Radioimmunoassay of Type I Collagen That Mainly Detects Degradation Products in Serum: 
Application to Patients with Liver Diseases

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This radioimmunoassay for type I collagen mainly detects degradation products of the molecule in human serum samples. Type I collagen antigenicity in serum can be separated into two peaks by gel-filtration chromatography. The larger form represents collagen molecules (as shown by immunoblotting experiments), and (or) type I collagen with aminoterminal propeptide or intact procollagen molecules. The smaller form, the exact nature of which is not known, is quantitatively the principal antigenic form and is derived from degradation of type I collagen. The concentration of type I collagen in serum is increased mainly in cirrhotic patients, with or without active liver disease, but also somewhat in alcoholic patients without cirrhosis.

Fibrosis is characterized by excessive deposition of collagen and other components of the extracellular matrix, which leads to a disturbed function of the organs involved. Normal liver contains approximately equal amounts of type I collagen, type III collagen, and basement-membrane collagen (1–3). In fibrotic livers with cirrhosis, the total collagen content is increased by four- to sevenfold, all collagen types being increased by about the same extent (1, 3). The most reliable method for assessing and measuring hepatic fibrosis is histological examination of a liver biopsy sample. However, this procedure is invasive (4). Furthermore, the quantification of liver fibrosis by use of hepatic biopsies is not entirely reliable, given the small size of the sample, the heterogeneous distribution of fibrosis (5, 6), and the practical difficulties of using objective and quantitative methods such as colorimetry in these procedures (7). Iterative biopsies are ethically questionable and only dubiously reliable for measuring the progression of liver fibrosis. Markers in serum would be very useful for assessing fibrosis or fibrogenesis in patients with liver diseases and for monitoring drug trials.

Since 1979, several attempts have been made to evaluate the metabolism of human connective tissue proteins by radioimmunoassay techniques (for reviews, see 8–12). Much has been published on the N-terminal propeptide of type III procollagen (PIIINP)3 (13–21) but some also on the N- and C-terminal propeptides of type I procollagen (20, 22–25), on basement membrane components (type IV collagen and its amino- and carboxyterminal fragments—7S and NC1, and fragments P1 or P2 of laminin) (26–31), and on type VI collagen (32). The principal methodological problems in these assays are the very small amounts of these connective tissue proteins in the various biological fluids and the size heterogeneity of a given circulating antigen (13, 15).

Apart from the C- and N-terminal propeptides of type I procollagen (PICP and PINP) (20, 22–25), no other antigenic forms of human type I collagen, in particular none containing the triple helical domain, have been found in serum thus far, probably because assays are not sensitive enough (10). We have now developed a sensitive RIA for human type I collagen with an antibody raised against a fully processed molecule, and we have applied it to measure liberated antigens in the serum of patients with various liver diseases. Moreover, we analyzed the sera biochemically to characterize the size distribution of the recognized antigens. We found that the assay detects mainly degradation products of type I collagen. Quantifying the degradation of the extracellular matrix rather than only its synthesis is quite important, because the dynamic remodelling of connective tissue as it occurs during fibrogenesis or fibrolysis is the net result of synthesis and degradation (12).

Materials and Methods

Isolation, purification, and characterization of acid-soluble human type I collagen. Skin specimens taken during surgical procedures from healthy donors (ages 30–50 years, with no connective tissue disease) were ground under liquid nitrogen. All subsequent procedures were carried out at 4°C. Centrifugations were at 30,000 × g for 1 h. After delipidation with methanol/chloroform (2/1, by vol), the skin was extracted twice for 12 h with 50 mmol/L Tris hydrochloride buffer, pH 7.5, containing 0.1 mol of NaCl per liter. After centrifugation, the pellet was resuspended in 0.5 mol/L acetic acid with agitation for 12 h. The resulting supernatant, collected after re-centrifugation, contained acid-soluble collagen, which was precipitated by 0.7 mol/L NaCl solution. Acidic contaminants were removed by ion-exchange chromatography at 10°C on a 5 × 20 cm column of diethylaminoethyl-cellulose (Whatman Ltd., Springfield Mill, U.K.), equilibrated and eluted with Tris buffer containing 2.0 mol of urea and 0.2 mol of NaCl per liter. The eluted fraction was dialyzed against 0.5 mol/L acetic acid and further purified by fractionation with NaCl, 0.7 mol/L. The resulting precipitate was dissolved in 0.1 mol/L acetic acid, extensively dialyzed against 0.1 mol/L acetic acid, and lyophilized.

