Radioimmunoassay for the Carboxy-terminal Cross-linking Domain of Type IV (Basement Membrane) Procollagen in Body Fluids

Characterization and Application to Collagen Type IV Metabolism in Fibrotic Liver Disease

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Abstract

The carboxy-terminal cross-linking domain (NCl) of type IV procollagen was isolated from human placenta and used for the production of polyclonal and monoclonal antibodies. Purity of the antigen and specificity of the antibodies were verified by Western blotting and radioimmunoassays.

A radioimmunoassay was developed using rabbit antiserum. Intra- and interassay coefficients of variation were 4.7% and 5.8%, respectively; recovery of NCl added to serum and bile was 95–105%. NCl concentration in sera of healthy volunteers was 6±2.9 ng/ml (mean±2.5 SD) and was elevated up to 18 ng in sera of patients with autoimmune or metastatic tumor disease and up to 240 ng in sera of patients with fibrogenic liver disease. Substantial amounts of antigen were also found in bile, urine, and ascites. 67% of serum antigens eluted from an agarose A5M column with an apparent molecular weight of 60 kD and 23% with a molecular weight of 90 and 150 kD, well below the molecular weight of type IV procollagen (550 kD). Serum NCl is apparently derived from the degradation of basement membrane collagen.

The time course of NCl concentrations in sera of patients with fibrogenic liver disease showed no correlation with the serum concentration of the amino-terminal procollagen type III peptide, a marker of hepatic collagen biosynthesis. A decline of serum NCl levels along with elevated serum procollagen type III peptides apparently indicates bad prognosis in fibrogenic liver disease. The radioimmunoassay for NCl is a useful tool for studying type IV collagen metabolism in conditions causing remodeling or breakdown of basement membranes.

Introduction

Basement membranes (BM)[1] are extracellular sheetlike structures that play a major role in cell differentiation and morphogenesis. They serve as flexible mechanical support for endothelial and epithelial cells and form a filtration barrier in the kidney glomerulus or between circulatory and interstitial compartments (for reviews, see References 1–3). BM architecture is destroyed or altered in states of inflammation (2), tumor invasion (4), or hepatic fibrosis (5). Recently, rapid progress has been made in the isolation and characterization of the major structural components of all BM (for reviews, see References 6 and 7): laminin, a cross-shaped glycoprotein with a molecular weight of 1,000 kD (8) that mediates cellular attachment to other BM molecules (9); heparan sulfate proteoglycan (10) which interacts with a domain of laminin (11); nidogen, a recently characterized glycoprotein whose function is yet unknown (12); and type IV (BM) collagen which represents the principal scaffold for the other BM structures.

Based on biochemical and electron-microscopic evidence, Timpl et al. (13) postulated a network model for BM collagen, in which four amino-terminal regions of type IV procollagen are linked together to form the 7-S domain (14) and the carboxy-terminal globular extensions of two molecules are combined to build the hexameric carboxy-terminal cross-linking (NCl) domain of type IV procollagen (13, 15). The isolated NCl globule has recently been investigated more thoroughly by biochemical and electron-microscopic methods (16).

Sensitive radioimmunoassays have been developed for mouse 7-S collagen (14) and pepsin fragments P1 and P2 of mouse laminin (17), and significant elevations of cross-reacting antigens in streptozotocin-diabetic rats were detected (18). The radioimmunoassays described for human 7-S collagen and the laminin P1 fragment (19, 20) either suffer from low sensitivity for measurements in normal human serum or from the nonparallelity of serum inhibition curves, respectively (our own unpublished results). Furthermore, it is not clear whether these circulating antigens reflect novel synthesis or degradation of BM structures, or both. In search for sensitive and precise serum parameters of BM metabolism in humans, we focused on fragment NCl, the carboxy-terminal noncollagenous domain of type IV collagen. This collagenase-resistant structure has recently gained widespread attention as the presumptive autoantigen of Goodpasture's syndrome (21–24). It was shown by Wieslander et al. (22) that the autoantigenic epitope is buried in the globular structure and only exposed upon denaturation (25).

In this report, we describe a sensitive radioimmunoassay for human NCl and present the first analysis of the antigen in serum and other biological fluids under normal and abnormal conditions.

Methods

Purification of fragment NCl. Human placenta was collected in 100 mM Tris, 4 N NaCl, pH 7.4, with 5 mM *N*-ethylmaleimide (NEM), 2 mM phenylmethylsulfonyl fluoride, and 20 mM ethylene diaminetetraacetate

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1. *Abbreviations used in this paper:* BM, basement membrane; NCl, carboxy-terminal cross-linking domain of type IV procollagen; NEM, *N*-ethyl maleimide; PIIIP, amino-terminal procollagen type III peptide; RIA, radioimmunoassay.

