Purification of Human Procollagen Type I Carboxyl-Terminal Propeptide Cleaved As in Vivo from Procollagen and Used to Calibrate a Radioimmunoassay of the Propeptide

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We purified human procollagen type I carboxyl-terminal propeptide (PICP) that had been cleaved as in vivo from procollagen. PICP in serum-free medium from cultured human fetal fibroblasts was purified by thiophilic adsorption chromatography, low-pressure gel filtration, and HPLC gel filtration. The purity and homogeneity of the protein was verified by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Amino-terminal amino acid sequencing showed that the sequences of the α1 and α2 chains of this PICP were identical to those of the PICP produced in vivo. The monocOMPONENT PICP thus purified was used as calibrator in a simple equilibrium-type RIA of PICP with polyclonal antibodies raised in rabbits. The measuring range is 0.15–3.75 nmol/L, and the assay detection limit is 0.03 nmol/L. The within-run and total CVs are 2% and 4%, respectively. The reference interval for the plasma concentration of PICP in healthy women of ages >30 years is 0.36–1.44 nmol/L (geometrical mean 0.72 nmol/L, n = 154).

Indexing Terms: bone metabolism/proteins/chromatography, adsorption/chromatography, gel filtration/reference intervals/age-related effects/menopausal status

Type I collagen accounts for >90% of the nonmineral component of bone. It is synthesized intracellularly as a large molecule, type I procollagen, which contains amino- and carboxyl-terminal globular extensions (propeptides) (1). Before mature collagen fibrils are formed, these propeptides are cleaved from type I procollagen by specific extracellular tissue endopeptidases (2). Procollagen type I carboxyl-terminal propeptide (PICP) is a trimeric globular glycoprotein consisting of two α1(I) and one α2(I) polypeptide chains of 246 and 247 amino acid residues, respectively, with intra- and interchain disulfide bonds (3).\textsuperscript{4} PICP is released into the extracellular fluid and is found in blood, where it is a well-established biochemical marker of type I collagen synthesis (4).

In children the serum concentration of PICP reflects growth, and PICP measurements can be used to monitor normal and abnormal growth and the efficacy of treatments of abnormal growth (5), or to indicate early adverse effects on bone formation of (e.g.) treatment with glucocorticoids (6). In adults the serum concentration of PICP correlates with the rate of bone formation, as measured histomorphometrically (7), and deviates from normal in metabolic bone diseases (8). Reported RIAs of human PICP (9–11) all use PICP purified from procollagen isolated from the medium of various cultured fibroblasts and treated with bacterial collagenase. However, in the PICP isolated by these methods, the amino-terminal amino acid sequences of its component chains differ from those for PICP produced in vivo (11). Use of such PICP as calibrator in an immunoassay may lead to inaccurate results, owing to possible immunochemical differences between the assay calibrator and the analyte.

We report here a method of purifying a monocomponent preparation of intact human PICP cleaved as in vivo from procollagen (i.e., with amino-terminal sequences of its component polypeptide chains identical to those of PICP produced in vivo). PICP was purified directly from the medium of cultured human fetal fibroblasts, thereby avoiding the procollagen isolation step and subsequent treatment with bacterial collagenase. We also report the application of purified PICP as tracer and primary calibrator in an RIA of PICP. The assay is simple, reliable, and suitable for the further clinical and biochemical investigation of circulating PICP.

Materials and Methods

Materials

Shodex HPLC gel filtration column Protein WS-803 was purchased through Waters/Millipore (Milford, MA). Affi-T thiophilic agarose was purchased from Kem-En-Tec (Copenhagen, Denmark), Sephacryl S-300 high resolution from Pharmacia (Uppsala, Sweden), Sac-Cel solid-phase second antibody-coated cellulose suspension, donkey anti-rabbit serum from IDS (Boldon, UK), and \textsuperscript{125}I from Amersham International (Bucks, UK). Cell-free human amniotic fluid from amniocenteses was obtained from The Kennedy Institute (Glostrup, Denmark). Growth media, solutions, and buffers for cell culture were from Gibco BRL (through Life Technologies, Paisley, Scotland) and from Biological Industries (Kibbutz Beth Haemek, Israel). The chemicals used were of analytical grade or better from Sigma Chemical Co. (St. Louis, MO), Merck (Darmstadt, Germany), and Bio-Rad Labs. (Hemel Hempstead, UK). Amino-termi-
nal protein sequencing was performed on a 477A Protein Sequencer with an on-line 120A Analyzer (Applied Biosystems, Foster City, CA) and the manufacturers' recommended chemicals. Quantitative amino acid analysis was performed by the Picotag method (Waters/Milipore) with the manufacturers' recommended chemicals. Radioactivity was measured with an RT 100 solid-phase scintillation counter (Osteometer, Rødovre, Denmark). A commercial kit for measuring PICP was obtained from Orion Diagnostica, Turku, Finland.