The type I collagen thus obtained was assessed for purity

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3 Nonstandard abbreviations: type I pN-collagen, type I collagen bearing its amino-terminal propeptide; PICP, carboxy-terminal propeptide of type I procollagen; PIIINP, amino-terminal propeptide of type III procollagen; Coll 1, globular domain obtained from PIIINP by digestion with collagenase (EC 3.4.24.3); 7S, amino-terminal fragment of type IV collagen; NC1, non-collagenous carboxy-terminal fragment of type IV collagen; PBS, phosphate-buffered isotonic saline; and BSA, bovine serum albumin.

Received September 20, 1989; accepted November 27, 1989.
by amino acid analysis (JEOL 5AH automatic analyzer; Japan Electron Optics Laboratory, Tokyo, Japan) and by electrophoresis on 6.75% polyacrylamide gel as described by Laemmli (33). The gels were stained with Coomassie Blue or silver nitrate (silver stain kit; Amersham International Plc, Amersham, U.K.). The \( \beta/\alpha \) ratio was 1.22 and the \( \alpha_1/\alpha_2 \) ratio was 1.82 (\( \beta \) being a dimer of \( \alpha \) chains, and \( \alpha_1 \) and \( \alpha_2 \) the two different \( \alpha \) chains of the type I collagen molecule: molecular composition, \([\alpha_1(III)\alpha_2(III)]\). We ensured the absence of contaminating type III collagen by using interrupted gel electrophoresis according to Sykes et al. (34).

Preparation of antisera. Antibody to type I collagen was raised in rabbits as described previously (35). Antiserum to purified rabbit IgG was obtained from a goat by an established procedure.

Radiiodination. We radiolabeled type I collagen with \( ^{125}I \), using the Chloramine T method (36) with some modifications: 20 \( \mu \)g of collagen in phosphate-buffered saline (PBS; 0.05 mol/L phosphate buffer, 0.5 mol/L NaCl, pH 7.4) was reacted with 0.5 mg of [18.5 MBq] of Na\(^{125}I\) (NEN, Wilmington, DE; 15.5 \( \times \) \( 10^8 \) Bq/atom) and then 200 \( \mu \)L of 0.5 mol/L phosphate buffer, pH 7.4, and 10 \( \mu \)L of Chloramine T (1 g/L, in PBS) were added. The mixture was shaken for 2 min at room temperature, and the reaction was then stopped with 10 \( \mu \)L of cysteine (1.5 g/L) in PBS. Iodinated collagen was purified by gel-filtration chromatography, first on a 0.4 \( \times \) 10 cm column of Sephadex G 25 (Pharmacia, Uppsala, Sweden) and then on a 1.5 \( \times \) 90 cm column of Ultrogel AcA 54 (IBF, Paris, France), each eluted with PBS. Labeled collagen eluting in the void volume was diluted in PBS and stored in aliquots at \(-20^\circ \)C.

Determination of antibody titer. Rabbit antiserum to type I collagen was serially diluted in PBS. To 0.1 mL of the diluted antiserum we added 0.1 mL of normal rabbit serum (diluted 200-fold in PBS) and 0.1 mL of \( ^{125}I \)-labeled collagen (about 20,000 counts/min), then shook the tubes and incubated at 4 \( ^\circ \)C for 24 h. We then added 0.1 mL of goat anti-rabbit IgG (diluted 40-fold in PBS) and 1 mL of 15 g/L polyethylene glycol solution in PBS. After 30-min incubation at room temperature, the precipitate was collected by centrifugation at 5000 \( \times \) 90 for 30 min and the radioactivity measured. Nonspecific binding was measured by replacing specific antiserum with normal rabbit serum.