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(Na_2-EDTA) added to inhibit protease activity. After homogenization, the tissue was washed with 3 liters of 20 mM Na_2-EDTA, 1% Triton X-100, pH 7.4, per kilogram of wet weight, then washed extensively with distilled water, and finally preextracted with 5 liters of cold 0.5 N acetic acid. 1 kg of preextracted homogenate was suspended in 2 liters of 50 mM Tris, 5 mM $CaCl_2$, 2 mM NEM, pH 7.6, and digested with crude collagenase (Boehringer, Mannheim, Federal Republic of Germany, 5 mg/kg wet weight) for 40 h at 20°C. The supernatant was saved and the residue was extracted twice with 2 liters 0.5 N acetic acid containing 1 μg/ml pepstatin A (Sigma Chemical Co., Deisenhofen, Federal Republic of Germany) for 4 h at 20°C. The acid extracts were dialyzed against 50 mM Tris, 2 mM NEM, pH 7.6, and added to the supernatant of the collagenase digest. From the combined supernatants and extracts, type IV collagenous fragments were precipitated by the addition of solid NaCl to a final concentration of 2.2 N. The precipitate was dissolved in and dialyzed against 50 mM Tris, 2 M urea, pH 7.6, at 4°C and subjected to a DEAE-cellulose batch procedure. Type IV collagenous fragments were not bound and, after dialysis against 40 mM Tris, 2 mM NEM, 0.5 N NaCl, 5 mM $CaCl_2$, pH 7.6, were digested with 5 mg of purified bacterial collagenase (Worthington Biochemical Corp., Freehold, NJ, C1SPA) per kilogram of starting material for 6 h at 37°C. After extensive dialysis against 10 mM NH_4HCO_3, pH 7.9, the material was lyophilized and applied to a column (3.5 × 130 cm) of agarose A 1.5 M (Bio-Rad Laboratories, Munich, Federal Republic of Germany), equilibrated in 50 mM Tris, 1 M $CaCl_2$, pH 7.5. The major peak consisted of fragment NCl and was further purified by gradient elution on DEAE-Sephadex CL 4B (Pharmacia, Uppsala, Sweden) in 10 mM Tris, 2 M urea, pH 8.0, and chromatography on a column (2 × 110 cm) of Sephacryl S-200 (Pharmacia) equilibrated in 0.2 M NH_4HCO_3, pH 7.8.

Other proteins and peptides. Fibronectin was isolated by the procedure of Miekka et al. (26) from citrated plasma. Pepsin digestion of placenta was carried out as described in the literature in order to obtain laminin fragment P1 (20, 27), type IV collagenous fragments (27), 7-S collagen (14, 27), collagen type V (form AB_2, 27), and type VI collagen (28).

Analytical methods. Proteins were characterized by sodium dodecyl sulfate (SDS)-polyacrylamide slab gel electrophoresis prior to and after reduction with 5% mercaptoethanol (29). For amino acid analysis (D-500 analyzer, Durrum Corp., Palo Alto, CA), samples were hydrolyzed with 6 N HCl in the presence of 0.05% mercaptoethanol under N_2 for 20 h at 110°C. Values for threonine and serine were corrected for losses during hydrolysis by factors 1.08 and 1.21, respectively. Cysteine was determined after reduction and aminoethylation of the protein.

Electron microscopy. The globular domain used in this investigation was studied by the rotary shadowing technique modified for connective tissue proteins (30). This was carried out by Hanna Wiedemann and Klaus Kühn, Department of Connective Tissue Research, Max Planck Institute for Biochemistry, Martinsried, Federal Republic of Germany.

Antisera. To produce antisera against NCl, 0.3 mg of the antigen (subfraction *b*, see Results) was dissolved in 0.5 ml of 0.05 M NH_4HCO_3, pH 7.8, mixed with 0.5 ml complete Freund's adjuvant (Difco Laboratories, Detroit, MI) and injected subcutaneously into New Zealand White rabbits. The same amount of antigen mixed with incomplete adjuvant was given twice in 3-wk intervals. 2 wk after the last booster injection, the animals were killed by heart puncture. Rabbit antiserum against mouse IgG was obtained by a similar protocol. Goat antiserum against rabbit IgG was produced following a similar schedule using 4 mg of rabbit IgG dissolved in 2 ml phosphate-buffered saline (PBS) and mixed with the same amount of adjuvant. Blood (200–400 ml) was drawn from the jugular vein.

Affinity chromatography. For affinity purification (27), antiserum against NCl was passed over columns of immobilized human laminin P1 fragment, collagens type IV (triple helical and 7-S domains), V, and VI, and finally NCl fragment (fraction *b*). Briefly, 4–10 mg of protein were coupled to cyanogen bromide (CNBr)-activated Sepharose (Pharmacia) following the procedures supplied by the manufacturer. 4 ml of antiserum was pumped over the immobilized antigens at 3 ml/h. Recovery of NCl antibody eluted from its respective column with 3 M KSCN in PBS buffer/0.05% Tween 20 was 3.6 mg at a concentration of 300 μg/ml. This antibody solution was frozen in aliquots at −20°C and used in all experiments without further concentration.

Production of monoclonal NCl antibodies. Balb/c mice were immunized with 30 μg of NCl (fraction *b*), for a period of 6 wk at weekly intervals. The first injection (in 0.1 ml of 0.05 M NH_4HCO_3, pH 7.8, mixed with 0.1 ml of complete Freund's adjuvant) was administered intraperitoneally (i.p.), all subsequent injections were given with emulsions of 0.1 ml of aqueous antigen solution in an equal volume of incomplete Freund's adjuvant. Splenectomy was performed 3 d after the last injection. Fusion of splenocytes to NS-1 myeloma cells was carried out using the protocol of Oi and Herzenberg (31). Fused cells were washed and then distributed into 96-well plates containing hypoxanthine-aminopterinethymidine (HAT) medium. Macroscopic hybridoma growth was observed in most of the wells after 2 wk.

For initial screening and selection for cloning, supernatants from wells with hybrid growth were tested on tonsil sections for reactivity with connective tissue by immunoenzymatic staining using the alkaline phosphatase/monoclonal antialkaline phosphatase method (see below). Hybrids that produced immunoglobulin with probable reactivity for the immunizing agent were selected for cloning by limiting dilution in flat-bottomed 96-well plates in the presence of feeder cells. Selected clones were subsequently recloned and later maintained by i.p. injection of 50 × 10^6 cells into Nu/Nu mice of the CD-1 strain. The subclasses of ascites-derived monoclonal antibodies were determined by double immunodiffusion using subclass specific rabbit anti-mouse Ig reagents (Miles Laboratories, Elkhart, IN). Monoclonal antibody (IgG2b) 5A7 was used in this study.

