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Novel sandwich ELISA for detecting the human soluble (pro)renin receptor

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1. ABSTRACT

The (pro)renin receptor [(P)RR] plays a key role in the activation of the local renin-angiotensin system via interaction with renin and prorenin. A truncated form that is cleaved by furin, referred to as soluble (pro)renin receptor [s(P)RR], is secreted into the extracellular space. An accurate measurement of s(P)RR levels *in vivo* is an important issue in elucidating the roles of (P)RR in physiology and pathophysiology. To address this, we developed a sandwich ELISA that is applicable to human subjects. The standard curve of this ELISA showed a high linearity (125–8,000 pg/ml) with a correlation coefficient of >0.99. The recovery rate was approximately 90% in human blood and urine samples. The s(P)RR levels in plasma of healthy volunteers was in the range from 15.2 to 35.1 ng/ml (n = 20). Intra- and inter-assay coefficient of variations were less than 5.5% and 7.5%, respectively. It thus appears that this ELISA is a reliable tool for measuring s(P)RR levels in human subjects.

2. INTRODUCTION

The (Pro)renin receptor [(P)RR] is a 350-amino acids protein that contains a single transmembrane domain and binds preferentially to renin and prorenin(1). Once prorenin binds to (P)RR, it exerts two functions. First, the bound prorenin undergoes a conformational change without proteolytic cleavage of the prosegment and then develops renin activity, which catalyses the conversion of angiotensinogen to angiotensin I(2,3). This process plays a key role in the regulation of the tissue renin-angiotensin system (RAS). In the second phase, (P)RR induces its own intracellular signaling transduction that activates the MAP kinases ERK1/2 and p38 pathways. This contributes to fibril formation and proliferation in cells without angiotensin II generation(4).

It has been reported that the over-expression of (P)RR in transgenic rats resulted in the elevation of both blood pressure and heart rate(5). An enhanced mRNA

expression of (P)RR was also observed in an animal model with hypertension(6) and heart failure(7). Moreover, blockade with a (P)RR blocker inhibited the development and progression of nephropathy in a diabetes model rat(8). These findings indicate that (P)RR is involved in pathogenesis of hypertension and organ damage in diseases with over activated RAS such as diabetes and hypertension.

It was recently reported that (P)RR is cleaved into a soluble (pro)renin receptor [s(P)RR] and M8.9 which is a residual hydrophobic form by furin at the trans-Golgi network. The resulting s(P)RR, which is a truncated soluble 28kDa form, is secreted into extracellular space(9) and is ultimately found in plasma and urine(9,10). s(P)RR also binds to prorenin as well as the full-length (P)RR and mediates the activation of prorenin in a cell culture medium (*in vitro*)(11). These findings suggest that the circulating s(P)RR regulates the activities of RAS. Although the most studies to date have been focused on the full-length (P)RR, it has been suggested that assaying s(P)RR levels in biological fluids is essential in understanding tissue RAS and the pathogenesis of end-stage organ damage in patients with diabetes or hypertension(10). Biswas *et al* developed a s(P)RR enzyme-linked immunosorbent assay (ELISA) and determined the concentration of (P)RR in a cell culture medium(11). However, due to the lack of a s(P)RR quantitative system for human subjects, it was not possible to evaluate the kinetics of s(P)RR *in vivo*. Therefore, the objective of this study was to establish an ELISA system which is capable of accurately quantifying human s(P)RR in plasma.

