Measurement of \(\alpha\)-Thiol Proteinase Inhibitors in Human Serum by Laser Nephelometry

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SUMMARY The laser nephrometric quantitation of \(\alpha\)-thiol proteinase inhibitor (\(\alpha\)-TPI) in human serum was established by using a monospecific rabbit antiserum against human \(\alpha_2\)-TPI. The reaction was performed by incubating 100\(\mu\)l of 30-fold diluted serum and 200\(\mu\)l of 5-fold diluted antiserum in a cell at room temperature for 60 min. The accuracy of the method was determined to be 4.3% for the within-run and 8.7% for the between-run. The average \(\alpha\)-TPI level in sera from normal healthy individuals was 425\(\pm\)97 mg/l (mean\(\pm\)S.D., n=67). There was no statistical correlation between sex and age.

We observed a rough correlation between the \(\alpha\)-TPI level determined by laser nephelometry and by a single radial immunodiffusion (\(r=0.53, p<0.05\)), and a strict correlation between laser nephelometry and rocket immunodiffusion (\(r=0.75, p<0.01\)). We observed the remarkable changes of \(\alpha\)-TPI in sera from the patients with chronic renal failure, between before and after receiving hemodialysis. The patients with inflammatory diseases tended to show a lower level of serum \(\alpha\)-TPI within the normal range. We concluded that the laser nephelometry provides a more simple, rapid and sensitive procedure to determine serum \(\alpha\)-TPI, comparing with other immunochemical methods.

Introduction

It is well known that human plasma and serum contain the proteinase inhibitors specifically for thiol proteinases such as papain, ficin, cathepsins and calpain. \(\alpha\)-Thiol proteinase inhibitors (\(\alpha\)-TPI) are heterogenous glycoprotein consisting from at least three forms which differ in molecular size and isoelectric point. It was found that two of them migrate in \(\alpha_2\)-region (\(\alpha_2\)-TPI) and one in \(\alpha_1\)-region (\(\alpha_1\)-TPI) by immunoelectrophoresis with the same antigenicity. The physiological function of \(\alpha\)-TPI is still unknown, although its probable role has been suggested to be the regulators for thiol proteinases participating in the inflammatory processes. The \(\alpha\)-TPI level in human serum was preliminarily measured by rocket elec-
troimmunoassay\textsuperscript{3,5,10} and inhibitory assay to ficin\textsuperscript{11,12}. However, these procedures are not applicable for mass screening, because they need some complicated processes. It is necessary to establish a simple, reliable and reproducible method to determine the $\alpha$-TPI level in order to interpret the relationship between some pathological condition diseases and the levels of $\alpha$-TPI.

In this communication we developed a simple, rapid and sensitive method to determine the $\alpha$-TPI level in human serum by laser nephelometry using a monospecific rabbit antiserum against $\alpha_2$-TPI.

**Materials and Methods**

**Materials**

Human plasma and serum samples from healthy individuals as well as patients were obtained from central clinical laboratory in the hospital. Papain and ficin were purchased from Sigma Co. All chemicals used in the present study were reagent grade.

**Preparation of antigen and antibody**

Antigen, $\alpha_2$-TPI, from human serum was fractionated according to the method of Järvinen\textsuperscript{4} and was eluted from the Mono Q column (Pharmacia F.P.L.C. system) on the final step with a linear gradient of 0.05-0.25 M NaCl in 20mM Tris-HCl buffer, pH 8.0. The preparation showed a single band with molecular weight of 57,000 by SDS-electrophoresis. The concentration of $\alpha_2$-TPI solution was determined by the method of Lowry et al., using bovine serum albumin (Sigma) as a standard\textsuperscript{13}. Antiserum against $\alpha$-TPI was prepared by injecting the purified $\alpha_2$-TPI into two rabbits. $\alpha_2$-TPI was emulsified with an equal volume of Freund's complete adjuvant and 1 mg was of this emulsion injected into rabbit via intracutaneous route, four times at 7-day intervals. The rabbits were bled 7 days after the final injection.

**Laser nephelometry**

The quantitative determination of $\alpha$-TPI was performed with a Behring Laser Nephelometer, Module I and II (Hoechst Japan). The samples were diluted in the filtrated saline. After mixing with 100 $\mu$l of all samples and 200 $\mu$l of antiserum in a cell, the scattering light intensity by immunological reaction was measured in voltage when the reaction reached the end-point at room temperature (25°C). A secondary $\alpha$-TPI standard consisting of pooled serum was carefully calibrated against the pure $\alpha_2$-TPI as primary standard. The calibration curve was recorded in each measurements.

