A New Direct Radioimmunoassay for Human Renin Substrate and Heterogeneity of Human Renin Substrate in Pathological States

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The presence or absence of correlation in quantitation of human renin substrate by conventional indirect method (equivalent generated angiotensin I; AI) and newly-developed direct radioimmunoassay (immunoassayable substrate) has allowed us to screen heterogeneity of renin substrate in various pathological states, in conjunction with several procedures for protein analysis.

One major peak of substrate activity on polyacrylamide gel electrophoresis (PAGE) was found in normotensive and benign essential hypertensive subjects who gave a 1:1 correspondence and a good correlation between two substrate assays. In contrast, certain subjects on estrogen therapy, pregnant woman during the last trimester and some uremic patients demonstrated at least 3 different forms of renin substrate on electrophoresis (Rf of I = 0.65, II = 0.35, III = 0.16). Further analysis by isoelectric focusing PAGE presented additional findings on altered substrate type in patient with Cushing's syndrome (I = 4.7 and 4.3).

In these additional forms of renin substrate, structural or immunological difference was demonstrated by a lack of binding affinity to antiserum prepared against renin substrate from normotensive subjects.

Preliminary data on their AI generation rates in the in vitro incubation with added renin, implied altered kinetic characteristics in the in vivo renin-substrate reaction. These findings confirm the existence of multiple forms of human renin substrate, which physico-chemical and enzymological properties differ from each other. It is suggested that such proteins may be involved in abnormalities of renin-angiotensin system inducing hypertensive states.

We have isolated and partially characterized human renin substrate from normotensive plasma. Using this purified protein, we have also developed an antiserum in rabbits and established a direct radioimmunoassay for human renin substrate. It appears that this newly-developed substrate assay alleviates the problems of conventional indirect method.

Our previous report indicated that only in certain women on oral contraceptives is there a discrepancy between both direct and indirect methods for renin substrate. Initial data on protein analysis showed that such women might produce multiple forms of renin substrate, which

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were found in neither normal nor essential hypertensive subjects. By electrophoretic methods in conjunction with newly-developed antibody specific for normal renin substrate, we have obtained more evidence for heterogeneity of human renin substrate.

In this study we report further findings on multiple forms of renin substrate in patients under various conditions associated with an increase in renin substrate; some uremic patients with hypertension, those with steroid therapy and Cushing's syndrome patient, who are all exhibiting a great discrepancy in quantitation by both indirect and direct methods for renin substrate assay. We also demonstrate some physicochemical and enzymological properties of these different substrate types. Such study may provide further insight into understandings on etiology of hypertension, and give us precise diagnosis and treatment.

MATERIALS AND METHODS

Plasma samples

For this investigation the following patients, attending outpatient clinic or hospitalized, were examined. Many patients with benign essential hypertension (uncomplicated or complicated), malignant hypertension (n = 12), uremia treated with hemodialysis (n = 9), liver cirrhosis (n = 8), one Cushing's syndrome and those with steroid therapy (glucocorticoids n = 2, cancer on estrogen n = 1). In addition, 15 normotensive subjects including 10 healthy volunteers, 7 pregnant women (n = 2 in the first trimester, n = 4 in the last trimester, pre-eclampsia n = 1), one postpartum woman and 20 women taking oral contraceptives were examined. All plasma were collected into 15% NH₄-EDTA (final concentration 0.38%). The plasma samples after centrifugation were stored at -20°C. Plasma was no longer used after it was thawed and assayed once. A good reproducibility was found in the results of renin substrate assays during a few weeks.

Indirect assay for renin substrate (conventional method)

Total renin substrate (TS) or consumed angiotensinogen is defined as a quantity of generated angiotensin I (A I) on exhaustive incubation with excess "Haas" renin³ at pH 7.4, as determined by a radioimmunoassay for AI according to the method of Eggenga et al⁴.

Direct radioimmunoassay for renin substrate

Human renin substrate was isolated and purified from the plasma of normotensive subjects by our recently-described method¹ This procedure includes ammonium-sulfate precipitation, chromatography on Sephadex G150, DEAE cellulose and calcium phosphate gel followed by isoelectric focusing and preparative polyacrylamide gel electrophoresis. The purified renin substrate was used as an antigen. Antiserum was prepared by subcutaneous injection into New Zealand white rabbits with complete Freund's adjuvant (Cal Biochem.). Immunization was repeated six times at 2 week intervals. Animals were bled by cardiac puncture 3–5 days after the last immunization. Antiserum was aliquoted into 1ml fractions, frozen in dry ice-alcohol bath and stored at -40°C.

