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## Increased urine level of amino-terminal peptide derivatives of type III procollagen in patients with liver diseases.

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## Abstract

The amino-terminal peptides of type III procollagen (PIIIP) in the urine of 40 patients with various liver diseases were determined with a commercial radioimmunoassay kit. The level of urinary PIIIP (uPIIIP) was correlated well with serum PIIIP (sPIIIP) in 9 patients, the coefficient of correlation being  $r = 0.836$  ( $p$  less than 0.01) and the regression line being  $y = 1.42x + 24$ . Urinary PIIIP consisted of at least 4 different molecular species with molecular weights of 49 k, 18 k, 10 k and 4.6 k as estimated by column chromatography on Sephadex G-100. Furthermore, uPIIIP was found to be significantly elevated in acute hepatitis, chronic hepatitis, liver cirrhosis, hepatocellular carcinoma and other liver diseases, in which the elevation of sPIIIP has been reported by others. The mean values  $\pm$  standard deviations of uPIIIP were 44.0  $\pm$  32.0, 60.4  $\pm$  32.0, 62.0  $\pm$  46.5, 53.0  $\pm$  27.1 and 48.1  $\pm$  22.8 ng/ml for the respective liver diseases, and 13.2  $\pm$  4.5 for the non-hepatic disease group.

**KEYWORDS:** type III collagen, amino-terminal peptide, urinary peptide, molecular species, liver diseases

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## Increased Urine Level of Amino-Terminal Peptide Derivatives of Type III Procollagen in Patients with Liver Diseases

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The amino-terminal peptides of type III procollagen (PIII) in the urine of 40 patients with various liver diseases were determined with a commercial radioimmunoassay kit. The level of urinary PIII (uPIII) was correlated well with serum PIII (sPIII) in 9 patients, the coefficient of correlation being  $r = 0.836$  ( $p < 0.01$ ) and the regression line being  $y = 1.42x + 24$ . Urinary PIII consisted of at least 4 different molecular species with molecular weights of 49 k, 18 k, 10 k and 4.6 k as estimated by column chromatography on Sephadex G-100. Furthermore, uPIII was found to be significantly elevated in acute hepatitis, chronic hepatitis, liver cirrhosis, hepatocellular carcinoma and other liver diseases, in which the elevation of sPIII has been reported by others. The mean values  $\pm$  standard deviations of uPIII were  $44.0 \pm 32.0$ ,  $60.4 \pm 32.0$ ,  $62.0 \pm 46.5$ ,  $53.0 \pm 27.1$  and  $48.1 \pm 22.8$  ng/ml for the respective liver diseases, and  $13.2 \pm 4.5$  for the non-hepatic disease group.

*Key words* : type III collagen, amino-terminal peptide, urinary peptide, molecular species, liver diseases

One of the main features of liver fibrosis is the deposition of excess collagen (1). Type III collagen is a major collagen type deposited in such fibrotic liver and is also a major constituent of the extracellular matrix of normal liver (2, 3). A commercial radioimmunoassay (RIA) kit (Behringwerke) for serum PIII (sPIII) originally developed by Rhode *et al.* (4), is widely used for monitoring the synthetic activity of type III collagen. Studies in patients with various inflammatory liver diseases have shown that sPIII is a specific marker of fibroplasia associated with liver injury (4-8). A marked increase in sPIII was found in malignant liver diseases also (9), though the pathogenic mechanism is not well known. Studies

of molecular species of PIII in various body fluids have revealed that the major constituents of sPIII and urinary PIII (uPIII) are polypeptides similar to Cor 1-3 and Cor 1, respectively (4), which are generated by bacterial-collagenase digestion (10) of bovine amino-terminal peptides of type III collagen.

In this paper we measured PIII in the urine of patients with various liver diseases in comparison with serum PIII to verify their relationship and also analyzed molecular species of uPIII.

