Characterization of a Monoclonal Antibody Specific for Human Active Renin

Wen Min Zuo, Richard E. Pratt, Christoph H. Heusser, John P.A. Bews, Marc M. de Gasparo, and Victor J. Dzau

We have identified and characterized an anti-human renin monoclonal antibody Rl-20-5 that is selective for human active renin. Rl-20-5 binds active renin with a dissociation constant ($K_d$) of $2.5 \times 10^{-7} \text{ M/l}$ and inhibits renin enzymatic activity with an inhibitory constant ($IC_{50}$) of $1.4 \times 10^{-8} \text{ M/l}$. Rl-20-5 competes with a synthetic renin inhibitor for binding with renin, demonstrating further that it is binding to or close to the active site. This antibody does not bind prorenin in human plasma or recombinant prorenin expressed by L-929 fibroblasts transfected with human renin gene. Furthermore, trypsin activation of prorenin resulted in immunoreactivity of the activated prorenin toward the antibody. In addition, an immunoaffinity column of Rl-20-5 coupled to Sepharose retained active renin but had a low affinity for prorenin. A sensitive and rapid solid phase radioimmunoassay for active renin was developed using a "sandwich" technique employing Rl-20-5 and a second non-active site–directed monoclonal antibody to human renin. Renin levels in human plasma samples were determined by the standard enzymatic assay, and by the direct radioimmunoassay for active renin, before and after trypsin activation. Trypsin treatment of plasma resulted in parallel increases in both the plasma renin enzymatic activity and in the plasma active renin concentration as measured by the direct radioimmunoassay. Overall, plasma immunoreactive active renin concentration correlated significantly with plasma renin enzymatic activity ($r=0.96, p<0.001$). In summary, the monoclonal antibody Rl-20-5 is selective for human active renin and should be a very useful tool for studies of the active enzyme in humans. (Hypertension 1992;19:249–254)

KEY WORDS • renin • antibodies • immunoassay • monoclonal antibodies • renin inhibitors

Renin-angiotensin system plays an important role in the blood pressure, electrolyte, and fluid homeostasis. At least two forms of renin are found in human plasma (i.e., active renin and inactive renin). Recent data suggested that the latter is primarily the biological precursor of the active form (i.e., prorenin). Up to 90% of the renin in human plasma is present in the inactive form; and the level of active renin in human plasma is usually low. Plasma enzymatic activity is, at present, the most accepted method for the measurement of active renin. The assay of prorenin is indirect and requires prior activation of prorenin by trypsin, pepsin, acid, or cold treatment followed by assaying for renin enzymatic activity. All these activations are time consuming and have their technical limitations. For these reasons it is desirable to develop sensitive and rapid radioimmunoassays for active renin and prorenin. In the past few years, efforts have been made toward developing monoclonal antibodies that are selective for prorenin and active renin.

With one exception, current available monoclonal antibodies to renin cannot distinguish between prorenin and renin. Galen et al obtained an antibody that can preferentially bind active renin. This antibody, however, does not bind renin at its active site. In the present study, we obtained and characterized a monoclonal antibody (Rl-20-5) that selectively recognizes human active renin and established a direct radioimmunoassay for human active renin. This monoclonal antibody selective for active renin should also be useful as a probe for molecular and physiological studies of human active renin.

Methods

Monoclonal Antibodies

Production and characterization of the monoclonal antibodies for human renin used in this study were previously described. Antibodies developed were R3-36-16, R3-27-6, R3-47-10, R2-1-21, and Rl-20-5, which bind renin with $BC_{50}$ of $6.2 \times 10^{-11}$, $2.7 \times 10^{-10}$, $7.0 \times 10^{-11}$, $1.8 \times 10^{-9}$, and $2.5 \times 10^{-7}$, respectively, and inhibit plasma renin activity with $IC_{50}$ of $1.3 \times 10^{-11}$, $1.0 \times 10^{-10}$, $2.0 \times 10^{-11}$, $5.7 \times 10^{-10}$, and $1.4 \times 10^{-8}$ M/l, respectively. Monoclonal antibodies R3-36-16, R3-47-10, and R3-27-6 raised against pure human renin recognize both prorenin and active renin.