Indirect immunoenzymatic staining. For immunoalkaline phosphatase staining, the fixed tissue sections were incubated with monoclonal antibodies followed by incubation with a rabbit anti-mouse IgG and a complex of alkaline phosphatase and monoclonal antialkaline phosphatase antibody (32). To enhance the labeling, the incubation with rabbit anti-mouse IgG and alkaline phosphatase and monoclonal antialkaline phosphatase antibody was repeated once or twice. Between each incubation step, the slices were briefly washed with 0.05 M Tris HCl, pH 7.6. For visualization of the alkaline phosphatase activity, the slides were incubated with the alkaline substrate for 30 min with continued agitation. After the enzyme reaction, the slices were washed in Tris-HCl, pH 7.6, and counterstained with Mayer's Hemalum (Merck, Darmstadt, Federal Republic of Germany).

Western blotting. Peptides run on SDS-polyacrylamide gels (gradient 10–18% acrylamide) were blotted onto nitrocellulose (Trans Blot, Bio-Rad Laboratories) in transfer buffer containing 0.1% SDS, but otherwise following the method described by Dziadek et al. (33). After blocking with 2.5% (wt/vol) bovine serum albumin (Behring-Werke, Marburg, Federal Republic of Germany) in Tris-buffered saline (TBS), pH 7.4, the replica was incubated for 2 h at 25°C with rabbit NCl antiserum (diluted 1:200 in Tris-buffered saline), the affinity-purified polyclonal antibody or monoclonal antibody 5A7 (both at 3 μg/ml). Reaction with peroxidase-conjugated goat anti-rabbit IgG or rabbit anti-mouse IgG (Behring-Werke) was allowed to proceed for 2 h, and color was developed with a mixture of 25 mg of *p*-phenylenediamine, 25 mg of catechol (both Sigma Chemical Co.) and 15 μl of 30% hydrogen peroxide in 50 ml of 0.1 M sodium cacodylate, pH 6.0.

Radioimmunoassays. NCl fragment was labeled with ^{125}I using the procedure of Bolton and Hunter (34). ^{125}I was linked to other connective tissue proteins (see the above subsection "Other proteins and peptides") by the chloramine-T method (27, 28). Separation of bound from unbound label was achieved by dialysis against four changes of PBS containing 0.5 N NaCl and 0.04% Tween 20. The percentage of protein-bound label as determined by precipitation with 10% trichloroacetic acid in the presence of 5% serum was between 85% and 95%. Incorporated radioactivity ranged between 8,000 and 15,000 cpm/ng protein. Labeled peptide NCl, when chromatographed on a column of Sephacryl S-300 in PBS, 0.04% Tween 20, was eluted shortly before the albumin standard, with an apparent molecular weight of about 80,000 D.

All immunoassays were carried out in radioimmunoassay (RIA) buffer (PBS, pH 7.4, 0.04% Tween 20) at 4°C with duplicate or triplicate tubes

as described (27, 28). Binding of labeled antigen was determined by preincubating 0.2 ml of serial dilutions of the antiserum/antibody in RIA buffer containing 0.25% rabbit or mouse serum as carrier with 0.2 ml of RIA buffer alone for 20 h. 4 h after addition of 0.1 ml of labeled antigen (8,000–15,000 cpm, 1 ng protein), immune complexes were precipitated with 0.2 ml of a pretested dilution (usually 1:16) of goat antiserum to rabbit IgG or rabbit antiserum to mouse IgG.

In the inhibition assay (sequential saturation), 0.2 ml of the nonlabeled inhibitor (usually two to three sequential geometric dilutions per assay) were incubated with 0.2 ml of a specific antibody dilution sufficient to bind 40–50% of the labeled antigen. Labeled antigen and second antibody were added as outlined for the binding assay. The concentration of the standard inhibitor was determined by triplicate amino acid analysis. The concentration of antigen (nanograms per milliliter) in the unknown sample was obtained by the 50% intercept method (27, 28). The amino-terminal procollagen type III peptide (PIIIP) was determined with a commercial RIA kit (Behring-Werke).

Collection of serum, ascites, and bile. Blood was drawn from the antecubital vein after an overnight fast. Serum was obtained after clotting the blood samples at room temperature. Only sera from patients with a normal serum creatinine were included in the study. Ascites was drawn from the peritoneal cavity for diagnostic or therapeutic purposes. Bile was collected over periods of 24 h from patients with obstructive bile duct disease with and without impaired liver cell function after a nasobiliary tube (Krauth, Hamburg, Federal Republic of Germany) had been introduced into the main bile duct for therapeutic reasons. Urine was collected over 24 h and assayed after 10-fold concentration. Particulate material in ascites and bile was removed by centrifugation. All samples were stored at −20°C.

Patients and healthy controls. Serum NCl levels were followed-up in two patients with severe alcoholic hepatitis, a diagnosis supported by biopsy and clinical parameters. Both had a history of an alcohol intake of more than 200 g per day (patient A, age 36, male) and more than 150 g per day (patient B, age 48, female) for at least 5 yr. They apparently stopped drinking during the time of observation, being hospitalized most of the time. Patient A showed severe hepatic encephalopathy, but soon recovered and could be subjected to biopsy, which revealed a complete destruction of acini and severe pericellular and zone 3 fibrosis. His condition markedly improved, and a second biopsy, taken 9 mo later, showed complete inactive cirrhosis with regenerative nodules. Patient B remained icteric over the whole period of observation but demonstrated no signs of encephalopathy. Laparoscopy and biopsy could be performed shortly after admission to hospital, and severe alcoholic hepatitis and fibrosis were documented. Later, coagulation parameters did not allow a second biopsy. The disease took a progressive course and patient B died 9 mo after the first admission to the hospital. Postmortem liver histology showed active micronodular cirrhosis. Sera of 30 healthy adult volunteers, mainly laboratory personnel (18 male, 12 female, age 19–41 yr) were collected and served as controls.

