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Immunochemical Analysis of Human Kidney Reticulin

R. Fleischmajer,* L. Jacobs II,* J. S. Perlish,* B. Katchen,* E. Schwartz,* and R. Timpl†

From the Department of Dermatology, Mount Sinai School of Medicine, New York, New York, and the Max Planck Institute fur Biochemie,† Munich, Germany*

This study characterized the nature of reticulin fibrils from human kidney cortex by immunochemical analysis. Controls consisted of type I collagen fibrils derived from the kidney parietal capsule. Most of the fibrils in the capsule ranged in diameter from 60 to 80 nm whereas reticulin fibrils from the cortex ranged from 30–45 nm. Immunochemistry by light and electron microscopic examinations was carried out with antibodies directed against type I and type III collagens, their corresponding aminopropeptides, and decorin (PG-II). The ratio of type I to type III collagen was determined by cyanogen bromide peptide digests. This study showed that reticulin fibrils are hybrids of type I and type III collagens. Double immunoelectron microscopic examination showed that fibrils 20–25 nm consisted mainly of type I collagen some of which retained their aminopropeptide. Larger fibrils 30–35 nm labeled simultaneously for type I and type III collagens. However, most fibrils with diameters between 40–55 nm labeled for type III collagen and its corresponding aminopropeptide. No decorin was detected at the surface of reticulin fibrils. Purified reticulin consisted of 82% type III and 18% type I collagen whereas collagen derived from the capsule revealed 76% type I and 24% type III. The presence of the aminopropeptide of type III procollagen in reticulin fibrils is a striking feature and may play a role in regulating their diameter. (Am J Pathol 1992, 140:1225–1235)

Reticulin or reticular fibrils is a histologic term that identifies a major network of collagen fibrils with a distinct distribution and special tinctorial properties. These fibers are argyrophilic, and stain positively with the periodic acid-Schiff procedure. They are most commonly present in boundaries between epithelia and their surrounding connective tissue and in vital structures such as nerves (endoneurium) and blood vessels. They are particularly abundant in parenchymas, bone marrow, lymph nodes, and spleen. Reticulin fibrils have some distinct features that distinguish them from regular type I collagen fibrils. They are randomly arranged and are surrounded by an abundant ground substance and their diameter is restricted to about 30–40 nm. Reticular fibrils are markedly insoluble, which has hampered their purification and chemical characterization. Early biochemical studies showed that they consist of collagen with 4.2% hexose, small amounts of hexosamine, and 11% fatty acids, predominately myristic acid.[1] Immunofluorescence studies have also indicated that reticulin fibrils contain type III collagen[2–4] which appears to retain its aminopropeptide.[5] However, other studies have shown that type I and type III collagens codistribute with reticular fibers in the lung,[6] spleen,[7] and lymph nodes.[8] This could be explained by recent ultrastructural data suggesting that interstitial fibrillar collagens may be hybrids of type I and type III collagen molecules.[9,10]

The cortex of adult human kidney consists of a network of collagen fibrils around glomeruli, tubules, and blood vessels, which are thin, about 40 nm, and argyrophilic thus fulfilling the current criteria for reticulin fibrils. This study further characterized reticulin fibrils with light and electron microscopic examinations using immunochemical techniques with antibodies directed against type I and type III collagens, their corresponding aminopropeptides and a proteoglycan, decorin (PG-II). The ratio of type I to type III collagen was determined by measuring cyanogen bromide peptides.

Materials and Methods

Adult normal human kidneys were obtained from autopsy material. Areas corresponding to the visceral capsule (control) and the adjacent cortex were separated by dissection with a forceps. Electron microscopic (EM) exam-

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Address reprints requests to Dr. Raul Fleischmajer, Department of Dermatology, Mount Sinai School of Medicine, One Gustave L. Levy Place, New York, NY 10029.

ination showed that the capsule consisted mostly of thick collagen fibers of about 60–80 nm diameter, whereas the cortex contained a network of reticulin fibers of about 30–45 nm diameter surrounding glomeruli, tubules, and blood vessels. Trabeculae arising from the capsule, were not included in this study.

Source of Antibodies

Antibodies against a type I collagen structure were raised in rabbits using a synthetic peptide of 26 amino-acid residues corresponding to the sequence of the C-terminal telopeptide of the human alpha 1 (I) chain[11] as antigen. This antibody by Western blotting techniques, interacted with pro-collagen and alpha 1 (I) chains of human-derived type I collagen. Immunofluorescence studies showed that this antibody interacts with fibrillar collagen.[12] Controls for this antibody consisted of IgG from nonimmunized rabbits. Reagent for type III collagen was a monoclonal IgM antibody against human collagen.[13] The control for this antibody consisted of mouse IgM from nonimmunized animals. This antibody does not react with type I collagen fibrils generated *in vitro* and does not crossreact with type I collagen by ELISA techniques.[13] Antibodies against the aminopropeptide of type I procollagen were prepared with pN collagen from dermatosparaxis sheep skin, whereas pN collagen III from normal calf skin was used to prepare antibodies against the aminopropeptide of type III procollagen. Both antibodies were raised in rabbits and controls consisted of rabbit IgG from nonimmunized animals. These antibodies were rendered specific by affinity chromatography.[14] Small bone proteoglycans were purified from prenatal and neonatal human calvaria and an antibody against the protein core of human decorin (PG-II) was raised in rabbits as previously described.[15] All antibodies had a stock concentration of 0.5 to 1 mg Ig per ml and were used at 1 to 4 (IgG) or 1 to 20 (IgM) dilutions for immunoelectron microscopic examination. Antibodies (IgG) were diluted 1 to 10 and 1 to 30 (IgM) for immunofluorescence studies.