Sources of Human Renin

Pure human renin and partially purified human kidney renin (HKAS) (step 7 human kidney ammonium sulfate step) specific activity $8.3 \times 10^{9}$ ng angiotensin I...
Renin activity assay was assayed by incubation of 50 μl sample with 250 μl (1:3 diluted) anephric sheep plasma at pH 7.4, 37°C in the presence of 0.5 mM EDTA, 5 mM hydroxyquinolone, and 1.6 mM 2,3-dimercapto-1-propanol for 1 hour. Ang I generated was determined by radioimmunoassay.4 According to conventional nomenclature, the renin enzymatic activity assayed under this condition is termed "plasma renin concentration" (PRC).

Trypsin Activation

Trypsin ( Worthington Biochemical Corp., Freehold, N.J.) was coupled to CNBr-activated Sepharose 4B (Pharmacia Fine Chemicals, Piscataway, N.J.) at 20 mg/ml of packed gel.23 For prorenin activation, the trypsin-Sepharose (0–250 μl packed volume per ml) was added to the sample and rocked at 4°C for up to 6 hours. The trypsin-Sepharose was removed by brief centrifugation, and any residual trypsin was inhibited by the addition of 0.3 mg/ml soybean trypsin inhibitor, 0.06 mg/ml aprotinin, and 0.4 mM phenylmethylsulfonyl fluoride. The resultant renin activity was then determined as described above.

Direct Radioimmunoassay

The renin concentration was directly determined by a solid-phase radioimmunoassay according to Heusser et al19 with modification. Briefly, microtiter plates (96-well) (Dynatech Laboratories, Alexandria, Va.) were coated with 200 μl of an anti-renin monoclonal antibody (coating antibody: 10 μg/ml R3-27-6 or R2-1-21, or 50 μg/ml R3-36-16 as the developing antibody in all three cases). The assay with Rl-20-5 contained a wide range of prorenin levels (determined by trypsin activation) was added into tubes containing 50–100 μl aliquots of specific antibody Sepharose or BSA Sepharose and were washed three times with PBS, and the bound radioactivity quantitated. Antibodies were iodinated by the chloramine T method.24

Kinetics of Renin Inhibition by Monoclonal Antibody

The purified human kidney renin (25 pM) with or without Rl-20-5 (70 nM) or the competitive inhibitor CGP 29827 (1 nM) was incubated with hog (1.0–500 μM) tetradecapeptide (TDP) substrate in 0.1 M phosphate buffer containing 1% human serum albumin, pH 7.0. The reaction was performed at 37°C, and the incubation was stopped after 1 hour by adding an excess of cold buffer and transferring the tubes into ice. The Ang I generated was measured by radioimmunoassay as described previously.25 The concentration of the antibody was selected to produce a similar inhibition of Ang I production as 1 nM CGP 29287 at a concentration of TDP of 15 μM. The kinetic constants of the enzymatic reaction were determined for each curve by applying the Michaelis-Menten equation to the raw data. The dose–response curves were also forced to share common parameters and were analyzed with the ALLFIT program of De Lean et al26 as in Reference 20. To examine whether R1-20-5 and CGP 29287 bind at the same site, a preincubation of a constant amount of renin (25 pM) was performed for 1 hour at 37°C before adding the substrate (15 μM) and the test substance. The reaction was then pursued for 1 hour as usual.

CGP 29287

The CGP 29287, which was synthesized in the laboratories of CIBA-GEIGY (Dr. B. Riniker and Dr. P. Bühlmayer), is an analogue of the amino acid sequence of human angiotensinogen.21 It contains statine, the unusual amino acid from pepstatin, as a transition state mimic and has C-terminal protecting groups. It is a potent and specific competitive inhibitor of primate renin.25

Immunofinity Chromatography

BSA and monoclonal antibodies R1-20-5 and R3-47-10 were separately coupled to CNBr-activated Sepharose 4B (Pharmacia Fine Chemicals). The binding capacities of the antibody Sepharose column for active renin were 5.0×10^5 and 5.5×10^5 ng Ang I/hr/ml of gel, respectively.