Methods

Protein concentration and purity. During the purification of PICP the total protein concentration in various solutions was estimated either by absorbance at 280 nm, the Lowry method, or—if the solutions contained ammonium sulfate—the bicinchoninic acid procedure (12).

The protein concentration in the final preparation of purified PICP was quantified by the Picotag method. Amino acid analysis was performed by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS-PAGE) (14) with Coomassie Blue R-250 staining, and amino-terminal amino acid sequencing of protein bands from electrophorograms of SDS-PAGE gels (15).

Production of serum-free cell culture medium. Human fetal fibroblasts were cultured to confluence at 37°C in 5% (50 mL/L-enriched) humidified CO2 on petri dishes in an equimolar mixture of Dulbecco's modified Eagle's medium and Ham's F12, containing 100 mL/L fetal calf serum, 100 kIU/L penicillin, 100 mg/L streptomycin, 2 mmol/L L-glutamine, and 25 mmol/L HEPES. When the cells reached confluence, they were washed with phosphate-buffered saline and incubated for 24 or 48 h with the above-mentioned medium without fetal calf serum but with ascorbic acid added (50 mg/L). The confluent cells were then cultured for 2 days with medium containing fetal calf serum and then incubated with serum-free medium for a further 24 or 48 h; this cyclic treatment was repeated as many as eight times. The serum-free medium was collected, cells were removed by centrifugation, and bacteriostatic agents and protease inhibitors were added at the following final concentrations: 15 mmol/L sodium azide, 10 mmol/L EDTA, 1 mmol/L phenylmethylsulfonyl fluoride, 1 mmol/L N-ethylmaleimide, 1 mmol/L pepstatin A, and 0.1 mmol/L sodium iodoacetate.

Purification of intact PICP cleaved in vivo-like from procollagen. The serum-free medium was concentrated by ultrafiltration, dialyzed at 4°C against 50 mmol/L NH4HCO3, freeze-dried, and subjected to chromatography on a 2.5 × 8 cm column (for 500 mL of serum-free medium) of Affi-T thiophilic agarose (16). The column was eluted with 50 mmol/L sodium phosphate buffer, pH 7.5, containing the above-mentioned protease inhibitors at a gradient of 1.5–0.0 mol/L (NH4)2SO4 in 500 mL; the flow rate was 25 mL/h. The fractions containing PICP were identified by SDS-PAGE, pooled, dialyzed at 4°C against 50 mmol/L NH4HCO3, freeze-dried, and gel-filtered on a 1.6 × 35 cm column of Sephacryl S-300 (eluted with a solution of 0.25 mol/L NH4HCO3, 1 mmol/L EDTA, 0.2 mmol/L phenylmethylsulfonyl fluoride, 50 μmol/L sodium iodoacetate, and 8 mmol/L sodium azide at a flow rate of 6 mL/h). The fractions containing PICP were pooled and dialyzed at 4°C against 50 mmol/L NH4HCO3, freeze-dried, and finally purified by HPLC gel filtration on the Shodex Protein WS-803 column (eluted with 50 mmol/L sodium phosphate buffer, pH 7.0, containing 0.20 mol/L (NH4)2SO4, at a flow rate of 30 mL/h).

The resulting preparation of intact, monocomponent PICP cleaved in vivo from procollagen was quantified by amino acid analysis after acid hydrolysis and used to calibrate the RIA.

Production of antiserum to PICP. We immunized rabbits with a partly purified preparation of PICP (containing <60% PICP as assessed by SDS-PAGE with Coomassie Blue staining) from human amniotic fluid (from amniocentesis; results not shown), according to an established procedure with incomplete Freund's adjuvant (17).

