Association of Serum Anti-Prolylcarboxypeptidase Antibody Marker with Atherosclerotic Diseases Accompanied by Hypertension

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Abstract

Background: Atherosclerosis is known to be associated with various diseases such as Cerebral Infarction (CI), and Cardiovascular Disease (CVD), and closely related to Diabetes Mellitus (DM) and Chronic Kidney Disease (CKD). Biomarkers are useful but not sufficient for early detection. In the present study, we aimed to identify novel antibody markers for atherosclerosis-related diseases.

Methods: The protein array method was used for the initial screening, and a peptide containing a possible epitope domain was used to evaluate serum antibody levels using the Amplified Luminescent Proximity Homogeneous Assay (AlphaLisa) method.

Results: The protein array identified prolylcarboxypeptidase (PRCP) as one of the target antigens recognized by IgG antibodies in the sera of patients with atherosclerosis. We then prepared a possible antigenic peptide of amino acids 214-227 of PRCP. Results of AlphaLisa showed significantly higher serum antibody levels against the PRCP peptide in patients with DM, CVD, acute-phase cerebral infarction, transient ischemic attack or CKD, than in healthy donors. Furthermore, areas under the receiver operating characteristic curves of these antibodies were higher in patients with DM or CKD than in other patients. Spearman correlation analysis revealed that the serum anti-PRCP antibody levels were associated with hypertension, artery stenosis, and smoking habit.

Conclusion: The serum anti-PRCP antibody may be useful for early detection of atherosclerosis-related diseases, and may have a pathogenic role in the development of atherosclerosis.

Keywords: Prolylcarboxypeptidase; Angiotensin; Atherosclerosis; Hypertension; Serum antibody biomarker

Introduction

Recent progress in technology has revealed that humoral autoantibodies can develop against most, if not all, of proteins irrespective of intrinsic or extrinsic antigens. Therefore, an increasing number of antibody biomarkers have been reported for the diagnosis and recognition not only of cancers and autoimmune diseases but also metabolic and atherosclerotic diseases. For example, phospholipid [1], apolipoprotein A-1 [2,3], oxidized low-density lipoprotein [3,4], and heat shock proteins (Hsps) [3,5], for cardiovascular disease (CVD); Hsp60 for stroke [6]; insulin [7], glutamic acid decarboxylase (GAD) [8], and protein tyrosine phosphatase IA-2 [9,10], for diabetes mellitus (DM); and p53 for cancer [11].

We also identified the following autoantibody-recognized antigens by recombinant cDNA expression cloning (SEREX) method or protein array method: TACSTD2 [12], TRIM21 [13], SLCA21 [14], MKRN1 [15], ECSA [16], and CCNL2 [17] in esophageal squamous cell carcinoma; FIR/PUF60 in colon carcinoma [18]; SH3GL1 [19] and filamin C [20] in low-grade glioma; TLN1 in multiple sclerosis [21]; RPA2 [22] and SOSTDC1 [23] in ischemic stroke; NRD1 in acute coronary syndrome [24]; TUBB2C [25], GADD34 [26], and adiponectin [27] in DM; COPE in obstructive sleep apnea [28]; and ATP2B4 [29], BMP-1 [22,29], DHP5 [30], and SH3BP5 [31] in arteriosclerosis-related diseases.

The most prominent features of antibody markers are the large variation in the antibody expression levels affected by repeated exposure of small amounts of antigens, and an easy measurement of stable immunoglobulins. Thus, antibody markers are highly sensitive and can be used for diagnosis of early stages of cancers. Furthermore, autoantibodies cannot be developed immediately after the onset of acute-phase Cerebral Infarction (aCI) and Acute Myocardial Infarction (AMI) when many antigenic proteins are secreted and exposed. Therefore, antibodies specifically detected in patients with aCI or AMI immediately after the onset may be present before the onset, which raise the possibility that the antibody markers are useful to predict the onset.
In addition, we propose in the present study that the development of autoantibodies may have a causal role in the progression of diseases.