3. MATERIAL AND METHODS

3.1. Preparation of recombinant human (P)RR

To obtain a large amount of recombinant human (P)RR protein for immunogen, the protein was expressed using a baculo-virus-silkworm expression system (Sysmex Co. Ltd. Hyogo, Japan). Details of procedure have been described previously(12). Briefly, a (P)RR transfer vector pM03 (Sysmex Co. Ltd.) was constructed. (P)RR coding cDNA was obtained from the plasmid vector, pCAGGS-hProRenRc which was reported in a previous paper(13). This transfer vector was co-transfected with the BmNPV (CPd strain, Sysmex Co. Ltd.) into B.mori-cultured cells (BmN). After propagation of the recombinant baculovirus containing the (P)RR-His tag gene in BmN cells, they were used to infect silkworm pupae. Six days after injection, the pupae were homogenized in homogenous buffer (20mM Tris-HCl, pH8.0, 0.15M NaCl, 1mM EDTA, 1mM EGTA, 10% glycerol, 1mM DTT, 1mM phenylmethylsulfonyl fluoride, 10mM benzamidine) and centrifuged at 100,000×g for 1h 4°C and the supernatant was discarded. The pellet was resuspended in lysis buffer (20mM Tris-HCl, pH8.0, 150mM NaCl) containing 1 % triton X-100. After incubation with stirring for 1h at 4°C, the suspension was centrifuged at 100,000×g for 1h 4°C, and supernatant discarded. The pellet was then resuspended in lysis buffer containing 1 % N-lauroyl sarcosine. The suspension was incubated, centrifuged again in the same manner as above and the supernatant collected. The collected supernatant was applied to a Ni-agarose column and the column was then washed with wash buffer (50mM Tris-HCl, pH 8.0, 0.3M NaCl, 0.2% N-lauroyl sarcosine). The (P)RR-His tag protein was eluted with elution buffer (50mM Tris-HCl, pH 8.0, 300mM NaCl, 50mM Imidazole, 0.2% N-lauroyl sarcosine). The eluate was concentrated and replaced with 20mM NaP, 150mM NaCl, 0.2% N-lauroyl sarcosine, pH 7.5.

3.2. Construct and transfection

Total RNA was extracted from A-549 cells using illustra QuickPrep™ Micro mRNA purification Kit (GE Healthcare Japan, Tokyo, Japan), and cDNA was generated with a first-strand cDNA synthesis kit (GE Healthcare Japan). Human (P)RR cDNA was amplified by PCR, using forward 5'-AAGAATCCAGCCTGGACGAGTCCGAGCG-3' and reverse 5'-AACTCGAGATCCATTCGAATCTTCTGGTTT-3' primers, each of which contained the restriction sites for EcoRI and XhoI respectively. Following PCR amplification, the product was digested with EcoRI and XhoI, and inserted into the pcDNA3.1(+) vector which contained a histidine tag at the C-terminus. Subsequently, the construct was transfected into Chinese hamster ovary cells (CHO-K1), using lipofectamine 2000 (Invitrogen, CA, US) and stable transfectants were selected with 1 mg/ml G418 (PAA Laboratories GmbH, Pasching, Austria).

3.3. Preparation of anti (P)RR antibodies

Rabbits were immunized with synthetic peptides CSVTLRQLRNRLFQENSVL and CIGKRYGEDSEQFRDASKI which were coupled with bovine thyroglobulin as a carrier protein. These peptide sequences corresponded to the region S148 to L165 and I222 to I239 in the human (P)RR protein, respectively. The immunoglobulin (Ig) G fractions against these peptide sequences were obtained from the sera of immunized rabbits using columns of each antigen-coupled activated Thiol Sepharose 4B beads (GE Healthcare Japan), respectively. The resulting purified IgG were denoted as PoAb-148 and PoAb-222. The recombinant human (P)RR was subsequently injected into mice. Splenocytes from the immunized mice were fused with a myeloma cell line X63-Ag8.653. The s(P)RR, expressed in *Escherichia coli* as a glutathione S-transferase-tagged fusion protein, was purified by chromatography on Glutathione Sepharose 4B beads (GE Healthcare Japan). Using ELISA, the antibodies of supernatants of the hybridoma cells were screened for their reactivity to purified GST-tagged s(P)RR, and several positive clones were selected by the limited dilution method. Thus, a single clone 93A1B (subclass IgG1, kappa) was chosen for use in the present study.

3.4. Western blotting and immunoprecipitation analysis

The recombinant human (P)RR, CHO cells transfected with cDNA for (P)RR and its culture media were lysed with a solution containing 2 % SDS, 10 % glycerol, 50mM Tris-HCl (pH 6.8), 100mM dithiothreitol, boiled and subjected to a western blot analysis by Anti-His and PoAb-222. The reactivity of antibodies were assessed by immuno-precipitation western blotting. The supernatant of the (P)RR transfectant was incubated with PoAb-148 or 93A1B and then with added Protein-G sepharose (GE Healthcare Japan). After further incubation, it was

centrifuged and the resulting pellet was washed three times. The pellet was subsequently lysed and a western blot analysis preformed using 93A1B.