Recombinant prorenin (before or after trypsin activation) was added into tubes containing 50-μl aliquots of specific antibody Sepharose or BSA Sepharose and was rocked in the cold room for 5 hours. After centrifugation and removal of the Sepharose, the enzymatic activity of unbound recombinant renin remaining in the supernatant was determined.

Results

In these current studies, three sources of human renin (Table 1) with a wide range of prorenin levels (determined by trypsin activation) were used. Samples with high prorenin content (plasma KA and recombinant prorenin) were assayed by the direct radioimmunoassay using R1-20-5 or R3-27-6 or R2-1-21 as the coating antibody (with R3-36-16 as the developing antibody in all three cases). The assay with R1-20-5 underestimated consistently the total renin concentrations in the samples with high prorenin levels as compared with that with R3-27-6 or R2-1-21 (Table 1). In contrast, assays of HKAS, which contained almost exclusively active renin, yielded similar renin concentrations regardless of the coating antibody used. These results suggested that R1-20-5 might not recognize prorenin. To test this hypothesis, samples were treated with trypsin before the direct radioimmunoassay. This resulted in a dramatic increase of R1-20-5 immunoreactivity in the recombinant prorenin (Table 1). This increase in immunoreactivity paralleled the increase in
TABLE 1. Human Prorenin Levels

<table>
<thead>
<tr>
<th>Prorenin sources</th>
<th>% Prorenin</th>
<th>Immunoreactivity (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>R1-20-5</td>
</tr>
<tr>
<td>Partially purified renal renin</td>
<td>0-&lt;5%</td>
<td>38.5</td>
</tr>
<tr>
<td>(HKAS)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Plasma KA</td>
<td>85-90%</td>
<td>12.2</td>
</tr>
<tr>
<td>Recombinant prorenin</td>
<td>95-100%</td>
<td>5.5</td>
</tr>
<tr>
<td>Activated recombinant prorenin</td>
<td>0-&lt;5%</td>
<td>110.1</td>
</tr>
</tbody>
</table>

Three sources of renin were assayed for angiotensin I generating activity before and after activation of prorenin with trypsin. The percentage of prorenin was calculated as

\[
\frac{\text{Total renin}-\text{active renin}}{\text{Total renin}} \times 100\%
\]

These samples were also assayed for immunoreactivity (ng/ml) using the indicated antibodies as coating antibodies and R3-36-16 as the radiolabeled developing antibody.

renin activity in response to trypsin treatment. In contrast, no change in immunoreactivity of HKAS toward R1-20-5 was observed after trypsin treatment, nor was there a change in the immunoreactivity of any of the samples to the other two antibodies. Indeed, after trypsin activation, the three coating antibodies yielded similar results for the four samples.

To characterize further the antibody R1-20-5 for active renin, we coupled two different monoclonal antibodies to CNBr-activated Sepharose. Chromatography of recombinant prorenin before or after trypsin activation on R3-47-10-2-Sepharose showed that this antibody column would bind and retain prorenin (Figure 1A) equally as well as activated renin (Figure 1B). In contrast, R1-20-5-Sepharose bound activated renin to a much greater extent (Figure 1B) than prorenin (Figure 1A), suggesting that this antibody is highly selective for active renin or trypsin-activated prorenin but has a weaker affinity for native prorenin.

Kinetic studies showed that R1-20-5 is a competitive inhibitor similar to the peptidic transition state analogue CGP 29287 as analyzed using the Michaelis-Menten equation or constraining the data with the ALLFIT program (Table 2, Figure 2). The maximum rate \( (V_{\text{max}}) \) remained unchanged, whereas the Michaelis constant \( (K_a) \) increased threefold to fourfold, respectively. It was, therefore, of interest to analyze whether R1-20-5 competed with CGP 29287 for the same binding site. Preincubation of renin with R1-20-5 or CGP 29287 did not affect the subsequent inhibitory potency of the renin inhibitor or of the monoclonal antibody, respectively (Table 3). The combination of R1-20-5 with CGP 29287 produced a slight but significantly greater effect than that obtained with the two separately. These data suggest that R1-20-5 is specific for the active site of renin. We have shown previously that R3-36-16 induced an enhanced formation of Ang I from the reaction of renin with the synthetic substrate TDP. This effect was inhibited by CGP 29287. In the present study we examined whether R1-20-5 would also inhibit this reaction. Our results showed that preincubation of renin with R1-20-5 resulted in a twofold increase in Ang I production from TDP that was abolished by R1-20-5. This effect is similar to that of the transition state analogue CGP 29287, demonstrating further that R1-20-5 is directed against the active site. Furthermore, the data indicate that R1-20-5 and R3-36-16 have different binding sites and that there are no steric effects between them.