### Materials and Methods

*Subjects.* Forty patients with various dis-

eases aged from 34 to 65 who were admitted to the First Department of Internal Medicine, Okayama University Medical School Hospital from September 1982 to August 1983, were investigated. Thirty-one out of the 40 patients had liver diseases: 4 had acute hepatitis, 8 had chronic hepatitis, 6 had compensated liver cirrhosis, 6 had hepatocellular carcinoma and 7 had other liver diseases, including fatty liver, idiopathic portal hypertension, primary biliary cirrhosis and alcoholic liver injury. The remaining nine were the patients with non-hepatic diseases, including 3 with diabetes mellitus, 3 with essential hypertension, 2 with gall stone, and one with nephrosis, all of them having normal liver function tests. Subjects with chronic hepatitis and liver cirrhosis were all diagnosed histologically with a biopsied specimen obtained by the peritoneoscopy. The other liver diseases were diagnosed by means of laboratory tests and imaging examinations. All of the patients examined, except the one with nephrosis, were diagnosed free of kidney dysfunction.

**PfPpP determination.** PfPpP were determined by the 50% intercept method with an RIA kit (11), a Behringwerke product. The assay is based on the principle that PfPpP in the sample competitively inhibit the formation of an immune complex between  $^{125}\text{I}$ -labeled bovine Cor 1-3 and anti bovine Cor 1-3 rabbit antibody. Three dilutions, 1 : 5, 1 : 20 and 1 : 50 for serum samples and 1 : 2, 1 : 5 and 1 : 20 for urine samples, were used to obtain an inhibition curve. The assays were performed in duplicate.

**Molecular species of uPfPpP.** Sixteen liters of urine collected from 4 patients with liver cirrhosis was filtered with filter paper and saturated with ammonium sulfate. The precipitate was collected by centrifugation at  $10,000 \times g$  for 1 h, dialyzed against 0.01 M Tris-HCl buffer, pH 8.0, containing 0.15 M NaCl (TBS) using a Spectra/Por 6 dialysis tubing with molecular weight cutoff at 3,500 daltons (Spectro/Por 6, Spectrum Inc.) and lyophilized. The sample was redissolved in TBS and applied to a column of Sephadex G-50 ( $10 \times 1,200$  mm) equilibrated with TBS. The high molecular weight fraction was lyophilized and applied to a column of Sephadex G-100 equilibrated with TBS. Molecular weight markers were blue dextran, bovine serum albumin, cytochrome C and glucose.

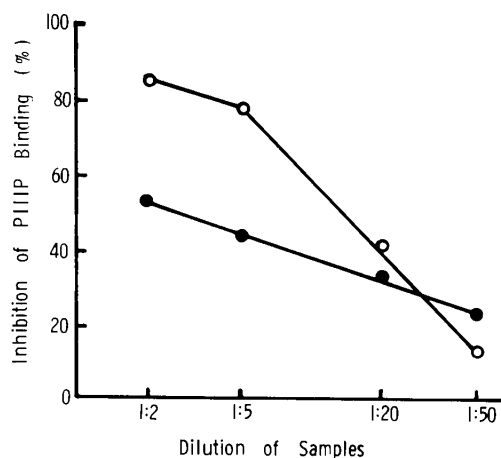
**Protein determination.** Protein was determined by the microbiuret method (12).

**Laboratory tests.** Tests performed were: Concentrations of serum bilirubin, albumin, gammaglobulin, CRP, blood urea N, creatinine and urinary protein; activities of serum aspartate aminotransferase (EC 2. 6. 1. 1), alanine amino transferase (EC 2. 6. 1. 2), and alkaline phosphatase (EC 3. 1. 3. 1); examinations of urine volume and urinary sediments.

**Statistic analysis.** Statistic analysis was performed by student's *t* test.

## Results

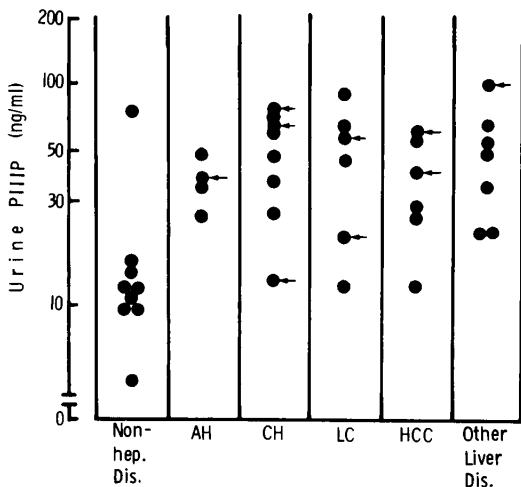
**Urinary PfPpP in liver diseases.** PfPpP were measured in the serum and urine which were collected in the early morning from one patient with liver cirrhosis. Titration curves were dose-responsively linear, at a dilution range of 1 : 5 to 1 : 50 for the serum and 1 : 2 to 1 : 50 for the urine. The inclination of the slope was smaller for the urine than for the serum, but the inhibition



