Radioimmunoassay of the Carboxyterminal Propeptide of Human Type I Procollagen

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Type I collagen is the most abundant collagen type in soft tissues and the only type found in mineralized bone. We established a rapid equilibrium radioimmunoassay for the carboxyterminal propeptide of human type I procollagen (PICP), to be used as an indicator of the synthesis of type I collagen. We isolated type I procollagen from the medium of primary cultures of human skin fibroblasts, digested the protein with highly purified bacterial collagenase, and purified PICP by lectin-affinity chromatography, gel filtration, and ion-exchange separation on HPLC. The purity of the protein was verified by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and by N-terminal amino acid sequencing of its component chains. The final radioimmunoassay was established with polyclonal rabbit antibodies. Material antigenically related to PICP is readily detected in human serum. There is only one form of the serum antigen, its molecular size and affinity to the antibodies being similar to those of the isolated propeptide. Intra- and interassay CVs are 3% and 5%, respectively. Preliminary reference intervals for healthy adults (18 to 61 years of age) are 38–202 μg/L for men and 50–170 μg/L for women; in men the concentration is inversely related to age. The serum antigen is stable during storage and after repeated thawing.

Additional Keyphrases: skin fibroblasts · bone · reference interval · sex- and age-related effects

Type I collagen is the most abundant collagen species in many soft tissues and accounts for >90% of the organic matrix of bone (1). The synthesis and (or) breakdown of this collagen type can be altered during the pathogenesis of many kinds of disease. Because bone is a metabolically active tissue throughout life, indicators of type I collagen turnover could be particularly useful as biochemical markers in metabolic bone disease. So far, the most important means of estimating the metabolic rate of bone collagen has been to quantify the urinary excretion of the amino acid hydroxyproline, which is derived from collagenous proteins. However, in clinical work this method is tedious, associated with several sources of error, and not specific for type I collagen (2).

Type I collagen is synthesized in the form of a larger protein, type I procollagen, which contains additional sequences at both ends (3); these sequences are removed by specific proteases before the collagen molecules are assembled into fibers. The part removed from the carboxyterminal end of the molecule, known as the carboxyterminal propeptide of type I procollagen (PICP), can be found in blood, where its concentration changes, e.g., during growth (4) and in metabolic bone diseases (5–7). A radioimmunoassay for this propeptide was described as early as 1974 (8). Originally the antigen was thought to be derived from the aminoterminus of the molecule, but it was subsequently found to be the carboxyterminal propeptide (5). Despite being a promising method with respect to monitoring metabolic bone disease, the assay of PICP has unfortunately not been available for general use.

Here we describe the purification and biochemical characterization of PICP and the development of a radioimmunoassay for it. The assay is rapid and suitable for clinical monitoring of bone metabolism.

Materials and Methods

Materials

Culture medium was collected from primary cultures of human skin fibroblasts, both confluent and subconfluent. The cells (5–10th passage) were grown in Dulbecco's modified Eagle's medium supplemented with newborn calf serum (100 ml/L), ascorbic acid (50 mg/L), penicillin (100 units/L), and streptomycin (100 mg/L). We used 10 ml of medium per 9-cm-diameter culture dish. Typically, we processed the conditioned medium in 15-L batches.

In the radioimmunoassay, which is available commercially from Farmos Diagnostica, Oulunsalo, Finland, the tracer and the antiserum were diluted with phosphate buffer (0.1 mol/L, pH 7.2) containing 9 g of NaCl, 5 g of bovine serum albumin, and 0.5 g of NaN₃ per liter. The separation agent (second antibody) was a suspension of goat anti-rabbit antibody covalently bound to solid particles in 0.1 mol/L Tris · HCl buffer, pH 7.4, containing 9 g of NaCl, 2 g of bovine serum albumin, 1 mL of Tween 20, and 1 g of NaN₃ per liter. We obtained this suspension, as well as a corresponding goat anti-mouse antibody reagent, from Farmos Diagnostica, Turku, Finland.

The monoclonal antibody M-36 (9) was purchased for research purposes from the Developmental Studies Hybridoma Bank (c/o Dr. Thomas August, The Johns Hopkins University School of Medicine, Baltimore, MD). Radioimmunoassays with this antibody were carried out as will be described for the rabbit anti-PICP, but with an anti-mouse separation reagent substituted for the anti-rabbit reagent.