The selective binding of active renin to R1-20-5 suggested that this antibody might be useful for the direct radioimmunoassay determination of active renin. We modified our previous RIA condition to increase the sensitivity of the assay for active renin by using R3-
### TABLE 2. Kinetic Constants of the Enzymatic Reaction

<table>
<thead>
<tr>
<th>Preincubation</th>
<th>Michaelis-Menten equation</th>
<th>ALLFIT analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>$K_m = 20.3 \pm 2.8$</td>
<td>$V_{max} = 1,330 \pm 46.3$</td>
</tr>
<tr>
<td>CGP 29287</td>
<td>$K_m = 61.9 \pm 12.4$</td>
<td>$V_{max} = 1,259 \pm 80.3$</td>
</tr>
<tr>
<td>R1-20-5</td>
<td>$K_m = 62.9 \pm 8.4$</td>
<td>$V_{max} = 1,183 \pm 50.2$</td>
</tr>
</tbody>
</table>

Values are mean±SEM (n=9). Effect of R1-20-5 or CGP 29287 on the Michaelis constant ($K_m$) and maximum rate ($V_{max}$) of the enzymatic reaction using the Michaelis-Menten equation or the ALLFIT analysis. The means (nine points per curve) were used to calculate the constants.

36-16 as a coating antibody and R1-20-5 as the developing antibody. The sensitivity of the assay (Figure 3) is 10 pg/ml and the interassay and intra-assay coefficients of variation were 7% and 9%, respectively.

Demonstration of the potential usefulness of this assay is shown in Figure 4. Human plasma samples from normal volunteers on random diets were collected and assayed for renin enzymatic activity, trypsin activatable renin activity, and for immunoreactivity as determined by the two assays using either R1-20-5 or R3-27-6 in combination with R3-36-16 as coating antibody. Trypsin activation resulted in an increase in renin enzymatic activity (Figure 4A). Similarly, trypsin activation resulted in a parallel increase in the R1-20-5 immunoreactivity in the samples (Figure 4B) but had no effect on R3-27-6 immunoreactivity (Figure 4C). Overactivation with trypsin resulted in a decrease in immunoreactivity to both antibodies as well as a decrease in renin enzymatic activity. At all degrees of activation, the increase in immunoreactivity to R1-20-5 paralleled the increase in renin enzymatic activity as demonstrated (Figure 5) by plotting Ang I generation versus R1-20-5 immunoreactivity, which demonstrated a significant correlation ($r=0.96, p<0.001$). As noted in Figure 5, the direct radioimmunoassay for active renin was unable to detect renin in plasma samples with enzymatic activity of less than 6.5 ng Ang I/ml/hr.

**Discussion**

The amino acid residues of renin's active site are completely conserved as in other aspartyl proteases. However, there is evidence for species specificity of renin for angiotensinogen. Three-dimensional molecular modeling of human renin predicts that renin's active site is derived in a cleft formed by association of two near-symmetrical lobes. The center of this deep binding cleft contains two catalytically important aspartic acid residues, Asp 38 and Asp 226. Overlying this cleft is a flap (residues 75 to 85) that is unique for renin. The edges of the cleft consist of basic amino acid residues distinctive from other aspartyl proteases. The edges of the cleft and the flap are thought to be involved in the substrate and species specificity.
Our study demonstrates that the monoclonal antibody R1-20-5 selectively binds human active renin but not prorenin. In contrast to R3-36-16 and R3-47-10, which noncompetitively stimulate or inhibit the production of Ang I from TDP-renin substrate, R1-20-5 is a competitive inhibitor of renin similar to a peptidic transition state analogue, CGP 29287. Both of these competitive inhibitors combine with renin in a manner that prevents substrate binding. Therefore, the antibody and substrate may compete for the same binding site. However, our data do not exclude the possibility that R1-20-5 may cause a conformational change in the enzyme that distorts or masks the substrate binding site. Indeed, the size of the antibody is larger than that of the synthetic substrate TDP. However, the competition observed between R1-20-5 and CGP 29287 suggests that R1-20-5 binds near or within the active site. The interaction of R1-20-5 with active site is further suggested by direct binding studies in which a competition between CGP 2987 and R1-20-5 was observed (data not shown).