Serum chromatography. Serum (2–4 ml) was cleared by centrifugation and directly applied to a column of Agarose A5M (2 × 110 cm) equilibrated in PBS containing 0.05% Tween 20. Fractions of 5 ml were collected and measured by RIA without prior concentration. The included volume of the column was determined using dinitrophenylalanine (DNP-L-Phe, Fluka AG, Neu-Ulm, Federal Republic of Germany) as an indicator.

Results

Isolation and characterization of the antigen. The total yield of fragment NCl after final purification was 20–25 mg/kg of prewashed placenta. When we used purified instead of crude bacterial collagenase for the first tissue digest at 20°C, the final yield was only 5–10 mg/kg of placenta. The latter preparation contained most of the antigen in the acid extract of the collagenase-digested residual tissue. The tryptic protease activities of the crude collagenase most probably led to better extractability of NCl by exposing more completely type IV collagenous sequences to collagenolytic attack. After a second digest with purified bacterial collagenase at 37°C, the carboxy-terminal type IV collagen helices, still attached to the NCl globule, were completely removed. The antigen appeared as a homogeneous peak on a column of agarose A 1.5 M, eluting at the position of an IgG marker. Three overlapping molecular-weight fractions, however, were obtained after dissolution in 0.2 M ammonium bicarbonate, 4 M urea, pH 7.8, at 20°C, and a final chromatography on a column of Sephacryl S-200. SDS-gel electrophoresis (Fig. 1) of the major fraction *b* prior to reduction showed two pairs of closely spaced doublet bands with molecular weights of 24 + 27 (monomer) and 50 + 55 kD (dimer, see Weber et al. [16]), respectively, relative to globular protein standards. Reduction of fraction *b* produced one major component of 35 kD. Fraction *a* contained, in addition, some material of lower mobility before reduction, whereas fraction *c* was mainly composed of the doublet with a molecular weight 24 + 27 kD (monomer). Amino acid analysis (Table I) revealed a composition for fraction *b*, which was very similar to that reported previously for NCl isolated from mouse tumor and placental basement membranes (13, 16). When dissolved in 0.1 N acetic acid and visualized by the rotary shadowing technique, fraction *b* consisted mainly of fragmented hexamers (not shown), similar to the forms described recently after dissociative treatment of NCl (16). Taken together, the characteristic pattern on gel electrophoresis, the amino acid composition and the rotary shadowing were in excellent agreement with the data reported previously on mouse and human NCl fragments (13, 16). Fraction *b*, which represented 65% of the total yield and was considered homogeneous, served as immunogen and standard antigen in all further experiments.

By Western blot analysis, NCl antiserum or the affinity-pu-

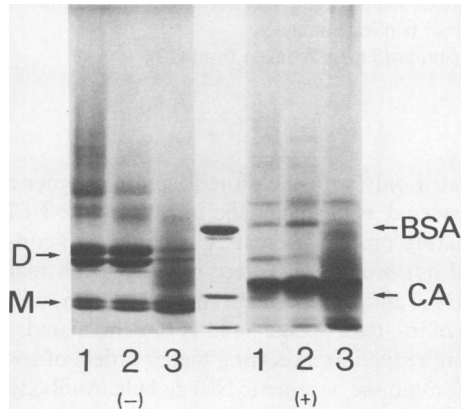

Figure 1. SDS-polyacrylamide slab gel electrophoresis (gradient of 5–15% acrylamide) of the three molecular weight fractions of fragment NCl obtained by a final chromatography on a column of Sephacryl S-200. Samples were applied to the gel prior to (−) and after (+) reduction in 5% mercaptoethanol. Lane *1*, high molecular weight fraction *a* (9% of total yield); lane *2*, medium molecular weight fraction *b* (65% of total yield); lane *3*, low molecular weight fraction *c* (26% of total yield); center lane, standards of bovine serum albumin (*BSA*, 68 kD) and carbonic anhydrase (*CA*, 29 kD). *D* and *M* denote the position of the unreduced dimer and monomer, respectively, each appearing as a characteristic doublet of bands. In fraction *a*, minor bands of more slowly migrating material were seen, possibly representing higher aggregates of the NCl monomer. After reduction, fractions *a* and *b* showed one major band of 35 kD and minor bands of 50, 70, and 85 kD.

Table I. Amino Acid Analysis of NCl Fractions

Amino acid	Fraction b	Fraction c	Mo*
	residues/1,000 residues		
Aspartic acid	72	88	84
Threonine	67	75	63
Serine	108	94	106
Glutamic acid	101	120	104
Proline	74	69	64
Glycine	97	108	112
Alanine	76	69	76
Half cysteine	43	29	36
Valine	51	62	46
Methionine	8	3	5
Isoleucine	41	36	41
Leucine	64	57	69
Tyrosine	43	33	25
Phenylalanine	40	25	41
Histidine	32	28	35
Hydroxylysine	4	4	7
Lysine	23	38	40
Arginine	60	63	46

Amino acid analysis for NCl isolated from human placenta. Three molecular weight fractions *a*, *b*, and *c* were obtained after chromatography on Sephacryl S-200. The composition of the high molecular weight fraction *a* (not listed) was essentially identical to that of the medium molecular weight fraction *b*. The low molecular weight fraction *c* contained significantly more polar residues and less cysteine than *a* and *b*. For comparison the amino acid analysis of hexameric mouse tumor NCl (Mo) is shown. Results are expressed as residues/1,000 amino acid residues. Tryptophane was not determined; compositions were derived from triplicate analyses.