Radioactive labeling of PICP. The PICP was labeled with 125I by a variation of the Chloramine-T method: We added 10 μL of PICP solution (0.8 g/L in 10 mmol/L sodium phosphate buffer, pH 7.4, containing 0.14 mol/L NaCl) to 250 μL of 0.3 mol/L sodium phosphate buffer, pH 7.4, containing 25 μg of Chloramine-T and 1 mCi of Na125I, and mixed. After 15 s we loaded the reaction mixture onto a 0.9 × 25 cm column of Sephacryl S-300 equilibrated with 10 mmol/L sodium phosphate buffer, pH 7.4, containing 0.14 mol/L NaCl and 15 mmol/L Na2SO4 (PBSA) and 1.0 g/L bovine serum albumin, and then eluted the column with this buffer at a flow rate of 6 mL/h. The specific radioactivity of the tracer produced was determined by the method of tracer self-displacement (18).

Procedure for RIA of PICP. All calibrators and samples were run in duplicate. Tracer, antiserum, and calibrators were diluted in PBSA containing 10 g/L bovine serum albumin; the tracer working solution was diluted to 50 000 counts/min in 200 μL and the antibody working solution was antiserum diluted 1:80 000.

To 100 μL of calibrator or serum sample we added 200 μL of tracer working solution and 200 μL of antibody working solution. After mixing the samples we incubated them for 150 min at room temperature, and then to each tube we added a 100-μL cellulose suspension of Sac-Cel anti-rabbit serum. After being mixed, the samples were incubated for 30 min at room temperature, and 1.0 mL of water per tube was added immediately before centrifugation (2000g, 4°C, 15 min). The supernates were discarded by decanting and the radioactivity of each precipitate was measured in a solid-phase scintillation counter. A calibration curve of B/B0 against log10[PICP] was constructed with use of a spline function.

Results

Production of serum-free cell culture medium. The repeated treatment of the confluent fibroblasts (2 days with medium containing fetal calf serum, followed by
incubation for 24 or 48 h with serum-free medium) gave relatively high concentrations of PICP in the serum-free medium (1–3 mg/L), even after the treatment had been repeated as many as eight times.

**Purification and characterization of PICP.** The Affi-T chromatography separated intact free PICP from most of the other proteins in the cell culture medium, including albumin and procollagen type III carboxyl-terminal propeptide (PIIICP), leaving osteonectin as the major contaminant in the fractions containing PICP (Fig. 1). The chemical nature of the proteins was assessed by SDS-PAGE, followed by electroblotting onto polyvinylidene difluoride membranes and amino-terminal sequencing of protein bands. The subsequent Sephacryl S-300 gel filtration was necessary to remove some very high-Mr, contaminating proteins from the PICP preparation, but did not appreciably separate PICP and osteonectin. HPLC gel filtration provided the necessary, final purification; the protein preparation isolated after this step gave one band on SDS-PAGE under nonreducing conditions with an apparent Mr of 117 000, and two bands of Mr 35 000 and 33 000 under reducing conditions (Fig. 2) with a relative staining intensity of ~2:1. The amino-terminal amino acid sequences were identical with the reported sequences of the in vivo-produced α1(I) and α2(I) chains of PICP (19, 20), i.e., DDANVRDRDLQVDTV- and DQPRASLSLRPKDYE- for the higher- and lower-mass bands, respectively.

**Radioiodination of PICP.** Determination of specific radioactivity (18) indicated that, on the average, 1.0 atom of $^{125}$I was incorporated per molecule of PICP (results not shown).

**Characteristics of the RIA and the serum antigen.** A typical calibration curve (Fig. 3) showed a 50% displacement point of ~0.70 nmol/L. With either serum samples or calibrator solution, nonspecific binding was ~1% of total. The detection limit (defined as the concentration corresponding to counts equal to the mean counts of the zero calibrator − 3 SD_zero calibrator) was 0.03 nmol/L.

The within-run CV was <2% and the total CV <4% (Table 1).

The RIA for PICP cross-reacted ~0.1% with PIIICP; i.e., a partially purified preparation of PIIICP containing ~250 nmol/L PIIICP (as assessed by Coomassie Blue staining of an SDS-PAGE gel) inhibited the binding of tracer to antibody to the same degree as a solution of purified PICP containing 0.25 nmol/L PICP.

Serum samples gave dilution curves that paralleled the calibration curve (Fig. 3), and purified PICP added to serum samples was completely recovered (Table 2).