Materials and Methods

Patient and Healthy Donor (HD) sera

This study was approved by the Local Ethical Review Board of the Chiba University Graduate School of Medicine (Chiba, Japan) as well as the review boards of co-operating hospitals. Serum samples were collected from patients who had provided written informed consent. Each serum sample was centrifuged at 3000 g for 10 min, and the supernatant was stored at -80°C until use. Repeated thawing and freezing of samples were avoided.

Serum samples from HDs and patients with DM or CVD were obtained from Chiba University Hospital. Subjects from Chiba Prefectural Sawara Hospital involved HDs and patients with aCI, transient ischemic attack (TIA), or deep and subcortical white matter hyperintensity (DSWMH). Samples from patients with chronic kidney disease (CKD) were obtained from the Kumamoto cohort [32,33]. Sera of HDs obtained from Chiba Prefectural Sawara Hospital were selected from among subjects who exhibited no abnormalities on cranial magnetic resonance imaging.

Preparation of an antigenic peptide

ProtoArray was carried out as described previously [23,30,31]. Possible epitope sites in prolylcarboxypeptidase (PRCP) protein were predicted using the program ProPred (http://www.imtech.res.in/raghava/propred/) as described previously [23]. After pre-testing antigenicity using crude/non-purified peptides, we selected a peptide of amino acid positions 214-227 of PRCP, of which amino-terminal browning (bPRCP-214) was obtained from the Kumamoto cohort [32,33]. Sera of HDs obtained from Chiba Prefectural Sawara Hospital were selected from among subjects who exhibited no abnormalities on cranial magnetic resonance imaging.

Amplified luminescence proximity homogeneous assay (AlphaLISA)

AlphaLISA was performed in 384-well microtiter plates (white opaque OptiPlate", Perkin Elmer, Waltham, MA) containing 2.5 µL of 1:100-diluted serum and 2.5 µL of the biotinylated PRCP peptide (bPRCP-214; 400 ng/mL) in AlphaLISA buffer (25 mM HEPES, pH 7.4, 0.1% casein, 0.5% Triton X-100, 1 mg/mL dextran-500, and 0.05% Proclin-300). The reaction mixture was incubated at room temperature for 8 to 11 h, following which anti-human IgG-conjugated acceptor beads (2.5 µL at 40 µg/mL) and streptavidin-conjugated donor beads (2.5 µL at 40 µg/mL) were added and incubated prior to another incubation at room temperature in the dark for 7 to 14 days. Chemical emissions were read on an EnSpire Alpha microplate reader (PerkinElmer) as described previously [22-31]. Specific reactions were calculated by subtracting the alpha counts of the buffer control without antigenic peptides, from the counts in the presence of bPRCP-214 peptide.

Statistical analyses

The Mann-Whitney U test and student’s t test were used to determine the significance of differences between the two groups. Correlation was examined with Spearman’s rank-order correlation analysis. All statistical analyses were performed using GraphPad Prism 5 (GraphPad Software, La Jolla, CA). The predictive values of putative disease markers were assessed via a receiver operating characteristic (ROC) curve analysis, and the cutoff values were set to maximize the sums of sensitivity and specificity. All the tests were two-tailed, and P values of <0.05 were considered statistically significant.

Results

Identification of PRCP as an antigen recognized by serum IgG of patients with atherosclerosis

ProtoArray loaded with 9375 proteins were used to select the antigens recognized by IgG antibodies in the sera of patients with atherosclerosis. PRCP (NCBI Accession Number: NM_005040.1) was found to react with three of the five serum samples from patients with atherosclerosis, and none of the five samples from HDs. Three epitope sites of PRCP protein predicted by using website were prepared, and one peptide, bPRCP-214, reacted well with serum IgG antibodies from patients with aCI.