Five or 2.5 μl (duplicate) of a 1:20 diluted plasma was directly aliquoted into Beckman biovials containing 0.2 ml of 0.1 M Tris-acetate buffer (pH 7.4, 0.1% lysozyme). A freshly prepared mixture (0.8 ml) of antiserum (1 : 30,000 titer) was added. This mixture also contained sufficient ¹²⁵I labelled, completely purified, human renin substrate, iodinated by the method of Hunter and Greenwood² to ensure at least 5000 cpm per tube. Samples were equilibrated for 24–36 hrs at 4°C. Separation of antibody bound and free substrate was attained by a second equilibration with goat anti-rabbit gamma globulin. 0.5 ml of supernatant containing unbound

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**Fig.1. Standard curve of direct radioimmunoassay for human renin substrate (1 : 30,000). Substrate standard is based on angiotensin generating capacity of the plasma, assuming 1 mol of angiotensin I per mol of substrate and a molecular weight of 110,000 for renin substrate.**

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substrate was taken from each tube and counted in Beckman Biogamma Spectrometer. The standard curve was sensitive from 5—100 ng of renin substrate (Fig. 1). Standard renin substrate was quantitated indirectly by measurement of generated AI. The mean substrate concentration in normal control plasma was 189 ± 7.0 S.E.M. µg/ml by this method; this result corresponds to 2.2 ± 0.1 µg of AI equivalents, based on our reported MW value of 110,000 daltons for normal human renin substrate.

Protein analysis for renin substrate types

Gel filtration chromatography and isoelectric focusing: Sephadex G150 was equilibrated in 0.05 M Tris-acetate buffer (pH 7.4). The column (5 × 80 cm) were standardized by using blue dextran 2000, aldolase, ovalbumin, chymotrypsinogen A and ribonuclease A (Pharmacia Co.). Molecular weight estimations of unknown proteins were determined by the method of Ackers et al. Individual column flow rates were 10—15 ml/hr and 5 ml fractions were collected.

Isoelectric focusing columns (LKB8100, LKB Prod. Co.) were run by the method outlined by Vesterberg and Svenson, using ampholines in pH 3—10 range.

Polyacrylamide Gel Electrophoresis (PAGE) and isoelectric focusing PAGE: Gel electrophoresis of plasma samples (0.1 ml) were run at pH 8.4 and 4.3 in a 7% polyacrylamide gel. Isoelectric focusing electrophoresis on 7% polyacrylamide gel (2% ampholine in pH 4—6 range) were performed in some plasma by the method of Lim and Tadayon. Sodium dodecylsulfate gel electrophoresis (SDS-PAGE) was used for MW determination as described by Ugel et al. These analytical electrophoresis were performed in the presence of 8 M Urea or 1% SDS to prevent the formation of protein aggregates.

RESULTS

Comparison between two assay methods for renin substrate

Comparison of two measurements obtained with the direct (Immunoadassayable substrate; I-RS) and indirect (Total Substrate Activity; TS) methods for human renin substrate is shown in Figures 2—4. The linear regression analysis demonstrates a 1 : 1 correspondence (y = 11.1x + 4.0 in Figure 2) and a good correlation (r = 0.780, p < 0.01, n = 28) in the results of two assay systems for plasma from 10 normotensives and 18 benign essential hypertensives.

In certain plasma, two types of dissociation between both results of two methods for renin substrate measurement were observed as shown in Figure 3 (Type I discrepancy: TS > I-RS) and figure 4 (Type II discrepancy: I-RS > TS).

"Type I" discrepancy in the results of two assays: TS values as determined with equivalent AI generated were higher than I-RS as measured...
greater discrepancy between two substrate values as shown in Figure 3 (TS; 610–1250 μg/ml transferred from generated AI equivalents vs. I-RS; 230–348 μg/ml by direct assay).

"Type II" discrepancy: It is, on the other hand, noteworthy that in most plasma from patients with renovascular hypertension (Plasma renin activity; 21.0–58.1 ngAl/ml/hr), I-RS measurements were always significantly higher than TS values as shown in Figure 4, (I-RS; 226–350 vs. TS; 104–228, p < 0.01, n = 11).