* Electrophoretically similar to form *b*, taken from (13).

rified antibody reacted only with the dimeric and monomeric doublet bands considered as characteristic for oligomeric NCl peptide. Contaminating protein present in a crude NCl-containing fraction did not produce a pattern different from that obtained with pure NCl antigen (Fig. 2 *A*). As with protein stain (Fig. 1) weak bands of tri-, tetra-, penta-, and hexameric material were observed before reduction indicating higher orders of covalent cross-linking within the hexameric NCl globule. Antibody reaction with the reduced bands of molecular weights 35, 70, and 85 kD was less pronounced, possibly due to their higher degree of unfolding and a higher affinity of the antibody for the native conformation.

Furthermore, a monoclonal NCl antibody (IgG2b) could be produced, which revealed a superimposable staining pattern on Western blotting with a lower affinity for penta/hexamers and reduced bands of NCl (Fig. 2 *B*), thus demonstrating the purity of the antigen and the specificity of the polyclonal antiserum and antibody. The monoclonal antibody, however, precipitated only 40% of radiolabeled NCl and could not be used for a sensitive radioimmunoassay (Fig. 3). When the antiserum, the purified polyclonal or the monoclonal antibodies were applied to cryostat sections of human skin or liver for indirect immunofluorescence, a sharp delineation of subepithelial and vascular basement membranes without any stromal reaction was observed (data not shown).

Radioimmunoassay. For the determination of NCl antigen in biological fluids, we developed an assay based on a rabbit antiserum to fragment NCl and ^{125}I-labeled peptide. Radioactive label was incorporated with the Bolton-Hunter reagent, because iodination by the use of chloramine-T seemed to destroy most of the antigenic determinants. Binding of labeled peptide to excess antibody was 80% and, with a sharp decline, reached 50% at antiserum dilutions of 1:20,000 to 1:50,000 (Fig. 3). Binding of ^{125}I-labeled collagens type I, III, V, and VI, 7-S collagen, and the aminoterminal propeptide of type III collagen to the antiserum was completely absent. Only negligible binding was observed with fibronectin and laminin P1 fragment. In that immunoassay results obtained with the purified polyclonal antibody were identical to those with the antiserum, the latter was used in the inhibition assay. Binding of ^{125}I-NCl to antibodies could be inhibited by unlabeled peptide showing a linear relationship ranging from 20% to 75% when plotted against the logarithm of inhibitor concentration. Standard antigen, kept in neutral buffer, yielded a highly reproducible 50% inhibition at a concentration of 4.4±0.2 ng/ml (mean±SD) in 10 experiments. With monomeric NCl (fraction *c*, Fig. 1) as inhibitor, an ~10-fold higher concentration was necessary (Fig. 4 *A*). Several human body fluids could act as inhibitors of the reaction between labeled NCl and the antiserum. Serial dilutions of 150 sera tested so far showed a slope completely parallel to the standard curve; samples of urine, however, were slightly less parallel (Fig. 4 *B*). Parallelity was also observed for supernatants of fibroblast cell cultures from human liver, whereas bile and ascites caused inhibition slopes intermediate between those of urine and those of serum.

NCl antigen levels in serum and other body fluids. NCl was measured in the sera of 30 adult healthy volunteers and found to be 6±2.9 ng/ml (mean±2.5 SD). In patients with fibrotic liver disease, however, we observed a dramatic increase of the antigen up to 240 ng/ml (Table II), correlating to some degree with the severity of the illness. Preliminary data (11 sera) showed that in systemic diseases like lupus erythematosus and metastasizing cancers, serum antigen was only slightly elevated (8–18 ng/ml). As mentioned before, inhibition was also detected in other body fluids (Table II), suggesting a considerable turnover of NCl and/or fragments thereof.

Course of serum antigen levels in fibrotic liver disease. In order to obtain a first idea about the diagnostic and prognostic significance of changing serum NCl levels, we observed, over a period of 5–8 mo, two patients with initially severe alcoholic hepatitis (see Methods). One patient (Fig. 5 *A*), who during several weeks of hospitalization rapidly recovered, demonstrated a slowly falling albeit elevated level of NCl antigen in serum. The other patient (Fig. 5 *B*) exhibited extremely high and fluctuating serum NCl with a sharp increase shortly before death. The amino-terminal procollagen type III peptide, now being widely recognized as a serum marker to assess fibrogenic activity in liver disease (35, 36) and measured concomitantly in both patients, took a different somewhat contrapuntal course (Fig. 5 *A* and *B*) and did not correlate to NCl antigen levels.

Reproducibility and precision of the assay. For 10 successive inhibition assays, the interassay coefficients of variation for the standard 50% intercept as well as the normal and the pathological control serum were below 5.8% (Table III *A*). The intraassay variation was tested with the two control sera and found to be less than 4.7% (Table III *B*). When standard antigen was added