Elevation of serum antibody levels against PRCP in patients with DM and CVD

We examined the levels of antibodies against the bPRCP-214 peptide in sera of HDs and patients with DM or CVD, using the supersensitive and stable AlphaLISA method [23-31]. All serum samples were obtained from Chiba University Hospital. The levels of serum antibodies against bPRCP-214 peptide (s-PRCP-Abs) were significantly higher in samples from patients with DM or CVD than in those from HDs (Figure 1a). At a cutoff value equivalent to the average plus two standard deviations (SDs) of the HD specimen values, the s-PRCP-Ab-positive rates in HDs, patients with DM, and those with CVD were 4.7%, 14.0%, and 10.4%, respectively (Table 1).

ROC analysis was performed to evaluate the abilities of these antibody markers to indicate the presence of DM. The area under the ROC curve (AUC) for s-PRCP-Abs was 0.6737 [95% confidence intervals (CI)=0.6111-0.7362] and 0.6209 (95% CI=0.5407-0.7011) for DM and CVD, respectively, yielding sensitivity and specificity values of 84.1% and 51.6%, for the diagnosis of DM and 76.1% and 50.8%, for the diagnosis of CVD (Figures 1b and 1c).

Figure 1: Comparison of the levels of serum anti-PRCP antibodies (s-PRCP-Abs) between HDs and patients with diabetes mellitus (DM) or cardiovascular disease (CVD). (a) AlphaLISA-determined s-PRCP-Ab levels (Alpha counts) are shown as box-whisker plots displaying the 10th, 20th, 50th, 80th, and 90th percentiles. P values calculated using Mann–Whitney U test are shown. The total (male/female) sample numbers, average ages of the subjects with standard deviations (SDs), average Alpha counts with SDs, cutoff values, positive numbers, positive rates (%), and P values are summarized and shown in Table 1. Receiver operating characteristic curve (ROC) analysis was performed to assess the abilities of s-PRCP-Abs to detect DM and CVD. Numbers in the figures indicate areas under the curve (AUC), 95% confidence intervals (CI), and the cutoff values for marker levels, and numbers in parentheses indicate sensitivity (left) and specificity (right).
Elevation of levels of s-PRCP-Abs in patients with TIA and aCI

We next examined antibody levels in the sera of patients with CKD because kidney plays an important role in essential hypertension [34]. CKD was divided into three groups as follows: type 1, diabetic kidney disease; type 2, nephrosclerosis; and type 3, glomerulonephritis. Samples from patients with CKD were obtained from the Kumamoto cohort, and those from HDs from Chiba University Hospital. Patients from all three groups of CKD had significantly higher serum levels of s-PRCP-Abs than HDs (Figure 3a). The s-PRCP-Ab-positive rates in HDs and patients with CKD type 1, type 2, and type 3 were 4.9%, 27.6%, and 15.6%, and 7.3%, respectively (Table 3). Such highly positive rates observed in patients with type 1- and type 2-CKD were consistent with the following ROC analysis. AUCs of s-PRCP-Abs for CKD type 1, type 2, and type 3 were 0.6644 (95% CI: 0.5924-0.7363) (Figure 3b), 0.7085 (95% CI: 0.6304-0.8136) (Figure 3c), and 0.6423 (95% CI: 0.5654-0.7192) (Figure 3d), respectively. Relatively few CKD type 3 samples may have resulted in high AUC value.

Elevation of levels of s-PRCP-Abs in patients with CVD

We then examined the levels of s-PRCP-Abs in the sera of patients with TIA or aCI. Sera of HD and patients with TIA and aCI were obtained from Chiba Prefectural Sawara Hospital. The AlphaLISA results demonstrated that the levels of s-PRCP-Ab were significantly higher in patients with TIA or aCI than in HDs (Figure 2a). Using cutoff values determined as described in the previous section, the s-PRCP-Ab-positive rates in HDs, patients with TIA, and those with aCI were found to be 3.6%, 9.1%, and 9.2%, respectively (Table 2). ROC analysis revealed that AUCs of s-PRCP-Ab were 0.6222 for TIA (Figure 2b), and 0.6163 for aCI (Figure 2c). Thus, bPRCP antibodies were associated with ischemic stroke but less useful as a diagnostic marker of TIA and aCI as compared with those as a marker of DM.