Comparison of renin substrate with plasma renin activity in essential hypertension: No significant differences (p < 0.05) in substrate levels were found between low renin (PRA; 0.3 ± 0.6 ng/ml/hr), normal renin (1.6 ± 1.8) and high renin (12.3 ± 12.0) as shown in Figure 5. In each subgroup of essential hypertension no correlation between renin substrate (both direct and indirect assays) and plasma renin activity was observed.

Influence of diuretic therapy on plasma renin substrate: In benign essential hypertensives, diuretic therapy with hydrochlorothiazide (HCT) and/or spironolactone raised two substrate values with a pretty good correlation as shown in Figure 6 (HCT; n = 6, HCT and/or Spironolactone; n = 5). Some plasma from patients administered with spironolactone seem to show somewhat dissociation in two substrate

![Graph](image)

**Fig. 4.** Renin substrate values in various clinical states (II). "Type II" discrepancy: I-RS > TS in certain plasma from uremic and some hypertensive patients.

**Fig. 5.** No significant difference in renin substrate between each subgroup of essential hypertension. Comparison of renin substrate values in low renin (LR), normal renin (NR), high renin (HR) hypertension and renovascular hypertension (RVH), classified by the level of plasma renin activity (PRA).

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values comparing to those treated only with HCT.

Decreased values of two substrate measurements in patients with liver cirrhosis: In comparison with normal range of plasma renin substrate, both substrate values in patient with liver cirrhosis (n = 8), associated with or without ascites, were relatively lower as shown in figure 4 (TS; 50–178 vs. I-RS; 42–192, PRA; 2.5–37.0).

Heterogeneity of human renin substrate

To elucidate whether or not heterogeneity in renin substrate could explain the dissociation in quantitation by both assay methods observed in certain patients, plasma from patients in various states were examined by the following techniques for protein analysis.

Polyacrylamide Gel Electrophoresis: One major peak and two minor peaks of substrate activity, as measured by equivalent Al generated on incubation of homogenized gel slices (fractionated on PAGE at pH 8.4), with excess renin, were determined in plasma from certain subjects on oral contraceptives and pregnant women in the last trimester as shown in figure 7-B. The electrophoretic mobility (Rf: relative distance vs. albumin-dye front) responsible for each peak of substrate activity was Rf = 0.65 ± 0.05 S.D. for major peak (I), 0.35 ± 0.05 (II) and 0.16 ± 0.02 (III), respectively. These three substrate components have been confirmed on acid PAGE at pH 4.3 as demonstrated; Rf = 0.06, 0.17, 0.29. Such additional substrate components were also found in plasma of uremic patients with type I discrepancy in both substrate assays as shown in figure 4. The affinity to our newly-developed antibody specific for normal renin substrate was demonstrated exclusively corresponding to one major peak (I) of substrate activity; the quantity of immunoassayable substrate (I-RS) was equivalent to the substrate value of generated Al (TS) as measured by indirect assay.

In contrast, control plasma showing a good correlation between two substrate assays demonstrated one major peak on PAGE with a correspondence in quantitation of substrate by each method. Trace quantities (<1%) of two minor components of renin substrate were also observed in normal subjects, as shown in figure 7-A. In most plasma from hypertensive patients, no distinct profile of multiple substrate components were detected with this analytical procedure. Plasma from postpartum woman.
who demonstrated multiple substrate components in the last trimester of pregnancy, showed no distinct additional peaks of substrate activity on PAGE analysis.

Isolelectric focusing at amphoteline pH 3.5–10 and isoelectric focusing PAGE at amphoteline pH 4–6: On isoelectric focusing at pH 3.5–10, oral contraceptive plasma showing high substrate value with “type I” discrepancy demonstrated only one broad component of substrate activity and also showed one component of immunoreactivity to substrate antibody. Upon isoelectric PAGE at pH 4–6, on the other hand, most plasma demonstrated various sawtoothed patterns of substrate activities, so that it is very hard to recognize them distinctly as specific substrate components. Regardless of those irregular profiles of substrate activities as determined by AI equivalents, such substrate peak seems to be composed of a single homologous component, because a direct immunoassay demonstrated one broad substrate component responsible for such sawtoothed pattern of substrate activity.