3.5. Establishment of an ELISA system for the quantification of s(P)RR

The ELISA assay system is finally designed as a kit (Immuno-Biological Laboratories Co., Ltd., Fujioka, Gunma, Japan, code # 27782) as follows.

3.5.1. Standard

The purity of the recombinant human (P)RR protein was demonstrated densitometrically with Multi gauge (Fuji Film, Tokyo, Japan), and the concentration of the protein was determined by comparison with bovine serum albumin as an indicator after electrophoresis. In addition, the culture supernatant of CHO-K1 cells that had been transfected with s(P)RR cDNA was used as a working standard for this ELISA system.

3.5.2. Sandwich ELISA

Microtiter plates (96 wells) were coated by adding 100µl of 100mM carbonate buffer (pH9.5) to each well that also contained 0.5ug of purified PoAb-148, followed by incubation overnight at 4°C. The plates were then washed with PBS-T and blocked with 200µL of 1 % (w/v) bovine serum albumin (BSA) in PBS containing 0.05 % NaN_3 per well overnight at 4°C. Following two washings with PBS-T, test samples and recombinant s(P)RR, as a standard, that had been serially diluted with 1% BSA in PBS-T per 100µL were added to the wells of the coated microtiter plate in duplicate, and incubated at 4°C overnight. After washing four times with PBS-T, 100µl of horse radish peroxidase (HRP)-conjugated 93A1B mouse IgG Fab' was added to each well and the samples incubated for 1h at 4°C. The wells were washed with PBS-T five times, and 100µl of freshly prepared tetramethyl benzidine solution was then added to each well as a substrate, followed by incubation in the dark for 30min at room temperature. The reaction was terminated by adding of 100µL of 1M H_2SO_4. Absorbance of the solution was measured at 450nm by means of an ELISA reader (E-Max; Molecular Devices, Sunnyvale, CA, USA).

3.5.3. ELISA validation

In order to assess the intra- and inter-assay precision for the ELISA, three Quality Controls (QC) were established and covering the high, middle and low range of the standard curves. Intra-assay was determined by 21 repeated measurements of each QC sample in a plate and inter-assay precision was determined by assessing each QC sample across six different plates with quintuple wells. Moreover, for assessing the recovery rate in blood and urine samples, different concentrations of recombinant (P)RR added to samples were measured, and the recovery rate validated as the differentiation between the measured concentration and the theoretical concentration. The sensitivity for this kit was determined based on the guidelines provided by the National Committee for Clinical Laboratory Standards (NCCLS) Evaluation Protocols.

3.6. Detection and quantification of the s(P)RR in culture medium and blood samples of normal healthy subjects

Each batch of cells (5×10^4), (P)RR transfectant and CHO, was seeded in TIL media (Immuno-Biological Laboratories Co., Ltd., Fujioka, Gunma, Japan) containing 10 % fetal bovine serum (PAA Laboratories GmbH, Pasching) and 24hours later the media was exchanged for serum-free TIL media. After culturing for 3days, the supernatants of each well were collected and measured. The culture media of the conditionally immortalized human podocyte cell line were prepared as described previously(14) and also measured. We determined the concentrations of s(P)RR in EDTA plasma and sera of normal healthy subjects. The blood samples were purchased from Veritas Corp(Tokyo, Japan).

3.7. Statistical analysis

Statistical analyses were performed using a Student's t-test. Columns describing means ±SE. $P<0.05$ was considered to be significant.

4. RESULTS

4.1. Identification of recombinant human (P)RR and (P)RR transfectant

To identify the recombinant human (P)RR, derived from silk-worm, and to confirm the expression of the (P)RR transfectant, we performed western blotting analysis. The molecular weight of (P)RR was estimated to be 35 kDa and its soluble form about 28 kDa. In the results of western blotting analysis, we were able to confirm these estimates (Figure 1A, B).

4.2. Characterization of antibodies against s(P)RR

For confirming whether the antibodies have ability to react against native s(P)RR in aqueous conditions or not, immuno-precipitation western blotting analysis was performed. Both PoAb-148 and 93A1B antibodies used for ELISA were able to immuno-precipitate s(P)RR originated from the medium of cultured s(P)RR transfectant (Figure 1C).