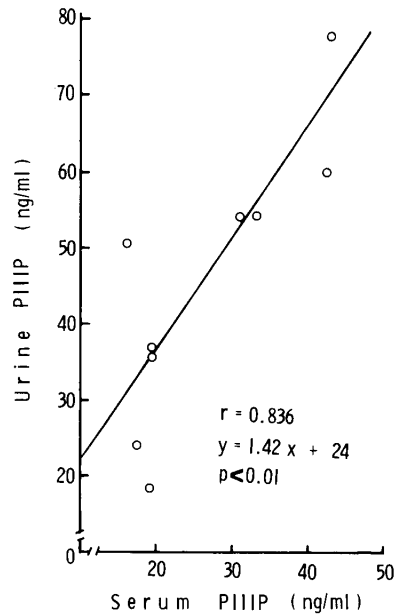
**Fig. 1** Titration curves of serum and urinary PfPpP. PfPpP in serum, ○—○, and urine, ●—●, of a patient with cirrhosis were assayed by RIA. The inhibition percent represents the activity of  $200 \mu\text{l}$  of samples to reduce radioactivity recovered in the immune complex formed by  $^{125}\text{I}$ -labeled PfPpP (bovine Cor 1-3) and anti PfPpP rabbit serum followed by the second antibody. The activity of fifty percent inhibition is equivalent to 1.36 ng/ml of standard PfPpP (11).

percentage was high enough to adapt to the 50% intercept method used for serum (Fig. 1). Thus, the above dilution range was used for determining uPIIIP in the other patients with various liver diseases. The mean values  $\pm$  standard deviations of the uPIIIP levels in the acute hepatitis, chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma patients were  $44.0 \pm 32.0$ ,  $60.4 \pm 32.0$ ,  $62.0 \pm 46.5$ ,  $53.0 \pm 27.1$  and  $48.1 \pm 22.8$  ng/ml, respectively. In the non-hepatic disease group, the uPIIIP level was  $13.2 \pm 4.5$  ng/ml as shown in Fig. 2. The values for liver diseases were significantly higher than that for non-hepatic diseases, except for the nephrosis case which showed a high value, 100 ng/ml.

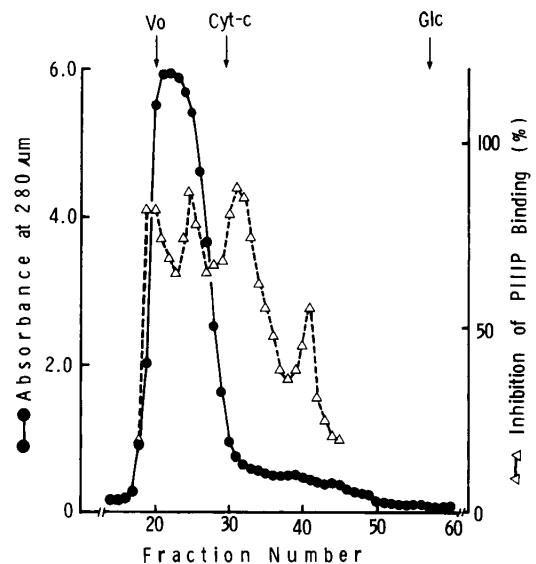
*Correlation between uPIIIP and sPIIIP.* Nine out of 30 liver disease patients (indicated by arrows in Fig. 2) were examined for sPIIIP also. Urinary PIIIIP correlated well with sPIIIP, the regression line for uPIIIP being  $y = 1.42x + 24$ ,  $r = 0.836$  ( $p < 0.01$ , Fig. 3). No significant relationship



**Fig. 2** Urinary PIIIIP in various liver diseases. Levels of urinary PIIIIP were measured in non-hepatic disease, (Non-hep. Dis.), acute hepatitis (AH), chronic hepatitis (CH), liver cirrhosis (LC), hepatocellular carcinoma (HCC), and other liver diseases (Other Liver Dis.) patients.



