLC-ESI MS/MS were essentially according to the previous report [14]. Proteins were identified in the NCBI databases based on MS/MS ion search with the search program MASCOT as described [14, 15].

**Western blot analysis**

After gel transfer, the membranes were soaked in Tris-buffered saline containing 5% nonfat dry milk powders and 0.1% Tween-20 (TBS-T) for one hour, followed by incubation with rabbit polyclonal antibody against CD14 (anti-CD14) (GTX101342; Genetex, San Antonio, TX, USA) overnight at 4°C. After three washes with TBS-T, the membranes were then incubated
with a solution of anti-rabbit Ig conjugated with horseradish peroxidase. After 1-h incubation at room temperature, the membranes were washed three times with TBS-T and the membrane blots were developed by using enhanced chemiluminescence substrate (ECL kit; Pierce, Rockford, IL, USA).

**Measurement of soluble CD14 levels in serum and urine**

Serum and urinary CD14 levels were measured by human CD14 enzyme linked immunosorbent assay (ELISA) kit (HK320; Hycult Biotechnology, Uden, Netherlands). Serum and urine samples were diluted and assayed according to the manufacturer’s instructions. Intra-assay variability was determined by evaluating 5 serum and 5 urinary samples under the same assay conditions, showing that a coefficient of variation (CV) between 5% and 9%. Inter-assay variability was determined by measuring 5 serum and 5 urinary samples in 5 consecutive assay runs, which showed a CV between 7% and 11%. The coefficients of variation for the intraassay and interassay of this AGT ELISA were found to be 4.8% and 5.3%, respectively. The detection limit for CD14 was 10 pg/mL.

**Flow cytometry of CD14+ monocytes**

We investigated CD14+ monocytes in 5 patients with angiographically proved CAD and 5 age- and sex- matched control subjects using flow cytometry. All of the patients had no prior history of active infectious disease, urinary tract infection, recent stroke or acute myocardial infarction in the past 6 months.

The surface expression of CD14 on peripheral blood mononuclear cells (PBMCs) was analyzed on the day of collection by direct staining with fluorescein isothiocyanate (FITC)-conjugated mouse anti-CD14 monoclonal antibody (clone 61D3; eBioscience, San Diego, CA, USA). PBMC were incubated with saturating concentrations of FITC-labeled anti-CD14 mAb for 30 min at 4°C in the dark. PBMCs were washed well with PBS and re-suspended in PBS with 1% FCS. Cells were analyzed by using a LSRII flow cytometer (Becton Dickinson, Heidelberg, Germany) and data were analyzed by FCS Express v3 Software System (Becton Dickinson, Heidelberg, Germany). The monocytes were specifically analyzed by selective gating based on parameters of forward and side light scatters. The absolute count of CD14+ monocytes was calculated from the total leukocyte count and the proportion of monocytes in the total leukocytes, as determined by light scattering.

**Quantitative real time polymerase chain reaction (qRT-PCR)**

qRT-PCR was employed to assay the CD14 levels of patients with and without CAD. PBMCs were isolated by centrifugation. The PRISM 7900 Sequence Detection System (Applied Biosystems, Foster City, CA, USA) was used for real-time PCR analysis. Specific primers were designed using Primer Express software (Applied Biosystems, Foster City, CA, USA) using sequences accessed through Gen-Bank and checked for specificity using Blast-search. CD14 sequence was used to design primers for qRT-PCR as follows: CD14 5'-TGCGACCACCGCCAGAAGC-3' (sense) and 5'-GCAGACGCCAGGGAATTCC-3' (antisense). GAPDH (endogenous control) sequence was used to design primers for qRT-PCR as follows: 5'-GTTGGTCTCCTGACTTCAACA-3' (sense) and 5'-GGTGCTGTAGCCAAATTTGTTG-3' (antisense). Real-time polymerase chain reaction (PCR) was performed using the 1× SYBR Premix EX Taq (Perfect Real Time) premix reagent (Takara, Japan), and added 500 ng cDNA to a final volume of 10 μl. Samples were run in triplicate. Real-time PCR performed on ABI PRISM 7900 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). Fluorescence was measured after each of the repetitive cycles. For each gene, cycle threshold values were determined from the linear
region of the amplification plot. Expression levels of mRNA were normalized relative to GAPDH mRNA levels. A melting point dissociation curve generated by the instrument was used to confirm that only a single product was present. Each PCR performed also included triplicate wells containing no template as controls in which nuclease-free water was added to reaction wells.

Coronary angiographic evaluation

Coronary angiography was performed for all subjects by the Judkin’s method. Two different scores were used to evaluate the angiographic severity and extent of CAD: the number of stenosed (> 50% reduction of luminal diameter) or occluded vessels (one- to three-vessel disorder states), and the complexity and severity scoring method according to the SYNTAX score [16]. Scoring of all coronary angiograms was done visually by one experienced observer who was blinded to the existing clinical and laboratory data. The intra-class correlation coefficient for intra-rater reliability was 1.0 (one to three vessel disorder score), and 0.93 (tertiles of the SYNTAX score). Multi-vessel CAD was defined as patients with two or more stenosed vessels or significant lesions at left main coronary artery.