Table 1: Comparison of the serum antibody levels of healthy donors (HDs) vs. those of patients with diabetes mellitus (DM) or cardiovascular disease (CVD). The upper panel indicates the numbers of total samples, samples from male and female subjects, and ages (average ± standard deviation (SD)) of HDs and patients with DM or CVD. The lower panel summarizes the serum antibody levels (Alpha count) examined by AlphaLISA. Synthetic bPRCP-214 peptide was used as an antigen. Cutoff values were determined as the average HD values plus two SD, and positive samples for which the Alpha counts exceed the cutoff value were scored. P values were calculated using the students’ t test. P values <0.05 and positive rates >10% are marked in bold. Box-whisker plots of the same results are shown in Figure 1.

Table 2: Comparison of the serum antibody levels of HDs vs. those of patients with transient ischemic attack (TIA) or acute-phase cerebral infarction (aCI). Upper panel indicates the numbers of total samples and samples from male and female samples as well as ages (average ± SD). The lower panel summarizes the serum antibody levels examined by AlphaLISA using bPRCP-214 peptide as an antigen as described in the legend of Table 1. Box-whisker plots of the same results are shown in Figure 2.

Figure 2: Comparison of s-PRCP-Abs levels between HDs and patients with transient ischemic attack (TIA) or acute cerebral infarction (aCI). Serum antibody levels were determined by AlphaLISA and are shown as box-whisker plots, as described in the legend of Figure 1. P values calculated using Mann–Whitney U test are shown. The same results are summarized in Table 2. Responses of s-PRCP-Abs to TIA (b) and aCI (c) were also evaluated using ROC analysis, and the numbers in figures are as described in the legend of Figure 1.
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and TIA but not with DSWMH than those in HDs (Table 4). The s-PRCP-Ab levels were then compared between male and female subjects; with or without diseases of DM, hypertension (HT), CVD, and dyslipidemia; and with or without smoking and alcohol intake habits. Significantly higher s-PRCP-Ab were observed in subjects with DM, HT, and smoking habit than their control groups (Table 4).

Table 4: Correlation analysis of s-PRCP-Ab levels with data of subjects in the Sawara Hospital cohort. The uppermost panel indicates the numbers of total samples and s-PRCP-Ab levels (average ± SD) of HD, aCI, chronic-phase cerebral infarction, TIA and deep and subcortical white matter hyperintensity (DSWH). P values of each patients vs. HD are also shown. In lower panels, the subjects were divided as follows: sex (male and female); presence (+) or absence (-) of complication of DM, hypertension (HT), CVD, or dyslipidemia, and life style factors (smoking and alcohol intake habits). Antibody levels (Alpha counts) were compared using the Mann–Whitney U test. Sample numbers, averages and SD of counts as well as P values are shown. Significant correlations (P<0.05) are marked in bold text.

Table 3: Comparison of serum antibody levels of HDs vs. those of patients with chronic kidney disease (CKD). CKD types 1, 2, and 3 correspond to diabetic kidney disease, nephrosclerosis, and glomerulonephritis, respectively. The upper panel indicates the numbers of total samples and s-PRCP-Ab levels (average ± SD) of HD, aCI, chronic-phase cerebral infarction, TIA and deep and subcortical white matter hyperintensity (DSWH). P values of each patients vs. HD are also shown. In lower panels, the subjects were divided as follows: sex (male and female); presence (+) or absence (-) of complication of DM, hypertension (HT), CVD, or dyslipidemia, and life style factors (smoking and alcohol intake habits). Antibody levels (Alpha counts) were compared using the Mann–Whitney U test. Sample numbers, averages and SD of counts as well as P values are shown. Significant correlations (P<0.05) are marked in bold text.