Procedures

Purification of the propeptide. We first purified type I procollagen from the culture medium of human skin fibroblasts, essentially as described previously (10). The procollagen was digested for 16 h at 30°C with highly purified bacterial collagenase (Worthington Biochemicals, Freehold, NJ) to 2 × 10⁻³ mg of enzyme per liter of original medium volume. PICP was purified from the digest by lectin-affinity chromatography on concanavalin A-Sepharose (Pharmacia, Uppsala, Sweden) in 50 mmol/L Tris · HCl buffer, pH 7.4, containing 22.2 g of NaCl, 1.26 g of N-ethylmaleimide, 52.2 mg of phenylmethanesulfonyl fluoride, and 73.1 mg of CaCl₂ · 2H₂O per liter. We eluted the propeptide which was bound to the column, with the above solution
plus 97.1 g of α-methylmannoside per liter. For further purification we used gel filtration with Sephacryl S-300 (Pharmacia), eluting with a solution containing 15.8 g of NH₄HCO₃ per liter, and anion-exchange HPLC (column: Protein-Pak DEAE-5PW; Waters, Milford, MA; mobile phase: 50 mmol/L Tris-acetate buffer, pH 8.0, flow rate 1 mL/min). In the latter purification, the bound propeptide was eluted with a linear gradient of NaCl (0–0.5 mol/L in 30 min).

Production of antiseraum. Polyclonal antibodies against the propeptide antigen were raised in New Zealand White rabbits by injecting the purified propeptide in complete Freund’s adjuvant, essentially as described previously for another procollagen propeptide antigen (11).

Chemical characterization of the propeptide. We verified the purity of the propeptide by sodium dodecyl sulfate-polyacrylamide electrophoresis (SDS-PAGE) in 125 g/L acryl- amide gels. The concentration of the final propeptide preparation was determined by quantitative analysis of amino acids after acid hydrolysis. For N-terminal acid sequencing we used a liquid-phase sequencer (Applied Biosystems, Foster City, CA).

Iodination of the tracer. We labeled the purified antigen with 125I by the Chloramine-T method, separating the labeled antigen from free iodine by gel filtration on a 1 × 30 cm column of Sephacryl S-300 (Pharmacia) equilibrated at room temperature in the eluent: phosphate buffer (0.1 mol/L, pH 7.2) containing 2 g of bovine serum albumin and 1 g of NaN₃ per liter. The calculated specific activity of the labeled antigen was about 15–20 Ci/g.

Assay procedure. Incubate 50- or 100-μL aliquots of standards or serum samples with 200 μL of the tracer solution (about 50,000 counts/min) and 200 μL of diluted antiserum for 2 h at 37 °C. Then add 500 μL of the solid-phase second-antibody suspension to each tube and vortex-mix. After 30 min at room temperature, separate the bound fraction by centrifugation (2000 × g, 15 min, 4 °C). Decant the supernate containing the unbound tracer, and count the radioactivity of the precipitate containing the bound tracer (we used a Clinigamma 1272 counter; Pharmacia-Wallac, Turku, Finland).

Gel filtration of serum samples. To test the antigen-form specificity of the assay developed, we separated the antigens in serum by gel filtration, using a 1.5 × 110 cm column of Sephacryl S-300 equilibrated in phosphate-buffered saline containing 0.4 mL of Tween 20 per liter.

Computer analysis of radioimmunoassay data. The slopes of the inhibition curves obtained with the reference preparation of PICP and with serum samples were calculated with a microcomputer (12; see 10). We used logit and log transformations to obtain a linear dose–response curve, then used iterated weighted-least-squares regression analysis.

Results

Purification and characterization of the antigen. The PICP obtained after the purification procedure gave one major band in SDS-PAGE, which upon reduction resolved into two components with an approximate ratio of 2:1 (Figure 1). The molecular mass of the unreduced compound was about 100 kDa and those of the reduced components were around 30 kDa, as determined by comparison with standard proteins.

In N-terminal amino acid sequencing of the purified propeptide, we identified two sequences (Figure 2). The origins of the component chains of the isolated PICP can be traced to the carboxyterminal region of type I procollagen, as also shown in Figure 2, the amino terminals being amino acids 1169 (13) and 1102 (14) for the pro2(I) and pro1(I) chains, respectively. The same sequences were obtained for the two different batches of PICP analyzed, which were prepared with two different concentrations of bacterial collagenase.