Radioimmunoassays. In the standard inhibition assay (sequential saturation type), 0.1 mL of a 400-fold dilution of antiserum capable of binding 50% of the \( ^{125}I \)-labeled collagen was incubated with 0.1 mL of diluted unlabeled standard human type I collagen in PBS or with 0.1 mL of human serum. After incubation for 24 h at 4 \( ^\circ \)C, we added \( ^{125}I \)-labeled collagen and continued the assay as above.

Gel-filtration chromatography. We chromatographed 2-mL serum samples on a 1.5 \( \times \) 130 cm column of Sepharcl SS00 (Pharmacia) equilibrated in PBS containing 0.4 mL of Tween 20 surfactant per liter, eluting at a flow rate of 20 mL/h. We collected and assayed 2-mL fractions. Some of the serum samples were exposed to bacterial collagenase from Clostridium histolyticum (Sigma Chemical Co., St. Louis, MO) according to Savolainen et al. (37) added directly to serum for a 24-h incubation at room temperature, before gel-filtration chromatography. Collagenase activity was stopped by the addition of EDTA (0.5 mmol/L final concentration).

Immunoblotting. For electrotransfer of proteins from polyacrylamide gels onto nitrocellulose (0.45-\( \mu \)m pore size; Schleicher & Schuell, Dassel, F.R.G.), we used a semi-dry electrophoretic apparatus (BioIyon, Lyon, France). Anodic pH 8.3 buffer contained, per liter, 25 mmol of Tris, 192 mmol of glycine, and 200 mL of methanol; the cathodic buffer was the same plus sodium dodecyl sulfate, 1 g/L, but no methanol. The transfer was carried out at 24 V for 1 h, after which the nitrocellulose was immersed in PBS containing bovine serum albumin, 30 g/L (PBS-BSA), at 40 \( ^\circ \)C for 1 h to prevent nonspecific binding and incubated at room temperature overnight with the total IgG fraction of the antibody diluted in PBS-BSA.

We washed the nitrocellulose five times for 6 min each in PBS containing Tween 20 (0.5 mL/L). The secondary antibody was biotinylated anti-rabbit IgG (heavy and light chains; Vector Laboratories, Burlingame, CA) diluted 100-fold in PBS. After washing, the nitrocellulose was incubated for 1 h with "ABC" (Avidin Biotinylated horseradish peroxidase Complex; Vector Laboratories), then washed again, and the collagen chains were immunodetected with 4-chloro-1-naphthol (Sigma Chemical Co., St. Louis, MO) as substrate, as described by Hawkes et al. (38).

Patients. Serum samples were obtained from 91 apparently healthy adults (39 men and 52 women, mean age 36 years, range 18–54) and 146 patients with various liver diseases (86 men and 60 women; mean age 50 years, range 17–88). Serum was separated by centrifugation after clotting and stored at \(-60^\circ \)C until used. Simultaneously, percutaneous liver biopsy by the Menghini technique (Hepafix 1.8-mm needle; B. Braun Melsungen AG, F.R.G.) was performed on all patients, and was indicated as part of the standard clinical management. All specimens were fixed in Bouin’s fluid, paraffin-embedded, and stained with hematoxylin and eosin, trichrome, and Sirius red techniques before histological examination. According to clinical and histological data, the patients were classified into eight groups: active cirrhosis (AC), alcoholic hepatitis with cirrhosis (C + AlcH), inactive cirrhosis (IC), acute hepatitis (AH), alcoholic hepatitis without cirrhosis (AlcH), chronic active hepatitis (CAH), chronic persistent hepatitis (CPH), and alcoholic minimal lesions represented by fatty liver with various degrees of fibrosis without cirrhosis or alcoholic hepatitis (AlcML). The etiology of the liver disease was alcohol-related in 54 cases, virus B-related in 35, drug induced in 18, autoimmune in five, and unknown in 14.

Statistical analysis. The differences between the groups were analyzed by one-way analysis of variance and, if a statistically significant level was reached, Student’s unpaired t-test with pooled variance was used. Data were normalized by logarithmic transformation before analysis.