Statistical analysis

Baseline characteristics were calculated for patients and controls. Numeric data are presented as means ± standard deviation (SD) when they are normal distributed, otherwise presented as median and interquartile range, and categorical data are presented as frequencies and percentages. Normal Quantile plot (Q-Q plot) indicated the log of urinary CD14 values was approximately normally distributed. Comparisons between three groups were analyzed by one-way ANOVA using a two-tailed test for variables with a Gaussian distribution. Comparisons between ratios were carried out using the χ² test. Furthermore, logistic regression analysis was performed to assess clinical determinants between independent variables and presence of CAD. We divided urinary CD14 levels into quartiles based on the distribution among all subjects. To evaluate the relationship between urinary CD14 levels and other risk factors, we calculated mean levels of risk factors per urinary CD14 quartile. Interquartile comparison was performed with the mean or median of each urinary CD14 quartile. To estimate the relative risk of prevalent CAD, we calculated odds ratios (OR) and corresponding 95% confidence intervals (95% CI). ORs were calculated using conditional logistic regression, taking into account the matching for sex and age, and was adjusted for smoking status, diabetes, body mass index (BMI), low density lipoprotein (LDL) cholesterol, high density lipoprotein (HDL) cholesterol, systolic blood pressure and high sensitivity C-reactive protein (hs-CRP) levels. Data analysis was performed with SPSS software (version 15; SPSS Inc., Chicago, IL, USA). A probability value \( P < 0.05 \) was considered to show statistical significance in the analyzed sets of data.

Quantitative shotgun proteomics

The methods for shotgun proteomics on urine samples were described separately in S1 Materials and Methods.

Results

Proteomic analysis differentiates urine of normal controls from CAD patients

To identify potential urinary markers for CAD patients, 1-D SDS– PAGE gel is an ideal method to compare the protein differentially expressed in urine. After protein extraction of urine
samples from incremental CAD severity of 8 subjects (SYNTAX score from 0 to 34), 10 μg of protein from each sample was subjected to SDS-PAGE analysis (Fig. 1). The major bands with intensity difference were excised from the gels and analyzed by LC-MS/MS, leading to identification of 26 proteins and peptides (Table 1). 4 top candidates, monocyte antigen CD14, prostaglandin D2 synthase, transforming growth factor-beta and CD40 ligands, were further validated using Western blot analysis (data not shown). However, only CD14, a 55 kDa glycoprotein, showed markedly increased intensities ($P < 0.001$) from the urine samples of CAD patients while the CD14 protein band was barely detectable in the urine of healthy controls (Fig. 2). These data illustrate the facile application of using proteomic approaches to identify differentially expressed proteins in urine, resulting in the identification of urinary CD14 as a potential biomarker for CAD patients.