Characteristics of the radioimmunoassay. The titration of a rabbit anti-PICP antiseraum with iodine-labeled PICP is shown in Figure 3 (left), together with the binding of the monoclonal antibody M-38 to the labeled antigen. As illustrated in Figure 3 (right), only the polyclonal antibodies could serve as a basis for a sensitive inhibition assay. In the latter, 50% inhibition of binding was achieved by using about 100 μg of PICP per liter.

For most human serum samples studied, the inhibition curve given for serial dilutions of a sample was superimposable with that of the standard antigen (Figure 3),
indicating the presence in serum of material that is antigenically similar to the standard PICP.

The intra- and interassay variations (n = 16 and 8, respectively) were tested with 100-μL human serum samples containing several different concentrations of the PICP antigen (Table 1). In our hands, the intra-assay CV is constantly around 3%, the interassay CV close to 5%. Use of 50-μL serum samples from infants and children gave no significant loss in precision.

The sensitivity (detection limit) of the PICP assay, defined as the detectable mass equivalent to twice the standard deviation of the zero binding value, was 1.2 μg/L.

Two serum samples with different concentrations of PICP were mixed in different ratios and analyzed. The mean analytical recovery was 99.2% (SD 5.7%, n = 15) within the assay concentration range 90 to 315 μg/L. No interference was seen in hemolyzed, lipemic, or icteric sera.

**Size and stability of the serum antigen.** The PICP radioimmunoassay detects one major peak of antigenicity in human serum (Figure 4). Its elution position in gel filtration exactly corresponds to that of the purified standard antigen.

To test the stability of the PICP antigen in serum, we separated the serum of a blood sample with a PICP concentration of 224 μg/L, froze part of the serum immediately and divided the rest into two portions for storage at 4 °C and at room temperature, respectively. Aliquots were removed and frozen from both portions at different times. There were no significant changes in the measurable PICP concentrations during storage for up to 15 days at either temperature. At −20 °C the samples could be stored for several months, with no loss of antigenicity upon repeated freezing and thawing.

**Reference values.** The reference values for serum PICP were calculated for healthy adults, based on samples from 75 blood donors (Figure 5). In men ages 20 to 60 years, the PICP concentration was inversely related to age, whereas in women no such relationship could be found. In both sexes, the distribution of the concentrations was somewhat skewed.

The difference between results for serum and plasma samples was tested by measuring both kinds of sample from eight healthy volunteers. The mean PICP concentrations were 124 (SD 49) μg/L for serum and 119 (SD 42) μg/L for plasma, significantly correlated (r = 0.997, P < 0.001).

**Discussion**

PICP is a trimeric, globular protein that is cleaved of type I collagen molecules before the latter are assembled into fibers. According to current knowledge, this cleavage must be complete to ensure correct fibril and fiber formation (15). Thus the amount of PICP released is directly related to the number of collagen molecules formed, and the assay for PICP can be used to quantify type I collagen synthesis, in a manner analogous to the use of the C peptide of proinsulin as a marker of endogenous insulin production. In the present study, we prepared PICP from human fibroblast culture medium and verified its identity by SDS-PAGE, by amino acid sequencing, and by assessing its binding to the monoclonal antibody M-38, which had previously been shown to react with the carboxyterminus propeptide of type I procollagen (9).

Our preparation of PICP appears to be more homogeneous than those reported previously (16), which consistently showed the presence of several polypeptide bands after reduction. This may be due to a more efficient removal of the corresponding sequences of type III procollagen by the present purification procedure or to a more controlled digestion by bacterial collagenase. However, we occasion

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**Table 1. Intra- and Interassay Variations of PICP Assay**

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<tr>
<th>Mean PICP, μg/L</th>
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**Fig. 3.** (Left) Binding of iodinated PICP to a polyclonal anti-PICP antiserum (●) and to the monoclonal antibody M-38 (○); (right) assay curves for PICP standard (●) (slope −1.084, 50% intercept 101 μg/L) and a human serum sample (▲) (slope −1.104, 50% intercept 25 μL, PICP 420 μg/L), as assayed with a polyclonal anti-PICP antibody, and an assay curve for PICP standard (○), as assayed with the monoclonal antibody M-38.