Both serum and plasma samples can be used for the assay, and the concentrations of PICP measured in serum and in heparin-treated plasma did not differ significantly ($r = 0.993$, slope = 1.07, nonsignificantly different from 1.00; n = 10). Citrated plasma gives values ~90% of those of serum, as would be expected from the 9:1 dilution introduced in taking the blood sample for
Table 1. Assay Imprecision.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Mean, nmol/L</th>
<th>CV, %</th>
<th>Mean, nmol/L</th>
<th>CV, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>0.329</td>
<td>1.6</td>
<td>0.329</td>
<td>2.7</td>
</tr>
<tr>
<td>B</td>
<td>0.690</td>
<td>1.2</td>
<td>0.691</td>
<td>1.5</td>
</tr>
<tr>
<td>D</td>
<td>0.870</td>
<td>1.2</td>
<td>0.875</td>
<td>2.1</td>
</tr>
<tr>
<td>K</td>
<td>1.070</td>
<td>1.0</td>
<td>1.074</td>
<td>2.2</td>
</tr>
<tr>
<td>C</td>
<td>1.075</td>
<td>1.5</td>
<td>1.066</td>
<td>2.3</td>
</tr>
<tr>
<td>E</td>
<td>1.926</td>
<td>1.3</td>
<td>1.931</td>
<td>2.2</td>
</tr>
<tr>
<td>F</td>
<td>3.237</td>
<td>1.8</td>
<td>3.223</td>
<td>3.5</td>
</tr>
</tbody>
</table>

Data were calculated from the results of 9 days of triplicate determinations of seven serum samples, with the same batches of tracer and antibody. CVs were calculated for mean of duplicates as follows: Within-run imprecision for single determinations were calculated for each sample in each run and the mean imprecision was calculated with data from all nine runs; the imprecision for a duplicate determination was then calculated as the imprecision of the single determination divided by √2. Total imprecision was calculated from the mean of the first two determinations in each run.

Table 2. Analytical recovery of purified PICP (n = 7).

<table>
<thead>
<tr>
<th>Added PICP, nmol/L</th>
<th>Recovered PICP, mean ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.173</td>
<td>0.166 ± 0.006</td>
</tr>
<tr>
<td>0.345</td>
<td>0.326 ± 0.013</td>
</tr>
<tr>
<td>0.690</td>
<td>0.688 ± 0.021</td>
</tr>
<tr>
<td>1.380</td>
<td>1.383 ± 0.029</td>
</tr>
</tbody>
</table>

The concentration range of PICP in the serum samples was 0.565–1.230 nmol/L.

citrated plasma (r = 0.993, slope = 0.86, nonsignificantly different from 0.9; n = 10).

The serum PICP antigen is stable in serum without any additives for at least 4 days at 4°C (percent of initial value, mean ± SD: 98.3% ± 3.0%; n = 7). Furthermore, the PICP concentration in serum samples is stable for months at −20°C (percent of initial value, n = 6: 96.1% ± 2.3% (after 95 days); 95.4% ± 4.3% (after 92 days); 97.5% ± 1.8% (after 189 days)]. Repeated freezing and thawing as many as five times does not significantly change the serum concentration of PICP (percent of initial value after five freeze–thaw cycles, n = 7: 98.8% ± 2.3%).

Reference values. The reference value for heparin plasma PICP in women was investigated in samples from 171 healthy blood donors (Table 3). The procedures followed in acquiring the blood samples were in accordance with the Helsinki II Declaration. The reference group comprised 116 premenopausal women, ages 22–52 years (median 40 years), and 55 postmenopausal women, ages 50–74 years (median 59 years). To obtain normality and homogeneity of variance, we logarithmically transformed the measured values before statistical analysis. Plasma PICP values were significantly higher in the under-30 age group (P < 0.01), but at >30 years the premenopausal values were not significantly different from the postmenopausal values. The reference interval [defined as the geometric mean (0.72 nmol/L ± 2 SD) for the plasma PICP concentration in healthy women >30 years was 0.36–1.44 nmol/L. Plasma PICP values were significantly correlated with age (r = −0.18, P = 0.02) only when values from women <30 years were included in the calculations.

Comparison with the commercial assay. We analyzed 22 serum samples by both the assay presented here and a commercially available assay from Orion Diagnostica. The latter assay measures PICP in units of micrograms per liter. The mean and range of serum PICP (nmol/L) in the samples measured by the assay presented here were 0.832 and 0.495–1.486, respectively. By orthogonal linear regression analysis, the slope and intercept (± SE) were 5.98 ± 0.24 μmol/L and 0.067 ± 0.032 nmol/L, respectively. The standard error of the estimate (S_y) was 0.044 nmol/L and the correlation coefficient was 0.98.