**Urinary but not serum CD14 predicts the severity of CAD**

To confirm urinary CD14 being a verifiable biomarker, we further perform ELISA to screen clinical samples in a large-scale study of patients with differential severities of CAD. S1 Table shows the baseline biochemical data regarding CAD patients and control subjects. The prevalence of traditional risk factors such as age, gender, history of diabetes mellitus and serum creatinine was higher in patients with single- or multi-vessel CAD than in controls ($P < 0.05$). White cell counts, hs-CRP, fibrinogen levels, urinary microalbumin and UACR were not significantly different among CAD patients and controls. However, urinary CD14 levels measured
| Spot no. | Function                      | Protein name                          | GenInfo ID | Accession no. | Peptide queries matched | Cov (%) | pIc  | M.W. d  | Score |
|---------|-------------------------------|---------------------------------------|------------|---------------|------------------------|---------|------|---------|-------|
| 1–1     | Energy metabolism             | Transferrin                           | gi| 37747855     | AAH59367               | 27      | 32%  | 6.81   | 77030 | 529  |
| 2–1     | Transport                     | Serum albumin                         | gi| 28592        | CAA23754               | 63      | 50%  | 6.05   | 69321 | 1093 |
| 3–1     | Serine protease inhibitor     | Serine proteinase inhibitor           | gi| 50363217     | NP_000286              | 43      | 56%  | 5.37   | 46707 | 817  |
| 3–2     | Serine protease inhibitor     | Alpha1-Antitrypsin                    | gi| 157831596    | 1KCT_A                 | 43      | 60%  | 5.37   | 44223 | 817  |
| 3–3     | Serine protease inhibitor     | Serpin peptidase inhibitor            | gi| 15080499     | AAH11991               | 41      | 56%  | 5.36   | 46693 | 804  |
| 3–4     | Humoral immune system         | Chain H, Intact Human IgG B12         | gi| 15825647     | 1HZH_H                 | 16      | 19%  | 8.59   | 50385 | 222  |
| 3–5     | Antigen binding               | Immunoglobulin heavy chain            | gi| 272982598    | ACZ97422               | 15      | 16%  | 8.13   | 51775 | 204  |
| 3–6     | Antigen binding               | Immunoglobulin With A Hinge Deletion  | gi| 494350       | 1MCO_H                 | 13      | 17%  | 9.04   | 46823 | 191  |
| 3–7     | Antigen binding               | IgG2 heavy chain                      | gi| 14030849     | AAG00910               | 10      | 14%  | 7.94   | 49488 | 150  |
| 3–8     | Antigen binding               | IgG4 heavy chain                      | gi| 9857759      | AAG00912               | 7       | 19%  | 5.72   | 42895 | 130  |
| 3–9     | Inflammatory response         | Monocyte antigen CD14                 | gi| 3983127      | AAC83816               | 6       | 18%  | 5.85   | 40065 | 136  |
| 4–1     | Serine protease inhibitor     | Plasma serine protease inhibitor      | gi| 180550       | AAA35688               | 6       | 14%  | 9.38   | 45772 | 347  |
| 4–2     | Structural constituent of cytoskeleton | Beta-actin                           | gi| 14250401     | AAH08633               | 9       | 25%  | 5.56   | 40978 | 314  |
| 4–3     | Phosphoglycerate kinase activity | Phosphoglycerate kinase               | gi| 4505763     | NP_000282              | 3       | 10%  | 8.3    | 44586 | 121  |
| 6–1     | Regulation of immune system process | Alpha-1-acid glycoprotein 1           | gi| 112877       | P02763                 | 14      | 7%   | 4.93   | 23497 | 252  |
| 6–2     | Immune response               | Immunoglobulin kappa light chain VLJ region | gi| 21669329    | BAC01689               | 9       | 24%  | 6.73   | 28497 | 234  |
| 6–3     | Immune response               | Immunoglobulin kappa light chain      | gi| 110626504    | ABG79005               | 9       | 30%  | 7.85   | 23552 | 234  |
| 7–1     | Antigen binding               | Immunoglobulin light chain            | gi| 149673887    | BAF64541               | 20      | 47%  | 6.97   | 23380 | 684  |
| 7–2     | Immune response               | Human monoclonal IgM Cold agglutinin  | gi| 10835792     | 1QLR_A                 | 17      | 51%  | 5.75   | 23266 | 584  |
| 7–3     | Prostaglandin-D synthase activity | Prostaglandin D2 synthase             | gi| 54696702     | AAV38723               | 7       | 21%  | 7.66   | 21015 | 305  |
| 7–4     | Regulation of immune system process | Alpha-1-acid glycoprotein 1           | gi| 112877       | P02763                 | 5       | 7%   | 4.93   | 23497 | 133  |
| 7–5     | Antigen binding               | IgG kappa chain                       | gi| 4176418      | BAA37169               | 11      | 47%  | 6.92   | 23404 | 580  |
| 7–6     | Growth factor activity        | Transforming growth factor-Beta       | gi| 21579474    | 3EO0_A                 | 11      | 47%  | 6.19   | 23390 | 561  |
| 7–7     | Anti tumor necrosis factor    | Anti-TNF-alpha antibody               | gi| 11275306     | BAB18253               | 11      | 48%  | 7.75   | 23365 | 456  |
| 7–8     | Inflammatory response         | CD40 ligand                           | gi| 20150126     | 1BR_L                  | 14      | 39%  | 6.48   | 23843 | 335  |
| 7–9     | Serine-type endopeptidase activity | Protease, serine, 2 (trypsin 2)       | gi| 74353564     | AAJ03998               | 1       | 4%   | 4.78   | 26455 | 48   |

\* The number correspond to those showed in Fig. 1.
\* According to the NCBI database.
\* Theoretical pI.
\* Theoretical molecular mass.

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by ELISA in patients with single or multi-vessel CAD were significantly increased in comparison with patients with normal coronary angiography (Fig. 3). In contrast, there is no significant difference of serum CD14 levels among these three groups (S1 Table). Taken together, these results indicate that the level of urinary CD14 reflects the severity of CAD at different stages, supporting its application as a biomarker.

ROC curve analysis assesses urinary CD14 as a biomarker for CAD

As shown in S2 Table, The association of urinary CD14 quartiles with CAD was not attenuated after adjusting for CVD risk factors. Only age and SYNTAX score were associated positively with urinary CD14 levels. In logistic regression, quartiles of urinary CD14 levels were positively associated with SYNTAX score when compared with normal controls following adjustment for age, gender, medications, and all traditional CVD risk factors, and even with further adjustment for serum creatinine and hs-CRP levels (S3 Table). The urinary CD14 levels above the median were strongly associated with patients of CAD in fully adjusted models that included traditional CVD risk factors, serum creatinine, and hs-CRP (odds ratio 3.336 95% CI: 1.232 to 9.032; \( P = 0.018 \)). Using a threshold value of 3.51 μg/mL, urinary CD14 has a sensitivity of 0.838 and a specificity of 0.703 for the prediction of CAD, implicating CD14 exhibiting as a fairly exceptional and significant biomarker for CAD (Fig. 4).
Proportion of CD14+ monocyte is higher in CAD patients than in normal controls