4.3. Establishment of the quantitative ELISA system for the s(P)RR

In order to measure the s(P)RR concentration, we developed a sandwich s(P)RR ELISA system, which is combination of PoAb-148 and 93A1B. PoAb-148 was used as the capture antibody and 93A1B was used as the detection antibody. The standard dose-response curve for the s(P)RR ELISA system exhibited a linear shape when plotted on a log/log scale over a range from 125 to 8,000 pg/mL and the linearity was excellent (R^2= 0.99) (Figure 2). The precision was determined with three spiked QC controls (high (H), middle (M) and low (L)). The intra-assay precision exhibited coefficient of variation(CV) of 3.1% in H, 5.4% in M and 5.5% in L (Table 1). Additionally, the inter-assay results for CV of 3.0% in H, 2.4% in M and 7.5% in L, respectively (Table 2). Thus, the ELISA system can be considered to be fully reliable from the standpoint of precision. In the recovery validation test, the recovery rates were >86.8% for human EDTA plasma

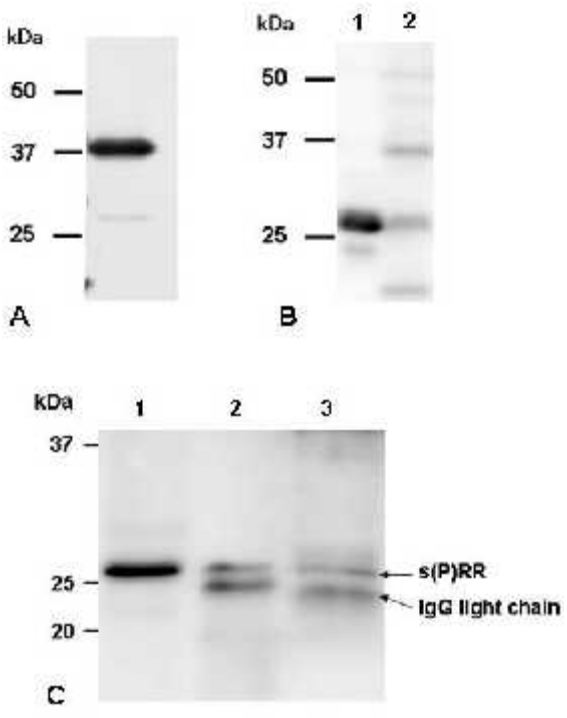

Figure 1. Identification of the recombinant human (P)RR and (P)RR transfectant by western blot analysis. A: Recombinant human (P)RR derived from silkworm was blotted by anti-His. B: The 20-fold concentrated culture supernatant of CHO cells transfected with cDNA of (P)RR (lane1) and total cell lysates of the transfectant(lane2) were blotted by PoAb-222. C: The 20-fold concentrated culture supernatant of CHO cells transfected with cDNA of (P)RR (lane1), s(P)RR protein were immunoprecipitated from culture supernatant of (P)RR transfectant with PoAb-148 (lane2) or 93A1B (lane3), and were detected by immunoblotting with 93A1B.

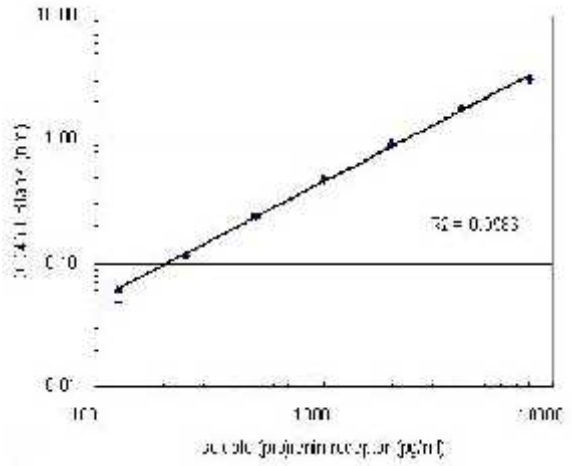

Figure 2. Characteristic of the human s(P)RR ELISA system. Standard curve for calculation of s(P)RR. The linearity of range for the s(P)RR is from 125 to 8,000 (pg/ml).

samples at 50 times dilution, >91.2% for human serum at 50 times dilution, >91.0% for human urine samples at 10 times dilution and nearly 100% for TIL media supplemented with 10% FCS at two times dilution. The assay sensitivity was calculated to be 24 (pg/ml) using NCCLS methods.