Results

Radioimmunoassay. With rabbit antiserum against human type I collagen we obtained a typical binding profile with the \( ^{125}I \)-labeled antigen. More than 85% of the total radioactivity was precipitated in the antibody-excess zone, indicating intact immunoreactivity of the radioiodinated type I collagen. The antiserum showed a strong reaction with the immunizing antigen and a weak one, if any, with other human collagens, PIINP, elastin, or fibronectin (Figure 1).

We established the inhibition assay for type I collagen, using a 400-fold dilution of the hyperimmune serum and 0.1 mL of sample (Figure 2). No significant inhibition was observed with other collagenous or noncollagenous connective tissue proteins (not shown).

The minimal detectable dose for the assay was estimated
at 600 pg by extrapolating the imprecision profile (Figure 3).

Type I collagen can be routinely detectable (coefficients of variation of replicates lower than 10%) in the concentration range between 30 and 1000 µg/L (Figure 3).

The precision was also estimated from the inter-assay coefficients of variation (Table 1).

Analytical recovery of known amounts of type I collagen, 120–700 µg/L, added to human serum was 102 ± 7% (mean ± SD).

Dilution of normal or pathological sera showed a nonparallelism with the standard curve (Figure 2).

Detection of type I collagen-related antigens in sera of healthy subjects and patients with liver disease. Table 2 gives the reference values for serum type I collagen from 91 healthy adult subjects. The upper limit of the reference interval (mean ± 2 SD) was 197 µg/L (Figure 4). No sex- or age-related differences in type I collagen concentrations in serum were noted.

Figure 4 depicts the results for patients with liver diseases. Type I collagen concentrations were significantly higher than in normal subjects in the following groups: AC (P <10^{-4}), C + AlcH (P <10^{-4}), IC (P <10^{-4}), AH (P <10^{-2}), AlcH (P <10^{-4}), and AlcML (P <10^{-4}).

A significant difference between the eight groups of patients was noted for serum type I collagen (F = 13.1, P < 10^{-4}). The results of this statistical comparison between groups are reported in Figure 4.

Molecular mass determination of immunoreactive components in serum. Gel-filtration chromatography of various normal or pathological serum samples showed two fractions reacting with anti-type I: a high-molecular mass fraction eluting in the position of standard type I collagen and a low-molecular-mass fraction, which quantitatively was the main antigen (Figure 5). When the serum was incubated with collagenase before chromatography, the type I antigenicity was recovered only in a fraction with molecular mass <15 000 Da (Figure 5).
Fig. 4. Distribution of type I collagen concentrations in sera from patients with various liver diseases (spelled out in text)
The solid line indicates the 95% confidence limit of the reference interval.

Fig. 5. Distribution of type I collagen antigen in human serum (from a patient with alcoholic cirrhosis) after chromatography on a Sephadryl S500 column before (A) and after (B) collagenase treatment (see Materials and Methods) (computer-generated plot). Inhibitory activity was measured in 2-ml individual fractions of the eluent by direct radioimmunoassay. The column was calibrated with $^{150}$I-labeled type I collagen, and nonlabeled PIIINP and Coll. V5 and V6 denote the void and total volumes of the column.

Sodium dodecyl sulfate/polyacrylamide gel electrophoresis and immunoblotting. The analysis of the high-molecular-mass fraction from serum (pool from several runs) by sodium dodecyl sulfate/polyacrylamide gel electrophoresis showed, after silver staining, the presence of two components co-migrating with $\alpha_1(1)$ and $\alpha_2(1)$ chains (Figure 6B).

Fig. 6. (A) Immunodetection on nitrocellulose with antibody to human type I collagen and biotin/avidin system (Vectorstain kit, Vector Laboratories): (1) high-molecular-mass fraction; (2) same as in 1, but reduced with 1,4-dithiothreitol; (3) low-molecular-mass fraction; (B) sodium dodecyl sulfate–polyacrylamide gel electrophoresis (on 6.75% polyacrylamide gel, with silver-staining): (1) high-molecular-mass fraction, (2) same as in 1, but reduced with 1,4-dithiothreitol in B, the migration of $\alpha_1$ and $\alpha_2$ chains of standard type I collagen is indicated on the left.

One of these components reacted better with the anti-human type I collagen antibody, as shown by immunoblotting experiments (Figure 6A). The low-molecular-mass fraction contained several components recognized by anti-human type I collagen antibody.