To investigate the potential mechanism leading to higher level of urinary CD14 in CAD patients, we hypothesize that it may be related to the number of CD14+ monocytes. To this end, we determined the proportions of CD14+ monocytes in peripheral blood samples from 5 CAD patients and 5 age-matched healthy controls using flow cytometry. Although we performed the flow cytometry study only in 5 patients and 5 controls, their clinical and biochemical characteristics were well controlled between these two groups (S4 Table). Hence, the expression of CD14+ monocytes can be distinguished between patient and control groups.

Fig. 5 showed representative staining patterns of surface expression of membrane-bound CD14 on monocytes from a patient with CAD and a healthy individual. The proportion of CD14+ monocytes

Figure 3. ELISA measurements of urinary and serum CD14 among subjects with different severity of coronary artery disease. There were significant differences in urinary CD14 levels in patients with different severity of CAD.

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Figure 4. Receiver-operating characteristic (ROC) plots of urinary CD14 for diagnosis of coronary artery disease. Using a threshold value of 3.51, urinary CD14 has a sensitivity of 0.838 and a specificity of 0.703, a fairly good and significant biomarker for the prediction of CAD.

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was significantly increased in CAD patients (59.7 ± 3.6%) as compared with healthy controls (14.9 ± 2.1%) (P < 0.001). To further confirm this result, we isolated peripheral blood mononuclear cells and examined their CD14 mRNA expression level by semi-quantitative RT-PCR. As shown in Fig. 6A, CD14 mRNA was found to be up-regulated in CAD patients. Moreover, to accurately quantify the levels of CD14 mRNA expression, 10 samples consisting of 5 controls and 5 CAD patients were further analyzed by real-time PCR. The amount of CD14 mRNA was found to be more than 15-fold higher in CAD patients than in controls. Statistical analysis revealed that this up-regulated CD14 mRNA expression observed in CAD patients was significant (P < 0.01) (Fig. 6B). Of note, although we found the proportion of CD14+ monocytes was
significantly increased in CAD patients as compared with healthy controls, we did not observe significant correlation between CAD severity and the number of CD+14 monocytes or CD14 mRNA in the patients. Together, these results indicate that CAD patients tend to have more CD14+ monocytes than normal controls, which suggests that the prospective mechanism for the increased release of urinary CD14 in CAD patients may be attributable to the up-regulation of CD14 mRNA expression resulting in higher expression of membrane-bound CD14 antigens on monocytes from CAD patients as compared to healthy controls.

Figure 6. Induction of CD14 gene expression in peripheral blood mononuclear cells from healthy subjects and CAD patients. (A) RT-PCR (reverse transcription-PCR) amplification of total RNA from CAD patients reveals CD14 mRNA product (284 bps) corresponding to the whole CD14 gene (356 bps) in genomic DNA. Amplified product of GAPDH was utilized as a reference mRNA marker. (B) Quantitative real-time PCR (qRT-PCR) was performed using primers specific for CD14 and GAPDH. Transcript levels were normalized to that of GAPDH mRNA. The CD14 mRNA expression is shown as the folds of transcript levels in CAD patients relative to healthy subjects.

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Shotgun proteomics reveals CD14-associated inflammatory response networks in CAD

In order to systematically map the protein networks related to urinary CD14 and identify potential mechanisms, we performed shotgun proteomics coupled with stable isotope labeling for quantitative analysis. As shown in S5 Table, more than 100 identified proteins in the MS/MS database with differential expression based on stable isotope (deuterium/hydrogen, D/H) labeling. Similar to previous result of 1-D gel proteomic analysis, CD14 had dramatically higher level in the mixed urine of five CAD patients as compared to five normal controls based on the obtained D/H quantification ratio of 9.682 (S5 Table). In addition, these identified proteins showing up- and down-regulation between normal and diseased patients were further mapped to the canonical pathways from Ingenuity Pathways Analysis library (Fig. 7). Interestingly, several important inflammation regulators, such as nuclear factor kB (NF-κB) and IL-8, are involved in CD14-related inflammatory response networks. These results attest to the observations that the high level of urinary CD14 or the increased number of CD14+ monocytes may result from the chronic inflammation in CAD patients.