**Fig. 3** Correlation between urinary PIIIIP and serum PIIIIP. Patients indicated by arrows in Fig. 2 were examined for serum PIIIIP and used in the correlation study.



**Fig. 4** Elution profile of uPIIIP on Sephadex G-50 column chromatography. The amount of uPIIIP was figured as the inhibition percent of 100  $\mu$ l of each fraction. Elution positions of the markers (Vo, blue dextran and bovine serum albumin; Cyt-c, cytochrome c; Glc, glucose) are indicated by arrows.

was found between uPIIP and any laboratory test. The high uPIIP value obtained for the nephrosis patient was thought to be related to the amount of urinary protein, though the correlation between the protein amount and uPIIP was not statistically significant. The other laboratory tests of the urine, including urine volume and sediments, did not show a significant relationship either.

*Molecular species of uPIIP.* Molecular species of urine PIIP in the urine pooled from 4 patients with liver cirrhosis were analyzed by column chromatography on Sephadex G-50. As shown in Fig. 4, four peaks of PIIPs at different elution positions were detected by the RIA. Molecular weights were over 30k, 18k, 10 k and 4.6 k for the peaks "a", "b", "c" and "d", respectively. The "a" fraction was further analyzed on Sephadex G-100, resulting in one major peak at a molecular weight of 49 k and two minor peaks at higher molecular weights.

## Discussion

Recent investigations on the metabolism of type III procollagen have shown that PIIP are released during the processing of type III collagen before deposition in the extracellular matrix and have suggested that the serum level of PIIP is a marker of the collagen biosynthetic activity (13). On the contrary, PIIP is not only a biosynthetic marker but also a marker of the inflammation, since it has been shown that PIIP arise during the stage of active inflammation from type III procollagen molecules which had been already deposited in the extracellular matrix (14). Many studies on patients with liver diseases have indicated that the PIIP level rises in parallel with the activity of both fibrosis and inflammation in the liver (4-8). An RIA developed by Rhode *et al.* (4) has

been used for measuring the serum level of PIIP in most of the previous clinical studies. It has been suggested that the RIA might be adaptable to the measurement of PIIP in the urine (4). Using the RIA, we measured PIIP in the urine collected in the early morning. The inhibition curve was linear even up to a two fold dilution and could be adapted to the 50% intercept method. Urea in the urine was expected to be high in concentration to loose linearity of the inhibition curve due to inhibition of immune complex formation, but such was not the case. As the urine collected during the day time could not be adapted to the 50% intercept method (data not shown), some enrichment of uPIIP is necessary to use the RIA (4). Thus, abnormal kidney function might affect the level of uPIIP as was seen in the case of nephrosis. The patients with various liver diseases examined in this study had normal kidney function and showed a correlation between uPIIP and sPIIP. The results indicate that the level of uPIIP reflects the level of sPIIP in liver diseases.

We analyzed the molecular species of uPIIP in the concentrated urine of cirrhotic patients and found the major component to have a molecular weight of 10 k as previously described (4). We found three other components with molecular weights of 49 k, 18 k, and 4.6 k. PIIP in the serum is known to consist of a 45 k-dalton peptide as the major component, which might correspond to Cor 1-3 (10), and two larger peptides (15) as minor components. The 49 k-dalton component we found might correspond to the Cor 1-3-like peptides. The 10 k-dalton peptide has been supposed to be a degradation product of the Cor 1-3-like peptides which correspond to Cor 1 (10). The other PIIP components have not previously observed in urine. However, these components might also be degradation products of Cor 1-3-like peptides, since they are smaller in mo-

lecular size and can be detected by an inhibition assay of the RIA. Further structural analysis of these peptides is necessary in order to clarify the degradation of serum PIII. Anyhow, the evidence presented in this report indicates that uPIII consist of 4 molecular species which might be different from those in serum. As mentioned above, sPIII were determined in patients with various liver diseases by many researchers and are known to be elevated in patients with acute hepatitis, chronic hepatitis, liver cirrhosis and hepatocellular carcinoma, which are usually associated with inflammation and/or fibrosis in the liver. We have shown that the PIII level in the urine of the patients with the same diseases was significantly elevated. From these results, we concluded that uPIII could not be used only as an alternate to sPIII in monitoring the synthetic activity of type III collagen but were possibly a marker of the degradation of PIII in relation to fibrosis and/or inflammation in the liver.

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