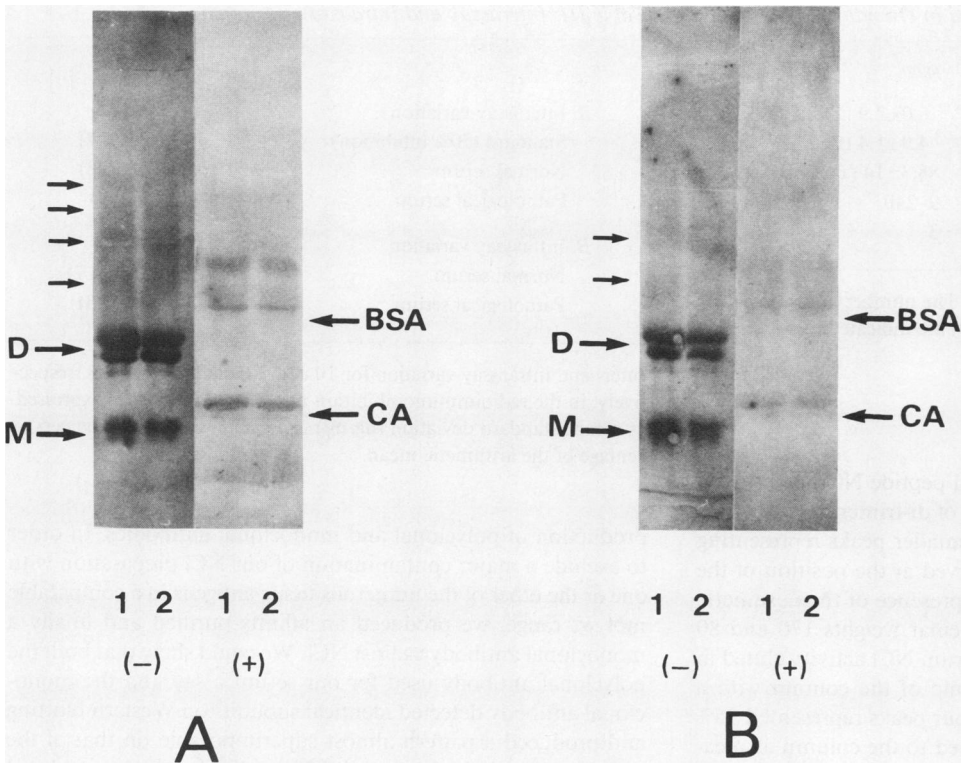

Figure 2. Western-blot analysis of NCl fractions with affinity purified polyclonal (*A*) or monoclonal (*B*) NCl antibody. Protein was run on a SDS-polyacrylamide gradient gel (10–18%) before (−) and after (+) reduction with 5% mercaptoethanol, blotted onto nitrocellulose, incubated with NCl antibody at 3 μg/ml, and color was developed as described in Methods. Lane *1*, NCl (fraction *b*), lane *2*, NCl-containing fraction obtained after the second collagenase digestion before further purification by molecular exclusion and ion-exchange chromatography and containing major high and low molecular weight impurities (see Methods). The relative mobilities of carbonic anhydrase (*CA*, 29 kD) and bovine serum albumin (*BSA*, 68 kD) are indicated. (*A*) Using the polyclonal antibody, weak staining of tri-, tetra-, penta-, and hexameric material in addition to the prominent dimer (*D*) and monomer (*M*) doublets was observed in the nonreduced gels. The major bands after reduction were of 35, 70, and 85 kD. (*B*) With the monoclonal antibody 5A7, a pattern virtually superimposable on that with polyclonal antibody of *A* was obtained. Reaction with oligomers of NCl before reduction was, however, weaker than in *A* with only tri- and tetramers (*arrows*) being stained.

to normal serum and bile in three different quantities to give a concentration of 55, 220, and 1,100 ng/ml serum or bile, 95–105% recovery by RIA measurement was observed in three different experiments. This indicates that apparently no serum or bile components interact with the globular domain to interfere with antibody binding.

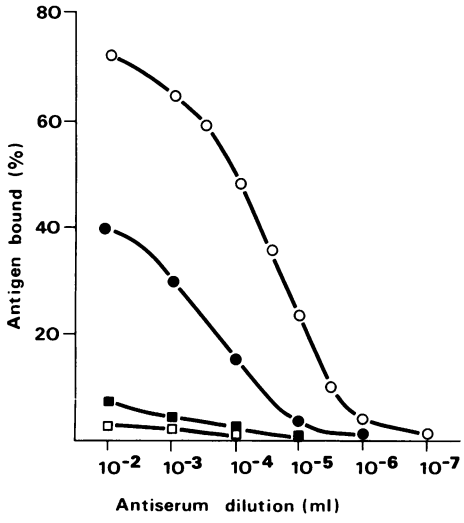

Figure 3. Binding profile of rabbit antiserum against NCl (fraction *b*, Fig. 1) to ^{125}I-labeled antigens: (○) NCl, (■) fibronectin, (□) laminin P1 fragment. Immune complexes were precipitated by goat antiserum to rabbit IgG. Binding of ^{125}I-labeled NCl to monoclonal antibody 5A7 (●; undiluted ascites containing 5 mg of IgG2b per milliliter) does not exceed 40%.

Molecular weight of serum NCl antigen. 2–4 ml serum of patients with alcoholic hepatitis containing 50–105 ng/ml of cross-reacting antigen was subjected to molecular sieve chromatography on a column of agarose A 5 M. Fractions of three individual runs were assayed by RIA for NCl, and a representative run is shown in Fig. 6. One major peak of inhibiting activity representing 67% of total serum antigen recovered from the column appeared at the position of a serum albumin standard with a molecular weight of ~60 kD (Fig. 6). This elution position

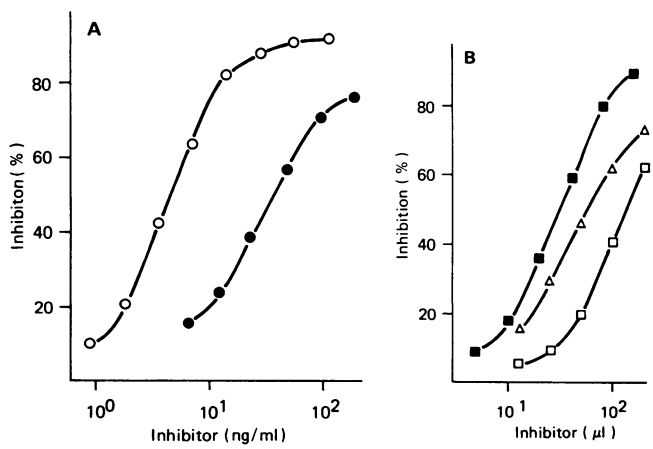

Figure 4. Radioimmune inhibition assay with the antiserum of Fig. 2 and ^{125}I-labeled NCl. Inhibitors were in *A* (○) NCl-standard (fraction *b*, Fig. 1), (●) NCl (monomeric fraction *c*, Fig. 1); in *B* (■) serum (hepatic fibrosis), (□) serum (normal), (△) normal urine (10-fold concentrated).