Discussion

Inflammation is contended to play a major role in the pathogenesis of atherosclerotic CAD. Atherosclerosis, the main cause of CAD, is now considered as an inflammatory disease in which immune mechanisms interact with metabolic risk factors to initiate, propagate, and activate lesions in the physiological artery system [1]. Atherosclerosis and its clinical complications are thus asserted to be an inflammatory syndrome in which an ongoing systemic inflammatory response is combined with the accumulation of immune myeloid cells in the atherosclerotic plaque. Above all, monocytes and macrophages play key roles in development and progression of the atherosclerotic plaque. Macrophages can differentiate into foam cells, which are the predominant cell population in the early stage of atherosclerosis [17–19]. In this study we demonstrated that CAD patients had significantly higher levels of urinary CD14 in spite of the fact that the levels of serum CD14 were found not to be different between CAD patients and healthy controls, which corroborated the previous study [20] in ruling out an independent relationship between CD14 genotypes or plasma levels of CD14 and risk of stable CAD in the studied cohort of population. In addition, we also established that urinary and not the plasma CD14 correlates with the severity of CAD, pointing to the potential significance of the expression and release of urinary soluble CD14 in relation to CAD.

Chronic inflammatory state induced by various infections within the vascular wall of animal models has long been suspected to be related to atherosclerosis, prompting researchers to make an extensive search for specific microbial products that may mediate arterial inflammation. Among them, bacterial endotoxin (e.g. lipopolysaccharide [LPS]) [21, 22] appears to play an important role in this aspect of endotoxin-induced pathological alteration in artery vascular system. In gram-negative bacterial infections, bacteria-derived LPS may complex with the serum derived LPS-binding protein. LPS-binding protein could then act as a lipid transfer protein that facilitates the binding of LPS monomers to cellular CD14 [23, 24]. CD14 appears thus to represent the principal binding element of the LPS receptor complex. After LPS binding to leukocyte CD14, there have been shown a cascade of an increase in intracellular Ca^{2+}, cellular tyrosine kinase phosphorylation, nuclear factor kB (NF-κB) activation, and cytokine plus chemokine production [25]. Both arms of the immune system, i.e. innate and adaptive immunity, have been implicated in contributing to essentially various stages of atherosclerosis, starting from stages of initiation and progression, and ultimately to the final stage of atherothrombotic complications. Physiological responsiveness to lipopolysaccharide may therefore be correlated...
with the risk of atherosclerotic disease. The molecular basis of this connection appears to lie in Toll-like receptors that are expressed on cells of the innate immune system. Toll-like receptors are found to bind to lipopolysaccharide, which then govern the strength of antibacterial immune responses exerted by the host [26]. Disparities and differences in the functions of various Toll-like receptors and their signaling pathways are now believed to play a critical role in determining the risk of atherosclerosis [27–29].

Several clinical studies have reported significantly elevated serum levels of CD14 under inflammatory conditions, such as Kawasaki disease [30], atopic dermatitis [31], rheumatoid arthritis [32, 33], and systemic lupus erythematosus [34]. However, the plasma levels of serum CD14 were not found to be different between CAD patients and controls according to the previous report [20]. This finding was in accordance with our results that showed no difference in
serum CD14 levels between CAD patients and control subjects (Fig. 3). Several previous studies showed that increased numbers of CD14+CD16+ monocytes are associated with CAD [35]. Our flow cytometric analysis also corroborated that CD14+ monocytes was significantly elevated in CAD patients as compared with healthy controls (Fig. 5). In addition to its function in lipopolysaccharide (LPS) signaling, CD14 may play a role in inflammatory diseases by regulating the innate immune system in response to endotoxin. A previous report [36] provided evidence that CD14 is shed from the cell surface of monocytes rather than internalized. The authors suggested that CD14 shedding induced by monocyte stimulation might play an important role in the regulation of surface CD14 expression. Nevertheless protease-mediated shedding of the mCD14 from leukocytes [37] or direct secretion by monocytes are not the only sources of CD14. CD14 has also been shown to be directly secreted by hepatocytes [38]. Although the relative contribution of CD14 in various cell sources has not been determined, the hepatocytes are probably an important source of circulating CD14. The concentrations of CD14 found in normal human plasma exceed by 1 or 2 logs that of the cell membrane-bound receptor [38]. Therefore we measured both the concentrations of serum CD14 and urinary CD14 in this study. It is of interest to find that there is a close association between urinary concentration of CD14 and the severity of CAD. To date this is the first report on the significant association between urinary CD14 and the risk and disease staging of prospective CAD patients. Further study along the line to look for disease mechanism underlying the correlation of CD14 in urine with the development and progression of CAD is currently in progress.

To date there are several biomarkers which have been used as relevant tools for differentiating patients with and without CAD. These biomarkers include lipocalin-type prostaglandin D synthase [39], electronegative low-density lipoprotein [40], oxidized-LDL/beta(2)-glycoprotein I complexes [41], monocyte chemo-attractant protein-1 [42], eosinophil cationic protein [43], high sensitive cardiac troponin T [44], and homocysteine [45]. All of these biomarkers are obtained and assessed on the day of patient enrollment in plasma or serum that may be interfered by a lot of unrelated factors in the plasma samples. It is worth noting that there are few studies to evaluate the association between urinary biomarker and CAD. According to Sonmez’s report, 24-hour urinary albumin excretion rates may be used to determine patients with a high prospect of CAD [46]. Fitzsimmons et al. [47] also showed that urine MMP-9 and TIMP-1 levels were elevated in patients with CAD as compared with healthy volunteers. However, the discriminative powers of these CAD biomarkers were not as high as urinary CD14 shown in this report. In addition, urinary levels of CD14 are generally stable under experimental conditions, implying that urinary CD14 may possess greater potential as a favorable biomarker than other existing biomarkers for epidemiological and clinical application.