**Other immunochemical methods**

The single radial immunodiffusion for quantitation of $\alpha$-TPI was performed according to the method by Mancini et al.\textsuperscript{14}, using glass plate, 1.2% agarose (Agarose L, Behring Institute) in 0.04 M veronal buffer, pH 8.6, and 3.0% antiserum. The volume of 5-fold diluted sample was 5 $\mu$l in 2.5 mm diameter hole in agarose plate. After diffusion for 48h at 37°C in a humid chamber, the area of the precipitin ring was measured. The rocket electroimmunoassay was carried out by the method as described by Laurell\textsuperscript{15}, using 1.0% agarose in 0.04 M veronal buffer, pH 8.6, and 2.0% antiserum.

**Inhibitory assay**

Inhibition of papain was measured with casein as substrate according to the procedure described by Arnon\textsuperscript{16} for the routine assay. The inhibitory assay for
the determination of the α-TPI level in serum was practiced according to the method of Minakata et al.11).  

**Results**

**Specificity of antiserum**

The antiserum against α2-TPI yielded the pattern of a complete fused precipitin line with serum and α-TPIs, but it did not distinguish between α1- and α2-TPI. This result indicates that it is possible to measure the total α-TPI level in serum with an antiserum against α2-TPI.

**Measurement of α-TPI in serum by laser nephelometry**

We examined the optimum conditions to determine the α-TPI level in serum by laser nephelometry. The scattering light intensity by immunological reaction reached the constant voltages at 60 min after mixing the samples and antiserum as shown in Fig. 1. The intensities of the samples continuously diluted α2-TPI and standard serum depended on the concentrations of antiserum that also continuously diluted. When 5-fold diluted anti-

![Fig. 1 Time dependency of the scattering light intensities by immunological reaction after mixing with samples and 5-fold diluted antiserum.](image)

![Fig. 2 The scattering light intensities by immunological reaction of α-TPI in continuously diluted serum (○—○), and continuously diluted α2-TPI (○—○) with 5-fold diluted antiserum.](image)

![Fig. 3 A typical calibration curve for the determination of the serum α-TPI level with standard deviations from nine measurements.](image)
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Table I Precisions of laser nephelometry for determination of $\alpha$-TPI level in serum.

<table>
<thead>
<tr>
<th>Assay</th>
<th>n</th>
<th>Mean $\pm$ D. (mg/l)</th>
<th>C. V. (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Within-run</td>
<td>10*</td>
<td>804$\pm$19</td>
<td>2.4</td>
</tr>
<tr>
<td></td>
<td>8**</td>
<td>576$\pm$24</td>
<td>5.8</td>
</tr>
<tr>
<td>Between-run</td>
<td>10</td>
<td>496$\pm$23</td>
<td>4.7</td>
</tr>
<tr>
<td></td>
<td>9**</td>
<td>593$\pm$52</td>
<td>8.7</td>
</tr>
</tbody>
</table>

*Serum from sepsis patient, **serum from a healthy individual.

Table II $\alpha$-TPI levels in sera from healthy individuals determined by laser nephelometry and other methods.

<table>
<thead>
<tr>
<th>Method</th>
<th>n</th>
<th>Mean $\pm$ S. D.</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Laser nephelometry (mg/l)</td>
<td>67</td>
<td>425$\pm$97</td>
<td>328–522</td>
</tr>
<tr>
<td>Men</td>
<td>31</td>
<td>415$\pm$94</td>
<td>322–510</td>
</tr>
<tr>
<td>Women</td>
<td>36</td>
<td>444$\pm$76</td>
<td>365–517</td>
</tr>
<tr>
<td>SRID (mg/l)</td>
<td>67</td>
<td>341$\pm$40</td>
<td>301–381</td>
</tr>
<tr>
<td>Electroimmunoassay (mg/l)</td>
<td>67</td>
<td>397$\pm$26</td>
<td>37l–423</td>
</tr>
<tr>
<td>Inhibitory assay (unit/ml)</td>
<td>67</td>
<td>0.36$\pm$0.08</td>
<td>0.28–0.44</td>
</tr>
</tbody>
</table>

*SRID, single radial immunodiffusion.

serum was used, there was no variation of intensities between the $\alpha_2$-TPI and standard serum except the high concentration of them (Fig. 2). Thus the routine measurement was performed at 60 min after mixing 100 $\mu$l of 30-fold diluted serum and 200 $\mu$l of 5-fold diluted antiserum. Figure 3 shows a typical calibration curve within the range of 8–60 mg/l $\alpha$-TPI in standard serum. The variation (n=9) at points measured on calibration curve shows bars in the figure, indicating that it can be used to determine the $\alpha$-TPI level in serum. The within- and between-run precisions of this method are summarized in Table I. The values in coefficient of variation indicated the satisfactory reproducibility of this method for the determination of the $\alpha$-TPI level. Furthermore, the recovery of the pure $\alpha_2$-TPI added into serum was almost perfect. Other remark for this method is to clear the sample solution. If necessary, they were filtrated with miliporefilter in the present study.