Table II. Levels of NCl Antigen Detected in Human Body Fluids

	ng/ml
Serum (normal, n = 30)	6.0±2.9 ($\bar{x}$±2.5 SD)
Bile (normal, n = 12)*	4.9±1.4 ($\bar{x}$±1 SD)
Ascites (alcoholic, n = 10)‡	88.3±14 ($\bar{x}$±1 SD)
Serum (liver fibrosis, n = 55)	9–240
Urine (normal)	3

Levels of NCl antigen in biological fluids. The number of samples measured (n) and standard deviations (SD) are indicated in parentheses.
* Range 2.5–6.3 ng/ml.
‡ Range 39.1–117.1 ng/ml.

Table III. Interassay and Intraassay Variations

	ng/ml (%)
A. Interassay variation	
Standard (50% inhibition)	4.4±0.25 (5.7)
Normal serum	6.0±0.35 (5.8)
Pathological serum	91.5±3.6 (3.9)
B. Intraassay variation	
Normal serum	5.15±0.29 (4.7)
Pathological serum	95.9±3.15 (3.3)

Inter- and intraassay variation for 10 and 20 identical samples, respectively, in the radioimmunoinhibition assay for NCl antigen, expressed as mean±standard deviation (ng/ml) and standard deviation as percentage of the arithmetic mean.

was slightly below that for the labeled peptide NCl used in this RIA, which consisted predominantly of di-trimeric units with a molecular weight of ~80 kD. Two smaller peaks representing 9% and 14% of total NCl were observed at the position of the IgG marker, possibly because of the presence of the hexameric and trimeric globular domain (molecular weights 170 and 80 kD, respectively), and only 10% of serum NCl activity eluted in fractions close to the excluded volume of the column with a molecular weight > 700 kD. These four peaks represented 95% of cross-reacting serum antigen applied to the column as measured by NCl RIA.

Discussion

We isolated the carboxy-terminal cross-linking domain (NCl) of type IV procollagen from human placenta and used it for the production of polyclonal and monoclonal antibodies. In order to exclude a major contamination of our NCl preparation with one or the other of the numerous tissue antigens in a comparable mol wt range, we produced an affinity-purified and finally a monoclonal antibody against NCl. We could show that both the polyclonal antibody used for our serum assay and the monoclonal antibody detected identical subunits on Western blotting and produced a pattern almost superimposable on that of the NCl bands stained for protein. Interestingly, the monoclonal antibody stained both bands of the doublets equally well (Fig. 2 *B*), indicating that each single band presents the same antigenic determinant of NCl. The monoclonal antibody was, however, not useful for a sensitive RIA owing to an incomplete precipitation of radiolabeled NCl. Using the polyclonal anti-NCl serum, the RIA established for the globular carboxy-terminal cross-linking region (domain NCl) of human basement membrane (type IV) collagen allowed a highly specific and reproducible measurement of this antigen in biological fluids. Serum levels in adult healthy donors were found in the narrow range of 6±2.9

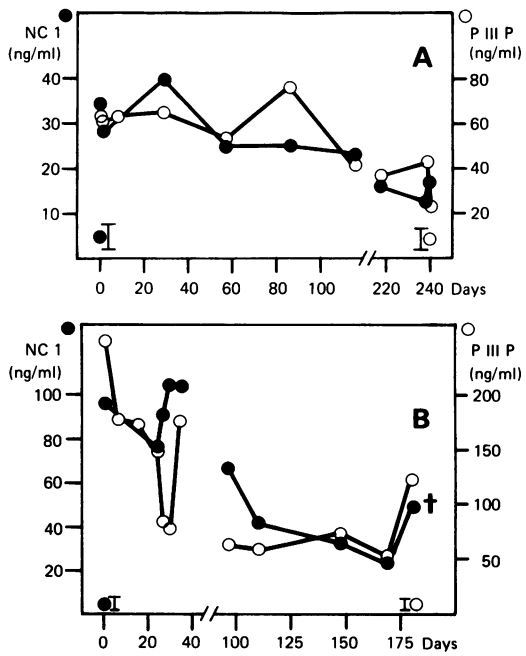

Figure 5. Time course of the serum levels of NCl antigen (●) and the amino-terminal procollagen type III peptide (○, PIIIP) in two patients with active fibrotic liver disease (see Methods); day 0 marking the time of first blood sampling; ●I and ○I indicating the range (mean±2.5 SD) of NCl and PIIIP antigen, respectively, in sera of normal young adults. Patient A recovered, whereas patient B finally died in hospital.