Correlation between PICP and osteocalcin. For premenopausal women in our reference population we found a significant correlation (r = 0.35, P <0.0002) between plasma PICP and plasma osteocalcin (21) values.

Discussion

Methods for the purification of PICP from the medium of cultured human skin (9, 22, 23) or lung (10) fibroblasts have been reported. They all call for the isolation of procollagen and cleavage with bacterial collagenase, followed by purification of the liberated PICP. The amino-terminal amino acid sequences of the component chains of PICP thus purified are different from those of PICP produced in vivo (11). This implies that an immunochromatography between calibrator and analyte could influence the assay performance. Furthermore, for the exact calibration of PICP assays, the advantage of a calibrator with physicochemical identity to the analyte is obvious. It would thus be beneficial to have a source of intact human PICP cleaved as in vivo from procollagen, and a method to purify the PICP thus produced.

In the present study, we found that the serum-free cell culture medium of human fetal fibroblasts contains most of its PICP not in the procollagen-bound form but in the free form, with amino-terminal sequences of its component chains identical to those of PICP produced in vivo. We purified PICP to homogeneity directly from this medium, avoiding the procollagen isolation step and subsequent cleavage with bacterial collagenase. The importance of the thiophilic adsorption chromatographic step lies in the fact that it separates free PICP from free PIICP, which closely resembles PICP—65.1%
amino acid sequence identity between their α1 chains (19, 24) and extensive structural similarities (24).

The PICP RIA presented here was sensitive and precise throughout the measuring range. The assay signal is independent of whether PICP is dissolved in assay buffer or in serum, as shown by the recovery experiment (Table 2) and the fact that dilution curves of serum samples are parallel to the calibration curve (Fig. 3); i.e., there is no matrix effect.

The cross-reaction with PIIIcP was very small, and, given that the partially purified preparation of PIIIcP used for testing the cross-reaction probably contained trace amounts of PICP, the true cross-reaction with PIIIcP seems negligible. Furthermore, the serum concentration of PIIIcP is probably lower than the concentration of PICP, the total body content of type I collagen being much higher than the content of type III collagen (1).

Because storage and repeated freezing and thawing of samples does not influence the PICP concentration, the assay can conveniently be used for clinical studies in which long-term serial measurements are used, e.g., in assessing the response of treatments influencing bone homeostasis.

The assay we present is closely and linearly correlated with the commercial assay. With our assay, the mean plasma concentrations of PICP in healthy women (ages 22–74 years) was 0.75 nmol/L. Assuming a molecular mass for PICP of 117 kDa (from Fig. 2), this translates to 88 μg/L. This is lower than the mean serum value of 110 μg/L for healthy women, ages 18–61 years, reported by Melkko et al. (11). The calibrator we used and that used in the assay of Melkko et al. were both quantified by amino acid analysis after acid hydrolysis; it is not clear why the results differ. It is unlikely that the different biological sources of the protein would account for a difference as large as 25%, although some difference would be expected.

The low mean value (42 μg/L) reported by Niitsu et al. (10) for normal subjects might be due to their use of the Lowry method for determining the concentration of PICP in their calibrator solution; this technique may give different results from those obtained by quantitative analysis of amino acids after acid hydrolysis (12).

Our data show that healthy women younger than 30 years have plasma PICP values ~40% greater than women older than 30 years (Table 3). This agrees with the serum concentration of osteocalcin, a marker of osteoblastic activity and thus of bone formation, which likewise reveals higher values in younger women than 30 years (25). We found that in healthy women >30 years the plasma PICP concentrations were not significantly correlated with age, and our data do not support a report (26) of an increase in circulating PICP at menopause. However, that reported increase in PICP was small, and there was a large overlap of pre- and postmenopausal values. Therefore, the particular populations of pre- and postmenopausal women investigated may determine whether a significant difference between pre- and postmenopausal PICP values is found.

PICP and osteocalcin values were correlated in the healthy premenopausal women included in our reference population, although the correlation was not as high as reported (27) for postmenopausal women with osteoporosis (r = 0.62, P < 0.001; n = 67). This is expected, however, because PICP and osteocalcin values in healthy individuals are represented in a narrower range than in individuals with osteoporosis or other metabolic bone diseases.

In conclusion, we report a new method of purifying PICP cleaved from procollagen as in vivo, and the development of a simple and reliable RIA of PICP, which is calibrated with the purified PICP. The assay is suitable for the further clinical and biochemical investigation of circulating PICP.

References
18. Morris B. Specific radioactivity of radioimmunoassay tracer.