Table 2: Comparison of s-PRCP-Ab Abs levels between HDs and patients with chronic kidney disease (CKD). s-PRCP-Ab levels were examined by AlphaLISA and were compared between HDs and patients with CKD types 1, 2, or 3 (a). Box-whisker plots are presented as described in the legend of Figure 1. P values of CKD types 1, 2, and 3 versus HD controls using Mann–Whitney U test are shown. The results are summarized and shown in Table 3. The abilities of s-PRCP-Ab to detect CKD types 1, 2, and 3were also evaluated using ROC analysis (b–d). The numbers in figures are as described in the legend of Figure 1.

Figure 3: Comparison of s-PRCP-Ab levels between HDs and patients with chronic kidney disease (CKD). s-PRCP-Ab levels were examined by AlphaLISA and were compared between HDs and patients with CKD types 1, 2, or 3 (a). Box-whisker plots are presented as described in the legend of Figure 1. P values of CKD types 1, 2, and 3 versus HD controls using Mann–Whitney U test are shown. The results are summarized and shown in Table 3. The abilities of s-PRCP-Ab to detect CKD types 1, 2, and 3were also evaluated using ROC analysis (b–d). The numbers in figures are as described in the legend of Figure 1.


**Discussion**

Our initial ProtoArray screening identified PRCP and subsequent prediction of the epitope domain identified bPRCP-214 as an antigenic peptide recognized by serum IgG in patients with atherosclerosis. Further analyses demonstrated higher levels of serum antibodies against the bPRCP-214 peptide in patients with DM, CVD, aCI, TIA, and CKD than in HDs (Figures 1-3) (Tables 1-3). Among diseases, DM, diabetic kidney disease, and nephrosclerosis were most closely associated with the s-PRCP-Ab levels. This suggests that the antibody marker is responsible to DM and/or atherosclerosis. Consistently, correlation analysis revealed that the levels of s-PRCP-Ab were significantly correlated with max IMT which reflects the atherosclerosis (Table 5). The correlation analysis also showed a highly significant association between the marker levels and HT and smoking habit, both of which are known to be typical risk factors of atherosclerosis [35,36] (Table 5).

There are some reports on the protein amounts and the activity of PRCP in blood. Xu et al. have demonstrated that plasma PRCP protein amounts were significantly correlated with obesity/BMI, DM/blood glucose, T-CHO, LDL-C, and TG [37]. Brouns et al. have examined the enzymatic activity of PRCP and reported the decrease of the activity in aCI [38]. The levels of s-PRCP-Ab showed significant association with DM and aCI (Figures 1 and 2) (Tables 1, 2 and 4), inverse correlation with T-CHO and LDL-C (Table 5), and no apparent correlation with BMI and TG. Thus, the development of anti-PRCP autoantibodies may not be attributable to the overexpression of PRCP protein. Although patients with DM showed significantly higher s-PRCP-Ab levels than HDs, blood test levels of DM markers such as HaA1c and blood sugar were not associated with s-PRCP-Ab levels (Table 5). This suggests that s-PRCP-Ab levels are not simply associated with the progress of DM but indirectly related to DM caused by kidney dysfunction and HT.

It is well known that blood pressure is regulated not only by renin-angiotensin system (RAS) but also by PRCP. Angiotensin II plays a main role in raising blood pressure. Angiotensin I was cleaved by angiotensin-converting enzyme (ACE) to generate active angiotensin II, which was then inactivated by removal of a carboxy-terminal amino acid by PRCP [39,40]. The other substrates thus far known are prekallikrein [41] and a-melanocyte stimulating hormone [1-13] (α-MSH) [42]. Digestive activation of prekallikrein by PRCP results in dilatation of blood vessels leading to decrease in blood pressure. Inactivation of α-MSH by PRCP regulates food intake [42]. As expected, it was reported that murine PRCP depletion induced HT and faster arterial thrombosis as well as thickening of Bowman capsule in the kidney [43], which might be leading to aCI, CVD, and CKD. Namely, the alterations caused by PRCP depletion were almost the same as those correlated with s-PRCP-Ab levels, suggesting that s-PRCP-Ab can inhibit PRCP activity. The credibility of this notion is further increased by the fact that PRCP works on cell surface where PRCP-Ab levels were highly associated with atherosclerotic diseases accompanied by hypertension. Therefore, PRCP-Ab may have a causal role in developing atherosclerosis induced by hypertension and kidney dysfunction.