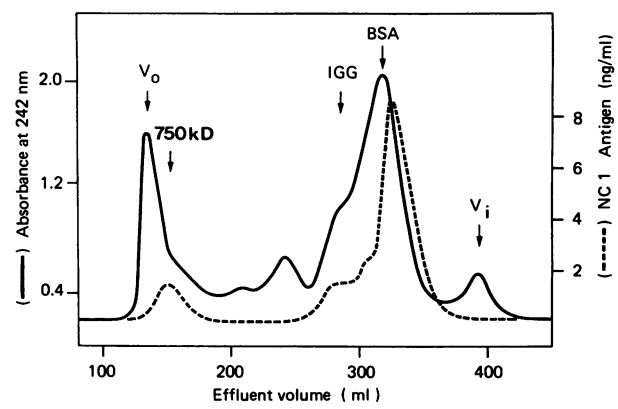

Figure 6. Chromatography of the serum of a patient with alcoholic hepatitis on a column of Agarose A5M (2 × 110 cm) equilibrated in PBS containing 0.05% Tween 20. The effluent of the column was monitored for protein absorbance (solid line). Fractions of 5 ml were collected and NCl serologic activity determined by inhibition assay (dash line). V_0 and V_i mark the excluded and included volumes, respectively. The elution of heat-denaturated type IV collagenous standards (750 and 320 kD), of IgG and bovine serum albumin (*BSA*) are indicated by arrows. Recovery of serum antigen applied to the column was 95%.

ng/ml (2.5 SD) well above the safe detection limit (3 ng/ml) of the assay. When serum of patients with alcoholic hepatitis was subjected to molecular sieve chromatography, ~70% of the antigenic material eluted with an apparent molecular weight of 50–60 kD equivalent to the dimeric fragment isolated by collagenase digestion from human placenta. Together with ~20% of the antigen, which presumably represents the hexameric and trimeric globular domain (molecular weights of 170 and 80 kD, respectively), this finding shows that ~90% of circulating NCl antigen have completely lost the long collagenous arms extending towards the amino-terminal 7-S domain. Thus we conclude that serum NCl is a result of and predominantly reflects degradation of basement membrane collagen.

The remaining 10% of serum antigen had a molecular weight > 700 kD as compared with collagenous standards. Previously, type IV procollagen from cell culture media could be visualized by electron microscopy as a triple-stranded molecule of 395 nm in length containing NCl as the trimeric carboxy-terminal globular domain (37). It was then shown by Duncan et al. (15) that the formation of the network of basement membrane collagen is initiated by the condensation of four type IV procollagen moieties at the aminotermini to the tetrameric 7-S domain. In contrast with these findings, Yurchenco et al. (38) reported that dimerization of procollagen IV at the carboxytermini (NCl domain) precedes its amino-terminal association to the 7-S domain. According to the latter report, the fraction with high molecular weight could consist of dimeric (1,100 kD) procollagen type IV, which, as a marker of biosynthesis, has escaped its integration into the extracellular matrix. Another interpretation is favored by an investigation of Fessler et al. (39), localizing the cleavage point of a purified murine tumor type IV collagenase at a distance 265 nm away from the carboxyterminus of procollagen IV. The primary cleavage product of the matrix type IV collagen containing the NCl globule should then have a molecular weight of 2 × 360 kD, also being consistent with a molecular weight > 700 kD. A definite answer to the question whether the high molecular weight fraction represents biosynthesis or degradation of basement membrane collagen can only be given by its isolation and electron-microscopic visualization.

It has been shown by immunofluorescence that interstitial and basement membrane proteins are intrinsic structures of the normal liver and are excessively deposited in the extracellular space even in early stages of hepatic fibrosis, probably owing to the activation of myofibroblasts (5). Although the deposition of collagen type III and collagen type IV might follow different kinetics, our own data show that their total tissue content correlates closely in early and advanced stages of hepatic fibrosis (40). As a serum marker, PIIIP is now generally considered as being indicative of connective tissue biosynthesis in hepatic fibrogenesis (36). In Fig. 5, we presented diagrams showing the course of serum PIIIP and serum NCl over several months in two patients with severe alcoholic liver disease. From these diagrams, it is evident that only little and an often inverse correlation exists between PIIIP and NCl serum levels in these patients. In the light of PIIIP as a marker of synthesis and NCl as a marker of degradation of the extracellular matrix, an increase in the ratio of NCl to PIIIP could indicate an overall removal of fibrotic liver tissue, whereas a decrease of that ratio could herald enhanced deposition of fibrous tissue in the liver. It is further remarkable that patient A in Fig. 5, who disclosed elevated but relatively constant and finally declining serum levels of both NCl and PIIIP, entered a stage of clinical stability in spite of the development of cirrhosis. Patient B, however, with highly elevated and fluctuating values died from hepatic failure.

NCl was also detected in bile, where its level rose up to 10 times above normal (4.9±1.4 ng/ml; mean±1 SD) in cases of biliary inflammation and/or obstruction. It remains to be established whether biliary NCl is primarily derived from the basement membrane underlying the biliary epithelium. Ascitic fluid contained very high levels of NCl, but no correlation with the concentration in serum, which was taken at the same time, could be observed. Appreciable amounts of antigen occurred in urine with a slope of inhibition slightly less parallel to the standard than that of serum. Whether the urinary and part of the biliary NCl peptides are more degraded products and similar to the less immunoreactive monomeric form of the antigen awaits further clarification. The use of Fab antibody fragments for RIA may help to measure smaller forms of NCl. An analogous approach has been successfully applied for the aminoterminal procollagen type III peptide (41).

Inasmuch as we expected serum NCl not only to be elevated in fibrogenic liver disease but also in conditions in which an increased turnover of basement membranes might be involved, we measured a collection of sera obtained from patients with autoimmune diseases (scleroderma, lupus erythematosus disseminatus) and liver metastasis of colonic adenocarcinoma. NCl was moderately elevated (1.5–3 times above normal) in most of these sera, but was normal in nonmetastatic colon carcinoma. Prospectively designed clinical studies are now feasible to validate the diagnostic and prognostic value of our NCl RIA. Its wide application should shed further light on the physiology and pathophysiology of type IV collagen metabolism in the human organism.

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