Study limitations

Our study is limited by the fact that we cannot provide direct evidence for an increased recruitment of CD14+ monocytes into coronary atherosclerotic lesions. In addition, we cannot definitely show whether the increase in CD14+ monocytes precedes the development of atherosclerotic lesions, or whether it results from inflammatory conditions in atherosclerosis. Interpretation of the present results is limited by the small number of patients we studied. However, the correlations we found may serve as a reliable marker in the prediction and evaluation of CAD severity.

Conclusions

The current study has demonstrated that release of CD14 in urine coupled with more CD14+ monocytes in CAD patients is significantly correlated with severity of CAD, pointing to the
potential application of urinary CD14 as a novel biomarker for screening and diagnosis of patients manifested with different extents of CAD. Moreover, the relatively low abundance of urinary CD14 in subjects with normal coronary conditions may suggest the diagnostic and prognostic potentials to apply this CD14 as a sensitive biomarker for the detection of early cardiovascular disorders in selected cohort of populations.

Supporting Information

S1 Materials and Methods.
(DOCX)

S1 Table. Clinical characteristics in normal subjects and patients with coronary artery disease.
(DOCX)

S2 Table. Urinary soluble CD14 and associations with baseline characteristics of the study population (both patients and controls).
(DOCX)

S3 Table. Association of presence of CAD with CAD risk factors and urinary soluble CD14.
(DOCX)

S4 Table. Baseline characteristics and biochemical parameters of patients for flow cytometry.
(DOCX)

S5 Table. The quantitative shotgun analysis provided more than 100 identified proteins with different expression.
(DOCX)

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Author Contributions

Conceived and designed the experiments: MYL CHH CJK CSL WTL SHC. Performed the experiments: MYL CJK. Analyzed the data: MYL CHH CJK CSL SHC. Wrote the paper: MYL CHH SHC.

References

1. Hansson GK (2005) Inflammation, atherosclerosis, and coronary artery disease. N Engl J Med 352: 1685–1695. PMID: 15843671

2. Smith JD, Trogan E, Ginsberg M, Grigaux C, Tian J, et al. (1995) Decreased atherosclerosis in mice deficient in both macrophage colony-stimulating factor (op) and apolipoprotein E. Proc Natl Acad Sci U S A 92: 8264–8268. PMID: 7667279

3. Zakynthinos E, Pappa N (2009) Inflammatory biomarkers in coronary artery disease. J Cardiol 53: 317–333. doi:10.1016/j.jcc.2008.12.007 PMID: 19477372
4. Afkarian M, Bhasin M, Dillon ST, Guerrero MC, Nelson RG, et al. (2010) Optimizing a proteomics platform for urine biomarker discovery. Mol Cell Proteomics 9: 2195–2204. doi: 10.1074/mcp.M110.000992 PMID: 20511394

5. Good DM, Zurbig P, Argiles A, Bauer HW, Behrens G, et al. (2010) Naturally occurring human urinary peptides for use in diagnosis of chronic kidney disease. Mol Cell Proteomics 9: 2424–2437. doi: 10.1074/mcp.M110.001917 PMID: 20616184

6. Haubitz M, Good DM, Woywodt A, Haller H, Rupprecht H, et al. (2009) Identification and validation of urinary biomarkers for differential diagnosis and evaluation of therapeutic intervention in anti-neutrophil cytoplasmic antibody-associated vasculitis. Mol Cell Proteomics 8: 2296–2307. doi: 10.1074/mcp.M800529-MCP200 PMID: 19564150

7. Chiu SH, Wu CY (2011) Clinical proteomics: current status, challenges, and future perspectives. Kaohsiung J Med Sci 27: 1–14. doi: 10.1016/j.kjms.2010.12.001 PMID: 21329886

8. MacCoss MJ, McDonald WH, Saraf A, Sadygov R, Clark JM, et al. (2002) Shotgun identification of protein modifications from protein complexes and lens tissue. Proc Natl Acad Sci U S A 99: 7900–7905. PMID: 12060738

9. Huang CH, Chiou SH (2011) Clinical proteomics identifies potential biomarkers in for gastrointestinal diseases. World J Gastroenterol 20: 1529–1536. doi: 10.3748/wjg.v20.i6.1529 PMID: 22487628

10. Ideker T, Thorsson V, Ranish JA, Christmas R, Buhler J, et al. (2001) Integrated genomic and proteomic analyses of a systematically perturbed metabolic network. Science 292: 929–934. PMID: 11340206