**Conclusion**

PRCP-Ab levels were highly associated with atherosclerotic diseases such as DM, CVD, aCI, TIA, and CKD. PRCP-Ab may have a causal role in developing atherosclerosis induced by hypertension and kidney dysfunction.

**Competing Interests**

This work was performed in collaboration with Fujikura Kasei Co., Ltd.

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**Table 5: Correlation analysis of s-PRCP-Ab levels with data on subjects in the Sawara Hospital cohort.**

| Variables                     | Subjects’ information | Abbreviation | Spearman r value | P value |
|-------------------------------|-----------------------|--------------|------------------|---------|
| **General**                   |                       |              |                  |         |
| Age                           | Years                 |              | 0.082            | 0.0344  |
| Body height                   | Height                |              | -0.030           | 0.4491  |
| Body weight                   | Weight                |              | -0.024           | 0.5447  |
| Body mass index               | BMI                   |              | -0.007           | 0.8644  |
| Blood pressure                | BP                    |              | 0.090            | 0.0241  |
| **Artery stenosis**           | Maximum intima-media  | max IMT      | 0.132            | 0.0047  |
| **Life style**                | Smoking habit duration| Years        | 0.217            | <0.0001 |
| Alcohol intake frequency      | (times/week)          |              | 0.056            | 0.1468  |
| **Blood test**                |                       |              |                  |         |
| Total cholesterol             | T-CHO                 |              | -0.122           | 0.0037  |
| LDL-cholesterol               | LDL-C                 |              | -0.135           | 0.0122  |
| Potassium                     | K                     |              | -0.076           | 0.0543  |
| HDL-cholesterol               | HDL-C                 |              | -0.078           | 0.1053  |
| Total protein                 | TP                    |              | -0.059           | 0.1381  |
| Blood sugar                   | BS                    |              | 0.056            | 0.1738  |
| Albumin                       | ALB                   |              | -0.054           | 0.1914  |
| Cholinesterase                | CHE                   |              | -0.058           | 0.1142  |
| Sodium                        | Na                    |              | -0.043           | 0.2764  |
| Uric acid                     | UA                    |              | 0.041            | 0.3692  |
| Blood urea nitrogen           | BUN                   |              | -0.035           | 0.3721  |
| Alkaline phosphatase          | ALP                   |              | 0.035            | 0.3982  |
| Alanine aminotransferase      | ALT (GPT)             |              | 0.031            | 0.4353  |
| α-Glutamyl transpeptidase     | α-GTP                 |              | 0.030            | 0.4539  |
| Hemoglobin A1c                | HbA1c                 |              | -0.033           | 0.4577  |
| Total bilirubin               | IBIL                  |              | -0.020           | 0.6209  |
| Chlorine                      | Cl                    |              | 0.018            | 0.6447  |
| Estimated glomerular          | eGFR                  |              | 0.020            | 0.6463  |
| filtrating ratio              |                      |              |                  |         |
| Albumin/globulin ratio        | A/G                   |              | -0.014           | 0.7262  |
| Creatinin                     | CRE                   |              | -0.003           | 0.9421  |
| Aspartate aminotransferase    | AST (GOT)             |              | 0.002            | 0.9661  |
| Triglyceride                  | TG                    |              | 0.001            | 0.9788  |
| Lactate dehydrogenase         | LDH                   |              | 0.001            | 0.9803  |
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