It is, however, noteworthy that another distinguishable component of substrate activity (Ip. = 4.3 vs. Ip. = 4.7 of major component) on isoelectric focusing PAGE at pH 4–6, but without immunoassayable substrate, was detected in plasma from Cushing’s syndrome patient as demonstrated in figure 8. No extra-components of renin substrate were observed on regular PAGE analysis in spite of a great discrepancy between two substrate values (TS; 368 vs. I-RS: 164).

Trials of molecular weight determination and kinetic study for different types of renin substrate: Using semipurified and concentrated multiple substrate components extracted from many elution pools of PAGE at pH 8.4 (n = 27 gel runs of “type I” oral contraceptive plasma), MW determination for such additional substrate forms were estimated with gel filtration method (Sephadex G150 Column Chromatography) and SDS-PAGE. Approximate MW for each substrate form was 8.7–11.0 x 10^4 (I), 3.5–4.7 x 10^4 (II), 4.3–5.9 x 10^4 (III) daltons, respectively. Kinetic characterization was also studied; they appear to have different enzymological reaction rate individually on incubation with the same amount of added renin. The reaction rate of each substrate form is defined as a percentage of AI generated on 20 min incubation with exogenous renin vs. total consumable substrate. In this system supposed of the first-order reaction, the peak III substrate in multiple forms on PAGE at pH 8.4 indicated a faster AI production rate comparing to other substrate forms and normal control plasma, whereas that of peak II substrate was distinctly less than others (II; 34.6 ± 3.2 S.D., III; 3.6 ± 1.7, I; 17.2 ± 4.8, Normal control plasma; 15.6 ± 4.7%).

DISCUSSION

To investigate more critically the physiochemical aspects of the renin substrate reaction in various hypertensive states, a homologous preparation of renin substrate has been needed. Plasma renin substrate activity measurement is widely performed as a useful test for evaluating the diagnosis and treatment of hypertension. Our previous study on purification and partial characterization of normal human substrate indicated that the protein appears a single homologous form with more than 95% purity on various analytical methods. Although several forms of renin substrate are reported in human plasma and in animal plasma our study demonstrated a single component of normal human renin substrate in all purification procedures. The molecular weight was determined as 110,000 daltons by gel filtration and SDS-polyacrylamide electrophoresis. The biological activity seems similar to “native” renin substrate since its kinetic parameters are the same as that reported for renin reaction in whole plasma.

Using this purified protein, we have recently established a direct radioimmunoassay for human renin substrate. The newly developed direct assay for renin substrate seems to be more useful as a clinical test than conventional method since it has several advantages over the conventional indirect method; because of eliminating such difficult problems as (1) suppressing angiotensinases, (2) achieving complete conversion of substrate to AI, (3) using a preparation of partially purified renin, in conventional assay system. No special precautions were taken to inhibit the action of renin in our assay system. The comparison of results in both assay methods for renin substrate in plasma from normal and essential hypertensive subjects demonstrated a 1:1 correspondence and good correlation, on the basis of molecular weight 110,000 and one mole generation of angiotensin per one mole of substrate. In contrast, certain women on oral contraceptives and pregnant women in the last trimester showed significantly greater substrate values as measured by the indirect assay comparing to those by the direct substrate assay (“Type I” discrepancy in figure 3). It was noteworthy that such plasma showing a dissociation between both substrate values had abnormally high values in both plasma renin activity and total substrate activity as measured by equivalent AI generated; it was suggested that such discrepancy might be due to the presence of different forms of renin substrate, which were capable of generating AI when incubated with renin enzyme, but not recognized by the antiserum prepared against normal human renin substrate.

Protein analysis by polyacrylamide gel electrophoresis (PAGE) demonstrated at least two additional forms of substrate in plasma from subjects indicating a lack of correlation between both assay methods. Multiple forms of renin substrate were also observed in a male patient with estrogen therapy against prostatic cancer. On the other hand, normal and essential hypertensive plasma contained one predominant form of renin substrate on PAGE analysis. Additional forms of substrate on electrophoresis had no affinity to antibody specific for normal human, which were not aggregation forms since this analysis was performed in the presence of 8 M Urea and 1% SDS. These data indicate that such additional forms of renin substrate are structurally, at least immunologically, different from normal human renin substrate. Thus, for exploring the multiple forms of substrate, the presence or absence of dissociation between both assay methods for renin substrate quantitation encouraged us to screen a number of hypertensive patients associated with abnormalities in renin angiotensin system.