**Fig. 4.** Gel-filtration analysis of a human serum sample on a column of Sephacryl S-300. The arrow indicates the elution position of isolated human PICP. (●●●), total protein.
ally saw the lower band, the proα2(I) chain, as a doublet in SDS-PAGE. This splitting could be due to (e.g.) microheterogeneity of oligosaccharide side chains. Both polypeptide chain types of PICP contain "high-mannose"-type oligosaccharides, and the concentration of mannose may even be higher in the proα2(I) chain than in the proα1(I) chains (17). We made use of the existence of these oligosaccharides by including concanavalin A lectin chromatography in the purification procedure. The physiological significance of these side chains seems to be to mediate the uptake and removal from blood of circulating PICP. In rats, labeled PICP is bound and internalized by the endothelial cells of the liver and the receptors involved are those that recognize terminal mannose residues (B. Smedsrød, J. Melkko, L. Risteli, and J. Risteli, in press, Biochem J).

The actual aminoterminal cleavage site in PICP, when this propeptide is prepared by bacterial collagenase digestion of the corresponding procollagen, has not been determined previously. In principle, only collagenous Gly-X-Y sequences should be susceptible to degradation by this enzyme. Surprisingly, in our hands this treatment reproducibly also removed the noncollagenous telopeptide sequences of the type I collagen α chains. Contamination by an unspcific proteolytic enzyme seems unlikely, because the carboxyterminal propeptide itself should be quite susceptible to the action of such enzymes. The results can be explained by assuming an exposed site at the junction of the C-propeptide and the telopeptide, which would be highly sensitive to bacterial collagenase. Such an assumption is in accordance with the earlier reported hydrophilic nature of this region (see Figure 2), which is also a hypervariable area within the structure of interstitial procollagens (18). When the propeptide is cleaved off during the physiological processing of type I procollagen, a strictly specific proteinase cuts the peptide bond between the propeptide and the telopeptide in a location close to, but not identical with, the cleavage sites obtained here (Figure 2).

Most of the type I collagen of the body is present in bone, where it forms the scaffold of the calcifying extracellular matrix. Thus a method that can accurately and reproducibly measure its rate of synthesis could prove quite useful for monitoring metabolic bone diseases noninvasively. Earlier work has suggested that PICP could serve as such a test, e.g., in Paget's disease of bone (5, 6), and its concentration in serum has been found to correlate with the rate of cancellous bone formation in a group of patients with various metabolic bone diseases (7). However, despite being a promising method, the PICP assay has been available in only one laboratory. Procollagens are difficult to handle, which easily leads to very low yields of the purified propeptide. We have now succeeded in redevelopment of the PICP method, and the characteristics of our assay seem to be fully comparable with those of the method reported previously (4–5). The present assay, based on a highly purified and well-characterized propeptide, with its small variations and rapid performance, seems well suited for the further studies that are required for establishing the clinical value of the PICP method. For instance, estrogen–progestogen therapy of osteoporosis, while slowing the bone turnover rate, causes a significant decrease in serum PICP (C. Hassager, L. T. Jensen, J. S. Johansen, et al., ms. submitted for publication). The assay could also be applied for detecting changes in growth rate in children, e.g., in response to treatment with exogenous growth hormone (4) or during the course of a chronic illness (19).

Several minor proteins, some of which are bone-specific, occur in the bone matrix. Specific immunoassays have been developed for some of these, the one most studied so far being osteocalcin, also known as bone gamma-carboxyglutamic acid-containing protein (20). However, the concentrations measured in different laboratories vary a lot because the assay is poorly standardized (2). In addition, the concentrations of osteocalcin in serum are significantly influenced by alterations in kidney function (20, 21), and even the proportion of synthesized osteocalcin that is released into the circulation is unknown. In comparison, the PICP assay has a sound biochemical basis as an indicator of the synthesis of type I collagen. However, because liver endothelial cells seem to be involved in the elimination of PICP from blood, the results of this test should be interpreted with caution in patients with decreased liver function (7). Indeed, increased concentrations of PICP in serum have been found in subjects with alcoholic liver disease (22).

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