11. Kolch W, Neussuss C, Pelzing M, Mischak H (2005) Capillary electrophoresis-mass spectrometry as a powerful tool in clinical diagnosis and biomarker discovery. Mass Spectrom Rev 24: 959–977. PMID: 15747373

12. Huang CH, Chuang MH, Lo WL, Wu MS, Wu YH, et al. (2011) Alkylhydroperoxide reductase of Helicobacter pylori as a biomarker for gastric patients with different pathological manifestations. Biochimie 93: 1115–1123. doi: 10.1016/j.biochi.2011.03.008 PMID: 21440595

13. Huang CH, Chiou SH (2011) Proteomic analysis of upregulated proteins in Helicobacter pylori under oxidative stress induced by hydrogen peroxide. Kaohsiung J Med Sci 27: 544–553. doi: 10.1016/j.kjms.2011.06.019 PMID: 22208537

14. Huang CH, Wang YT, Tsai CF, Chen YJ, Lee JS, et al. (2011) Phosphoproteomics characterization of novel phosphorylated sites of lens proteins from normal and cataractous human eye lenses. Mol Vis 17: 86–198. PMID: 21264232

15. Huang CH, Lee IL, Yeh LJ, Liao JH, Ni CL, et al. (2010) Upregulation of a non-heme iron-containing ferritin with dual ferroxidase and DNA-binding activities in Helicobacter pylori under acid stress. J Biochem 147: 535–543. doi: 10.1093/jb/mvp200 PMID: 19959503

16. Sianos G, Morel MA, Kappetein AP, Morice MC, Colombo A, et al. (2005) The SYNTAX Score: an angiographic tool grading the complexity of coronary artery disease. EuroIntervention 1: 219–227. PMID: 19758907

17. Frangogiannis NG (2008) The immune system and cardiac repair. Pharmacol Res 58: 88–111. doi: 10.1016/j.phrs.2008.06.007 PMID: 18620057

18. Nahrendorf M, Pittet MJ, Swirski FK (2010) Monocytes: protagonists of infarct inflammation and repair after myocardial infarction. Circulation 121: 2437–2445. doi: 10.1161/CIRCULATIONAHA.109.916346 PMID: 20530020

19. van der Laan AM, Hirsch A, Robbers LF, Nijveldt R, Lommerse I, et al. (2012) A proinflammatory monocyte response is associated with myocardial injury and impaired functional outcome in patients with ST-segment elevation myocardial infarction: monocytes and myocardial infarction. Am Heart J 163: 57–65 e52. doi: 10.1016/j.ahj.2011.09.002 PMID: 22172437

20. Koenig W, Khuseyinova N, Hoffmann MM, Marz W, Frohlich M, et al. (2002) CD14 C(-260)T polymorphism, plasma levels of the soluble endotoxin receptor CD14, their association with chronic infections and risk of stable coronary artery disease. J Am Coll Cardiol 40: 34–42. PMID: 12103253

21. Liao W (1996) Endotoxin: possible roles in initiation and development of atherosclerosis. J Lab Clin Med 128: 452–460. PMID: 8900288

22. Bhagat K, Moss R, Collier J, Vallance P (1996) Endothelial “stunning” following a brief exposure to endotoxin: a mechanism to link infection and infarction? Cardiovasc Res 32: 822–829. PMID: 8944812

23. Wright SD, Ramos RA, Tobias PS, Ulevitch RJ, Mathison JC (1990) CD14, a receptor for complexes of lipopolysaccharide (LPS) and LPS binding protein. Science 249: 1431–1433. PMID: 1698311

24. Schumann RR, Rietschel ET, Loppnow H (1994) The role of CD14 and lipopolysaccharide-binding protein (LBP) in the activation of different cell types by endotoxin. Med Microbiol Immunol 183: 279–297. PMID: 7541105
25. Pugin J, Kravchenko VV, Lee JD, Kline L, Ulevitch RJ, et al. (1998) Cell activation mediated by glycosylphosphatidylinositol-anchored or transmembrane forms of CD14. Infect Immun 66: 1174–1180. PMID: 9488411

26. Schroder NW, Opitz B, Lamping N, Michelsen KS, Zahringer U, et al. (2000) Involvement of lipopolysaccharide binding protein, CD14, and Toll-like receptors in the initiation of innate immune responses by Treponema glycolipids. J Immunol 165: 2683–2693. PMID: 10946299

27. Roy S, Karmakar M, Pearlman E (2014) CD14 Mediates Toll-like Receptor 4 (TLR4) Endocytosis and Spleen Tyrosine Kinase (Syk) and Interferon Regulatory Transcription Factor 3 (IRF3) Activation in Epithelial Cells and Impairs Neutrophil Infiltration and Pseudomonas aeruginosa Killing in Vivo. J Biol Chem 289: 1174–1182. doi: 10.1074/jbc.M113.523167 PMID: 24275652