Discussion
This reliable, reproducible radioimmunoassay for human type I collagen was found to be sufficiently sensitive to allow quantitative determination of related antigens released into the circulation in normal or pathological conditions. The type I collagen antigenicity appeared to be heterogeneous: nonparallel inhibition curves were produced by the biological samples as in other connective-tissue assays (13, 15, 28, 30), because the different peptides have different affinities for the antibody. We performed gel-filtration experiments to define the size of these antigens, the collagenous nature of which was demonstrated by susceptibility to collagenase. Type I antigenicity was found in two fractions (Figure 5).

The high-molecular-mass component was eluted by gel-filtration in the same position as acid-soluble human type I collagen and migrated in sodium dodecyl sulfate/polyacrylamide gel as $\alpha_1(1)$ and $\alpha_2(1)$ chains. It probably is intact, fully processed type I collagen, pC- or pN-collagen, procollagen itself, or some mixture of them.

The presence of such circulating high-molecular-mass antigens is not surprising, because (a) they also appeared on the gel-filtration profiles of human and rat sera for PIIINP (15, 37, 39, 40), (b) these kinds of compounds were described for type IV collagen and laminin (28, 30, 31, 37), and (c) we have found them in an analogous human type III collagen assay (Hartmann et al., in preparation).

The nature of the low-molecular-mass material is under investigation but it seems different from PIIINP according to
preliminary results evidencing different positions on gel-filtration and its absence of reactivity with an antibody directed to PICP (J. Risteli, personal communication).

The origin of these two antigenic fractions remains to be established. Because PICP (and PINP) is cleaved off when the collagen molecules are to be associated into fibrils, its measure estimates the amount of collagen that is being synthesized (12). Conversely, our assay probably will measure degradation products from the tissue form of type I collagen. At present, little is known about the excretion and elimination of extracellular matrix proteins from the serum. Some information has been published recently on the clearance of PIIINP. The liver seems to play a key role in its elimination from the blood via the hepatic endothelial cells (41, 42), which would also be the case for the other connective-tissue molecules, although the mechanisms may be different depending on the size of the antigen (41–43).

In fibrotic and cirrhotic hepatic tissue, the contents of all collagen types are increased. In cirrhotic livers with a low amount of fibrosis, the ratio of type I to type III remains similar to that found in normal liver, whereas in livers with a high amount of fibrosis, type I collagen is the prevailing type and the ratio is >1 (1). So, in tissues, type III collagen could be characteristic of "early" fibrosis, as in active diseases with inflammation and cell necrosis, whereas type I collagen could be a component encountered predominantly in "late" fibrosis (1). For serum, the PIIINP results have been interpreted in earlier studies (13–14) in terms of increased liver collagen synthesis and deposition. The relationship between PIIINP and aminotransferase concentrations in serum suggests in fact that the peptide may be released during the active inflammatory phase of the disease by cleavage of tissue type III procollagen and thus reflects inflammation and necrosis and not necessarily fibrosis (12, 44). By contrast, in this study, we have shown that the concentration of type I collagen in serum is predominantly increased in cirrhotic patients with or without active liver disease and thus is less related to liver activity than is PIIINP (Trinchet et al., ms. submitted for publication). Moreover, type I collagen was more increased in serum of alcoholic patients without cirrhosis (alcoholic hepatitis or minimal lesions) than in that of nonalcoholic patients (CPH, CAH, or AH) without cirrhosis; this fact may reflect the progressive weakly reversible fibrosis that characterizes alcoholic liver disease.

Owing to the heterogeneity of the liver diseases, further studies are needed to determine the significance of the respective different connective-tissue variables in serum.

We thank Drs. L. and J. Risteli (Department of Medical Biochemistry, Oulu) for their critical reading of the manuscript, Prof. C. M. Lapire and Dr. B. Nuqens (Dermatology Service, University of Liége) for the kind gift of PIIINP and Col I, and Mrs. C. Buffavant (Bioestica, Lyon) and Dr. D. Herbage (URA CNRS 244, Lyon) for their help in preparing human type I collagen.

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