The results in present study, in conjunction with additional techniques for protein analysis, have furthermore confirmed the heterogeneity of human renin substrate in certain pathological states. Some uremic patients showing a dissociation between both substrate assays have demonstrated multiple forms of renin substrate on PAGE at pH 8.4. In our study, several uremic patients with severe renal damage suspected from very low renin concentration and other clinical evidences showed higher levels of renin substrate by both assay methods. This high level of renin substrate is interesting in relation to a possible involvement of stimulatory mechanism of renin substrate synthesis in anephric subjects and hemodialysis patients in terminal stage of chronic renal failure. Increase in renin substrate after bilateral nephrectomy is a well known phenomenon, although its precise mechanism is still not clear. Some investigators suggest that elevation of renin substrate in nephrectomized animals may be due to a possible factor induced by nephrectomy which stimulates renin substrate synthesis in liver.

Present study has also demonstrated that not
only estrogen, but also glucocorticoid might be capable of inducing multiple forms of renin substrate both in patients with its therapeutic administration and in Cushing's syndrome where it is thought to be pathologically excess. In particular, patient with Cushing's syndrome, who showed a great discrepancy in both substrate assays, demonstrated another distinct peak of substrate activity on isoelectric focusing PAGE fractionation at pH gradient of 4 to 6.

The difference in renin substrates induced by two kinds of steroid may be due to the altered fashion of hormone action in protein synthesis by individual steroid. Furthermore, it strongly suggested the steroid induction of new forms of renin substrate that all subjects examined after cessation of oral contraceptives and in the postpartum, who had shown multiple substrate peaks, demonstrated no additional forms except one component identical to normally-occurring substrate form. It is also likely that the occurrence of these proteins depends on the susceptibility of individual subject responding to each steroid therapy as well as the prevalence of hypertension.

At present, it is not clarified how the absolute renin substrates could involve in prevalence of hypertension, but it is most likely that they may play, at least partly, a significant role since a cessation of steroid administration caused a rapid reduction both in blood pressure and in renin substrate level. Most hypertensive patients showed one major form of renin substrate identical to normal form on electrophoresis. However, certain patients with malignant hypertension and advanced essential hypertension indicated a pretty higher values in both substrate assays comparing to benign essential hypertensives. This may be a possible reflection of accelerated renin activation in such pathological state; the increased AI production may consequently induce a stimulation of renin substrate synthesis in liver. In contrast, some patients with renovascular hypertension have demonstrated the higher value in direct substrate assay comparing to total substrate activity as measured by indirect methods ("Type II" discrepancy); this may imply more increased formation of consumed renin substrate ("Des-angiotensin substrate") by very high renin produced in ischemic kidney, since "Des-angiotensin substrate" demonstrates a complete cross-reactivity to normal substrate antibody. Also, this protein may have a possible inhibitory effect on the

\textit{in vitro} renin-substrate reaction employed for this substrate assay system.\footnote{19}

In our study most plasma from normal and essential hypertensive subjects demonstrated only one major peak of substrate activity (Ip = 4.7) on isoelectric PAGE. This result is contrary to other report\footnote{20}; such a single component appears, nevertheless, homologous because a good affinity to normal substrate antibody was found corresponding to this peak. It is not clear whether multiple forms of renin substrate may be caused by an increase in normally occurring minor trace components seen in some patients with normal and hypertensive subjects, or due to the induction of structurally different forms responsible for an increased demand of substrate synthesis. At present, we are in the process of screening more patients with various hypertensive diseases and those in other pathological states, to examine if multiple forms of renin substrate exist, or if the profile of their concentrations is altered. We are also isolating and purifying these additional forms of renin substrate to establish more precise data on their physiochemical and enzymological characteristics. Our preliminary data on their kinetic properties indicated that one of these different substrate types had a faster rate of AI production, implying a higher affinity to renin on the \textit{in vivo} renin-substrate reaction in certain pathological conditions. It is reasonable to assume that such different forms of renin substrate with altered kinetic characteristics may, at least partly, involve in pathogenesis of hypertension associated with abnormality of renin angiotensin system.

In conclusion, using a newly-developed direct radioimmunoassay for human renin substrate and several techniques for protein analysis, we have established the heterogeneity renin substrate in certain human plasma.

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