4.4. Quantification of s(P)RR in the culture media of (P)RR transfectant and human blood

In order to determine if the ELISA assays are quantitative for native s(P)RR, we performed the following two experiments. We measured s(P)RR in the medium in which (P)RR cDNA transfected CHO cell were cultured and culture medium from CHO cell, which had not been transfected. The concentration of s(P)RR in the (P)RR transfectant cell culture supernatant was 126.3 ng/ml, which was much higher than the concentration in the culture media from CHO cell at 5.6 ng/ml, as expected (Figure 3A). Furthermore, we measured both podocyte culture supernatant samples with and without the transfection of siRNA. As this ELISA kit also detects s(P)RR originated from fetal bovine serum in the media, the values for each culture supernatant should be compared with the value of (non-cultured) media subtracted. The concentration of s(P)RR in the podocyte supernatant without the transfection of siRNA was 8.4 ng/ml, which was much higher than the result for the transfected podocyte supernatant at 0.4 ng/ml (Figure 3B). The concentrations of s(P)RR in human EDTA plasma and serum samples each (male; 10, female; 10) which were obtained from normal healthy subjects. EDTA plasma samples ranged from 15.19 to 35.11 ng/ml and the mean value was 22.31±5.19 ng/ml. On the other hand, serum samples were in the range from 12.61 to 33.06 ng/ml and the mean value was 22.34±5.25 ng/ml (Figure 4A). EDTA-plasma and serum have a correlation (R=0.757). No significant differences were found between males and females (Figure 4B). There were also no disparities in s(P)RR levels based on ages (Figure 4C).

5. DISCUSSION

In this study, we report on the development of a s(P)RR quantitative system that is applicable for use in human subjects. In a preliminary study, we raised three rabbit polyclonal antibodies against synthetic oligo-peptides, including PoAb-148, and established a sandwich ELISA using a combination of these antibodies. Although this ELISA could be used to quantitatively measure recombinant human (P)RR and s(P)RR in the culture supernatant of CHO cells, it was unable to detect s(P)RR in human subjects. We observed that these antibodies detected s(P)RR in a western blotting analysis, whereas the reactivity against native (P)RR protein was poor. Therefore, we attempted to establish mouse monoclonal antibodies using highly purified recombinant human (P)RR derived from silk-worms as an immunogen. In order to select a high reactivity clone against the whole native protein, we established a clone (93A1B) that showed a strong reactivity against recombinant (P)RR and GST-s(P)RR originated from *E.coli* by a sandwich ELISA using

Table 1. Intra-assay precision

QC	Measured values, pg/ml	SD	%CV	n
H	3,417.9	105.8	3.1	21
M	880.4	47.9	5.4	21
L	252.9	13.9	5.5	21

The intra-assay results were derived from analyses of 21 replicates of each QC sample. CV ; coefficient of variation.

Table 2. Inter-assay precision

QC	Measured values, pg/ml	SD	%CV	n
H	3,497.0	106.4	3.0	6
M	887.7	21.7	2.4	6
L	259.5	19.5	7.5	6

The inter-assay results were based on six separate measurements of each QC in quintuplicate.