Our previous data by direct antigen-antibody binding with a radioimmunometric assay showed that R1-20-5 and R3-27-6 cross-inhibited each other. On the other hand, R1-20-5 and R3-36-16 did not interfere with each other's binding to renin molecule. Similarly, no interference in the binding assay was seen between R3-27-6 and R3-36-16. Taken together, these data suggest that R1-20-5 recognizes an epitope that is in tertiary structure close but different to the epitope recognized by R3-27-6. On the other hand, R1-20-5 and R3-36-16 have different binding sites that are not close in tertiary structure. These binding characteristics should shed some light onto the binding site of R1-20-5. Our antibody R1-20-5 differs from the monoclonal antibody 4G1 reported by Galen et al; 4G1 is not directed against the active site. Data showed that 4G1 bound active renin preferentially over prorenin. Furthermore, 4G1 inhibited renin enzymatic activity in a noncompetitive manner, whereas R1-20-5 is a competitive inhibitor.

We investigated the possible use of this antibody in a radioimmunometric assay for human active renin. We used a sandwich technique rather than the more conventional competitive binding approach. Although the latter approach is thought to be very specific, the fact that the sandwich assay depends on the binding of two separate monoclonal antibodies, neither of which cross-react with other human aspartyl proteases guarantees a high degree of specificity in our assay. The sensitivity of our assay (10 pg/ml) is slightly better but comparable to that of the previously reported immuno-

| TABLE 3. Effect of Various Combinations of Antibodies and Inhibitors on Renin Activity |
|-----------------------------------------|-----------------|-----------------|
| Preincubation (60 min) | Test (60 min) | Angiotensin I produced* (ng/ml/hr) |
| Control | Buffer | 588±7.6 |
| R1-20-5 | Buffer | 234±5.1 |
| | CGP 29287 | 200±4.5* |
| CGP 29287 | Buffer | 279±10.2 |
| | R1-20-5 | 192±4.9* |
| R3-36-16 | Buffer | 1,143±28.6 |
| | R1-20-5 | 88±3.9* |

Values are mean±SEM (n=8). Effect of sequential combination of the antibodies and CGP 29287 with 15 μM tetradecapeptide and 25 pM renin. The test substance was added after 1 hour preincubation. Angiotensin I was measured after the second hour of incubation. *p<0.05 as compared with the corresponding control in buffer (Dunnett's test).
metric assay using monoclonal antibody 4G131-33 (sensitivity 16–20 pg/ml). The volumes of plasma needed and the times of incubation are also comparable. Thus, using a simple radioimmunoassay we can determine the concentration of inactive renin as the difference in immunometric assay measured by antibody R1-20-5 (active renin) and by antibody R2-27-5 (total renin) without artificial activation of plasma by a variety of complicated experimental techniques. However, neither assay at this point is useful for the detection of active renin in plasma samples with very low renin activity.

In summary, we have obtained and characterized a monoclonal antibody (R1-20-5) that binds specifically active human renin. This antibody reacts with the active site of renin. It behaves as a competitive inhibitor. We have established a sensitive radioimmunoassay for active human renin that may be suitable for the determination of plasma active renin concentration. Furthermore, this antibody may be useful as a molecular probe for studies of renin’s active site.

Acknowledgments

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References


Figure 5. Scatter plot shows relation between plasma immunoreactive active renin and renin enzymatic activity or "plasma renin concentration" (PRC) in various human plasma samples. AI, angiotensin I.
Characterization of a monoclonal antibody specific for human active renin.
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