The $\alpha$-TPI level in sera from healthy individuals is summarized in Table II, in which the differences of mean values between sexes as well as among ages seem to be not statistically significant (p>0.05).

Comparison between laser nephelometry and other methods

The $\alpha$-TPI levels determined by other three methods are given in Table II, in which the mean levels of $\alpha$-TPI determined by laser nephelometry show a higher estimate and its range showed a wider distribution compared with those determined by the two immunochemical methods. It was possible to determine the $\alpha$-TPI levels in serum with the larger
diluted sample, up to 100-fold, by laser nephelometry (Fig. 2), although the small amount of serum, 5 μl, was enough to determine it by three immunochemical methods. The detectable minimum amounts of α-TPI in serum was 8.5 mg/l by laser nephelometry, indicating that this method was sensitive by more than 100 times compared with other two methods. Furthermore, the α-TPI levels determined by laser nephelometry roughly correlated with those done by single radial immunodiffusion (r = 0.53, p<0.05) and rocket electroimmunoassay (r = 0.73, p<0.01), but not well with that by inhibitory assay method (r = 0.34, p<0.10). These results indicate that the determination of α-TPI by laser nephelometry is more sensitive and usefull than other methods.

Measurement of α-TPI in clinical samples

Laser nephelometry method was applied to the determination of α-TPI levels in sera from patients with chronic renal failure as well as CRP positive sera (2+ ~ 6+ by capillary method) from patients suffering from bacterial infection (Fig. 4). The α-TPI levels widely distributed in sera from patients with chronic renal failure, in which there was a significant difference in α-TPI level between before (481 ± 95 mg/l, n=18) and after (641 ± 134 mg/l, n=16) hemodialysis (p<0.01). On the other hand, the α-TPI levels in CRP positive sera were the lower one within normal range (372 ± 55 mg/l, n=32), but in some sera from patients suffering from serious sepsis, α-TPI levels is doubled compared with the normal.

Discussion

α-TPI is a dominant proteinase inhibitor in human plasma and serum which specifically inhibits thiol proteinases[17]. The biological method for the quantitative assay which is based on inhibitory effect to ficin was presented[11,12]. However, it is difficult to apply this method to determine the α-TPI levels in clinical samples, because the other kind of thiol proteinase inhibitor, which is a low molecular weight protein originated from spleen and liver, is present in serum from patients[10,18]. Other quantitative method by rocket electroimmunoassay is highly specific but time consuming and difficult to apply for long series of determination, keeping the same condition[5,10].

Laser nephelometry in the quantitative immunochemical method has been applied to the determination of several proteins in human plasma and serum[19].

Fig. 4 α-TPI levels in sera from : A ; healthy individuals, B ; patients with chronic renal failure, C ; patients with bacterial infection showing positive CRP. Open circles show the α-TPI levels in sera from three sepsis patients.
In the present study, we developed the method to determine the total α-TPI level in human serum by laser nephelometry. The result of immunodiffusion revealed that the rabbit antiserum against α₂-TPI identified all α-TPI molecular forms in human serum. We standardized the assay conditions with this monospecific antiserum. The developed method was more simple, rapid and sensitive than other immunochemical methods with respect to the procedures, precisions and recovery test. The defects of other immunochemical methods mentioned above may cause the weak correlations among the α-TPI levels determined by laser nephelometry and other methods. We applied the new method to determine the α-TPI levels in clinical samples from various patients. So far we detected the significant differences of the α-TPI levels in sera from patients suffering from chronic renal failure and bacterial infection as compared with that from healthy individuals. These results indicate that it is now possible to investigate the dynamics of α-TPI in acute and chronic diseases.

Recently, α-TPI was identified with plasma kininogens and/or its derivatives from the evidences of immunological properties, inhibitory profiles to proteinases and amino acid sequences²⁰⁻²². Although more detailed studies are necessary, this information suggests that the α-TPI level in serum determined by this method may be able to indicate the kininogen level in plasma.

Acknowledgements

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References