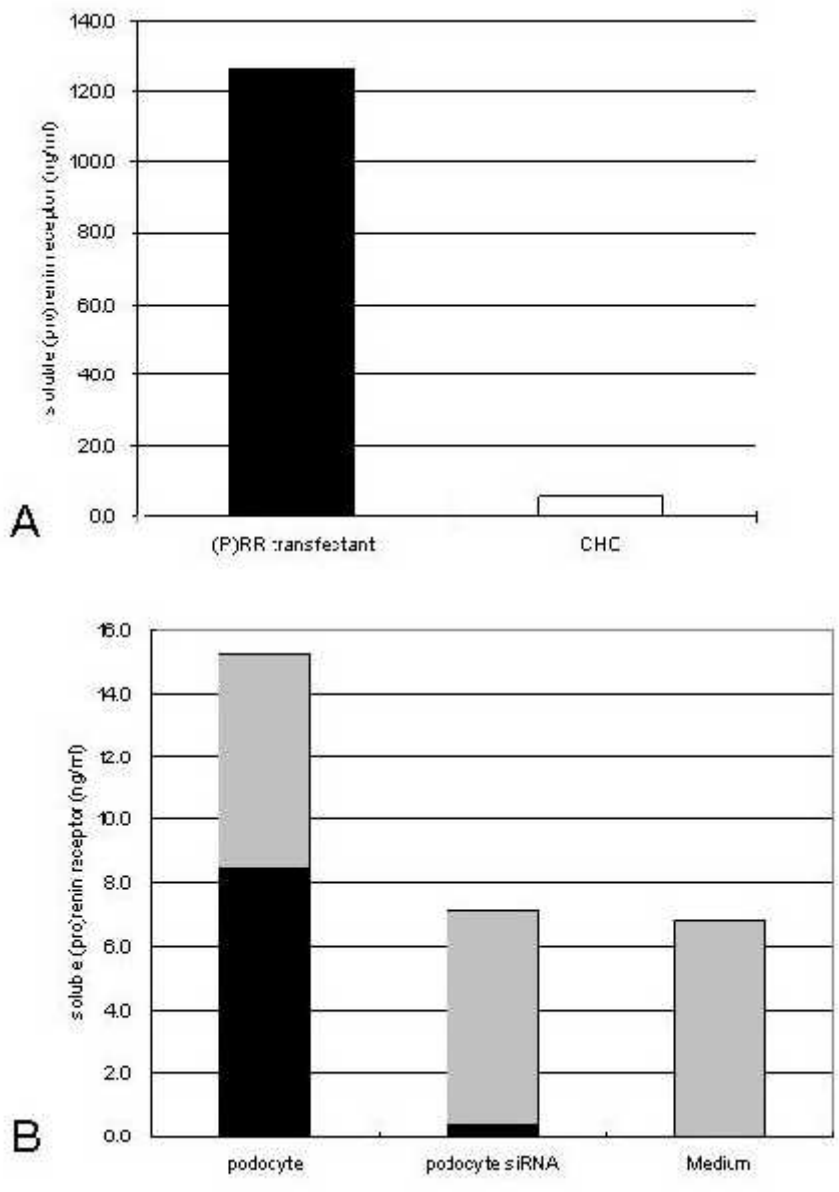

Figure 3. Concentrations of s(P)RR in cell culture media. A; CHO cells with transfection of (P)RR cDNA (left), and CHO cells without transfection (right). B; Podocyte cells (left), podocyte cells with transfection of siRNA (middle) and just only medium (right). The gray bar indicates the value of s(P)RR originated from fetal bovine serum in the media. The solid bar indicates the concentrations of s(P)RR that is thought to be originated from podocyte cells.

Table 3. Recovery

Sample	Theoretical value, pg/ml	Measured values, pg/ml	%
Human plasma	4,462.2	3,957.3	88.7%
	2,462.2	2,136.5	86.8%
	1,462.2	1,286.2	86.0%
Human serum	4,495.2	4,142.9	92.2%
	2,495.2	2,341.6	93.9%
	1,495.2	1,363.5	91.2%
Human urine	2576.2	2344.7	91.0%
	1826.2	1701.7	93.2%
	1451.2	1351.5	93.1%
TIL media supplemented with 10% FCS	2,488.5	2,496.3	100.3%
	1,738.5	1,667.5	95.9%
	1,363.5	1,331.4	97.6%

Different known concentrations of recombinant human s(P)RR standards were added to human plasma, serum, urine or TIL media supplemented with 10 % fetal bovine serum, and the recovery rates (%) were calculated from the corresponding s(P)RR concentrations in samples between with and without added s(P)RR standards.