28. Estruch M, Bancells C, Beloki L, Sanchez-Quesada JL, Ordonez-Llanos J, et al. (2013) CD14 and TLR4 mediate cytokine release promoted by electronegative LDL in monocytes. Atherosclerosis 229: 356–362. doi: 10.1016/j.atherosclerosis.2013.05.011 PMID: 23880187

29. Nair A, Kanda V, Bush-Joseph C, Verma N, Chubinskaya S, et al. (2012) Synovial fluid from patients with early osteoarthritis modulates fibroblast-like synoviocyte responses to toll-like receptor 4 and toll-like receptor 2 ligands via soluble CD14. Arthritis Rheum 64: 2268–2277. doi: 10.1002/art.34495 PMID: 22492243

30. Takeshita S, Nakatani K, Tsujimoto H, Kawamura Y, Kawase H, et al. (2000) Increased levels of circulating soluble CD14 in Kawasaki disease. Clin Exp Immunol 119: 376–381. PMID: 10652678

31. Wuthrich B, Kagi MK, Joller-Jemelka H (1992) Soluble CD14 but not interleukin-6 is a new marker for cardiovascular risk. Lipids 45: 619

32. Horner R, Uhl A, Schroder NW, Opitz B, Lamping N, et al. (2000) Involvement of lipopolysaccharide binding protein, CD14, and Toll-like receptors in the initiation of innate immune responses by Treponema glycolipids. J Immunol 165: 2683–2693. PMID: 10946299

33. Nockher WA, Wigand R, Schoeppe W, Scherberich JE (1994) Elevated levels of soluble CD14 in serum of patients with systemic lupus erythematosus. Clin Exp Immunol 96: 15–19. PMID: 7512005

34. Schlitt A, Heine GH, Blankenberg S, Espinola-Klein C, Dopheide JF, et al. (2004) CD14+CD16+ monocytes in coronary artery disease and their relationship to serum TNF-alpha levels. Thromb Haemost 92: 419–424. PMID: 15269840

35. Bazil V, Strominger JL (1991) Shedding as a mechanism of down-modulation of CD14 on stimulated human monocytes. J Immunol 147: 1567–1574. PMID: 1880416

36. Stelter F, Pfister M, Bernheiden M, Jack RS, Bufler P, et al. (1996) The myeloid differentiation antigen MPO-1 is a powerful biomarker for severity of stable coronary artery disease. Atherosclerosis 120: 835–839. doi: 10.1016/S0021-9150(96)00047-8

37. de Queiroz Mello AP, da Silva IT, Oliveira AS, Nunes VS, Abdalla DS, et al. (2010) Electronegative low-density lipoprotein is associated with dense low-density lipoprotein in subjects with different levels of cardiovascular risk. Lipids 45: 619–625. doi: 10.1007/s11745-010-3439-7 PMID: 20574778

38. Greco TP, Conti-Kelly AM, Anthony JR, Greco T., Doyle R, et al. (2010) Oxidized-LDL/β2-glycoprotein I complexes are associated with disease severity and increased risk for adverse outcomes in patients with acute coronary syndromes. Am J Cardiol Pathol 133: 737–743. doi: 10.1009/ AJCP8WRDDBAS PMID: 20395520

39. Harsimran K, Singh AA, Guruvinder S, Sharda S, Vasudha S (2009) Plasma monocyte chemoattractant protein-1 as risk marker in type 2 diabetes mellitus and coronary artery disease in North Indians. Diab Vasc Dis Res 6: 288–290. doi: 10.1177/1479164109346364 PMID: 20368224

40. Niccoli G, Ferrante G, Cosentino N, Conte M, Belloni F, et al. (2010) Eosinophil cationic protein: A new biomarker of coronary atherosclerosis. Atherosclerosis 211: 606–611. doi: 10.1016/j.atherosclerosis.2010.02.038 PMID: 20307883

41. Laufer EM, Mingels AM, Winkens MH, Joosan IA, Schellings MW, et al. (2010) The extent of coronary atherosclerosis is associated with increasing circulating levels of high sensitive cardiac troponin T. Arterioscler Thromb Vasc Biol 30: 1269–1275. doi: 10.1161/ATVBAHA.109.200394 PMID: 20299689
45. Schroecksnadel K, Grammer TB, Boehm BO, Marz W, Fuchs D (2010) Total homocysteine in patients with angiographic coronary artery disease correlates with inflammation markers. Thromb Haemost 103: 926–935. doi: 10.1160/TH09-07-0422 PMID: 20216983

46. Sonmez K, Eskisar AO, Demir D, Yazicioglu MV, Mutlu B, et al. (2006) Increased urinary albumin excretion rates can be a marker of coexisting coronary artery disease in patients with peripheral arterial disease. Angiology 57: 15–20. PMID: 16444452

47. Fitzsimmons PJ, Forough R, Lawrence ME, Gantt DS, Rajab MH, et al. (2007) Urinary levels of matrix metalloproteinase 9 and 2 and tissue inhibitor of matrix metalloproteinase in patients with coronary artery disease. Atherosclerosis 194: 196–203. PMID: 16942771