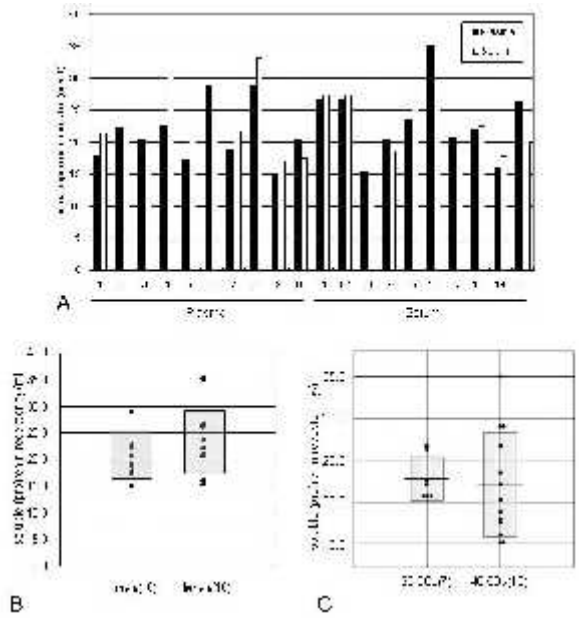

Figure 4. Measurement of s(P)RR in plasma and serum. A: The s(P)RR concentrations in plasma and serum obtained from 20 normal healthy subjects. Samples were applied to the ELISA system in duplicate, and averaged values were obtained. B: Comparison of s(P)RR concentration in EDTA-plasma between male and female. C: Comparison of s(P)RR concentration in EDTA-plasma between 20-30s and 40-50s.

PoAb-148 which shows the highest reactivity among three different types of peptide antibodies. We confirmed that these two antibodies (PoAb-148 and 93A1B) were reactive against native s(P)RR protein in an aqueous environment by immunoprecipitation analysis. Based on these facts, we then developed a new sandwich ELISA using these unique antibodies PoAb-148 as a capture antibody and 93A1B as a detection antibody. We measured s(P)RR in culture supernatants of (P)RR cDNA transfected CHO cells and in culture supernatants that had not been transfected. The findings confirmed that the transfection of (P)RR cDNA resulted in an increase in the concentration of s(P)RR in culture supernatant and also confirmed that the ELISA reflects the amount of s(P)RR present in the solution. The most notable advantage of this ELISA is that it can be used to quantify s(P)RR levels in plasma, serum and urine of human subjects. The range of measurement of this ELISA was determined to be 125- 8,000 pg/ml ($R2 = 0.99$) and its lowest detection limit was 24 pg/ml. The sensitivity of the ELISA is approximately 100 times higher than the ELISA established in our preliminary study using rabbit polyclonal antibodies against synthetic oligo-peptides. The sensitivity of the ELISA should be sufficient to meet the requirements for measuring s(P)RR in human subjects. Furthermore, we performed a validation test to verify the reliability and stability of this system. In the recovery test with human subjects, the recovery rate was approximately 90% in human blood samples as shown in Table.3. It was also equivalent in human urine. In addition, we performed intra- and inter-reproducibility tests, and the results confirmed the reproducibility of the method, as shown in Table 1, 2. The results of the validation tests suggest that the ELISA system can be reliably used to measure native s(P)RR levels in human subjects.

The presence of s(P)RR in human plasma was demonstrated by Causin *et al* in a coprecipitation analysis using His-tagged renin(9) . However, there was no quantitative data on s(P)RR levels in the circulating system. Whereas, we determined these values in plasma and serum samples obtained from twenty healthy subjects. The concentration in the plasma and serum samples was similar and showed well correlation and these values were unaffected by age and gender (Figure 4B,C).

Studies have suggested that a relationship exists between (P)RR and organ damage caused by the over-activated RAS in hypertension and diabetes. Enhanced mRNA expression of (P)RR was observed in animal models with hypertension and heart failure(6,7). Since the enzymatic activity of prorenin is increased when it binds to (P)RR, the expression levels of (P)RR appears to be important in investigating the contribution of tissue RAS to diseases. The (P)RR is cleaved into a 28 kDa soluble form and a 8.9 kDa hydrophobic form. The soluble form of (P)RR is secreted into the extracellular space(9). Thus, an accurate measurement of s(P)RR may permit (P)RR expression to be estimated and will be an useful tool for assessing the pathological condition and the mechanism of pathogenesis.

Additionaly, the residual hydrophobic domain, termed M8.9, was reported as a constituent of vacuolar H^+-ATPase (V-ATPase)(15,16). The V-ATPase is an essential protein unit that is associated with cellular pH homeostasis(17). The expression of the protein is increased in cancer cells, suggesting that the V-ATPase is involved in proliferation and metastasis of cancer cells(18,19). s(P)RR levels may also be associated with the activity of V-ATPase or/and malignancy of cancer cells.

In conclusion, we report on the development of a novel system for quantifying s(P)RR using a sandwich ELISA which is applicable for human subjects. It is expected that the use of this reliable human s(P)RR ELISA will contribute to a better understanding of the fields of hypertension, diabetes and cancer.

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