Immunofluorescence Microscopic Examination

Indirect immunofluorescence microscopic examination was completed in kidney specimens that contained the visceral capsule and adjacent cortical tissue. Frozen sections, 2–4 microns thick, were fixed for 5 minutes in 100% acetone and washed with phosphate-buffered saline (PBS) (pH 7.4). The primary antibody was placed on the sections for 40 minutes, washed with PBS, and followed by an incubation for 40 minutes with fluorescein-conjugated goat anti-rabbit (IgG) or anti-mouse IgM serum. After extensive washes with PBS, all specimens were counterstained with propidium iodide to visualize the nuclei of the cells.

Immunoelectron Microscopic Examination

All steps of indirect immunoelectron microscopic examination were carried out at 4°C except for dehydration and

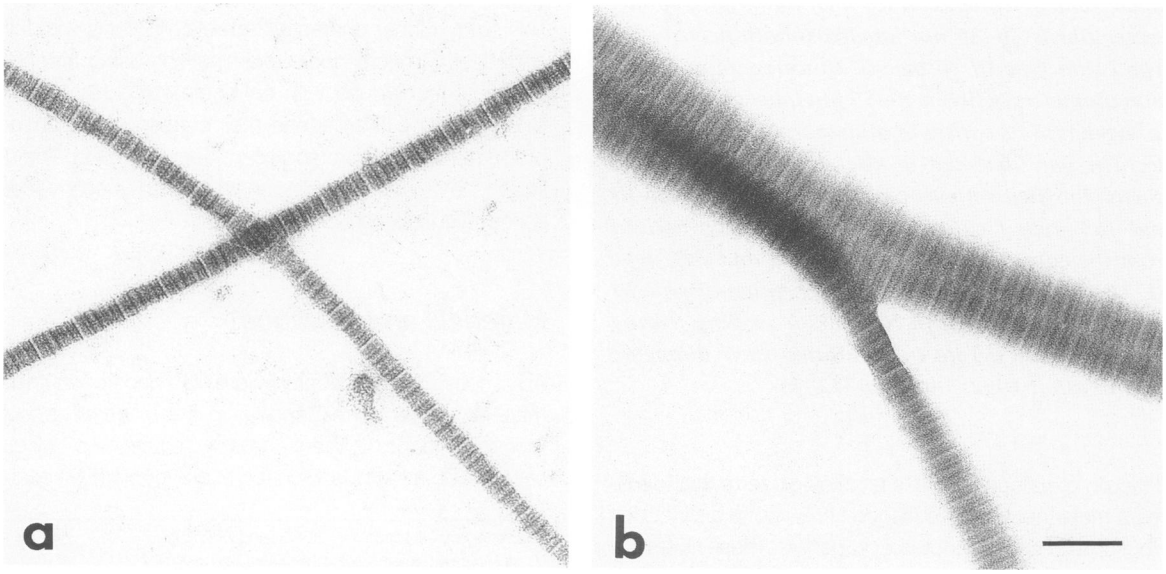

Figure 1. *Kidney collagen fibrils isolated from* (a) *cortex and* (b) *capsule. The collagen fibrils were stained with 2% aqueous uranyl acetate. Note that fibrils from the capsule are thicker and have a tendency to coalesce. Bar = 100 nm.*

embedding. Specimens from the capsule and cortex, about 0.2–0.5 mm thick were washed with PBS (pH 7.4) and exposed to the primary antibody for 24 hours with continuous agitation. For double-labeling techniques, tissues were exposed simultaneously to type I (IgG, rabbit) and type III (IgM, mouse) collagen-specific antibodies. After extensive washings with PBS, a secondary antibody consisting of either 5 or 10 nm gold conjugated goat anti-rabbit IgG or goat anti-mouse IgM (Janssen Life Sciences, Piscataway, NJ) was diluted in 1% bovine serum albumin (BSA), 20 mmol/l TRIS buffer (pH 8.0) that contained 0.9% NaCl, which was added for 24 hours. After washing with PBS and cacodylate buffer (pH 7.4), the blocks were fixed in Karnovsky's solution for 1 hour and postfixed in 1% osmium tetroxide for 1 hour. The tissue was stained *en block* with 1% aqueous phosphotungstic acid for 1 hour and with 2% aqueous uranyl acetate for 1 hour.[16] After dehydration with graded series of ethanol and propylene oxide, specimens were embedded in Spurr's resin, sectioned, and examined in a JEOL-100-CX electron microscope (Japan Electron Optics Laboratory, Tokyo, Japan).

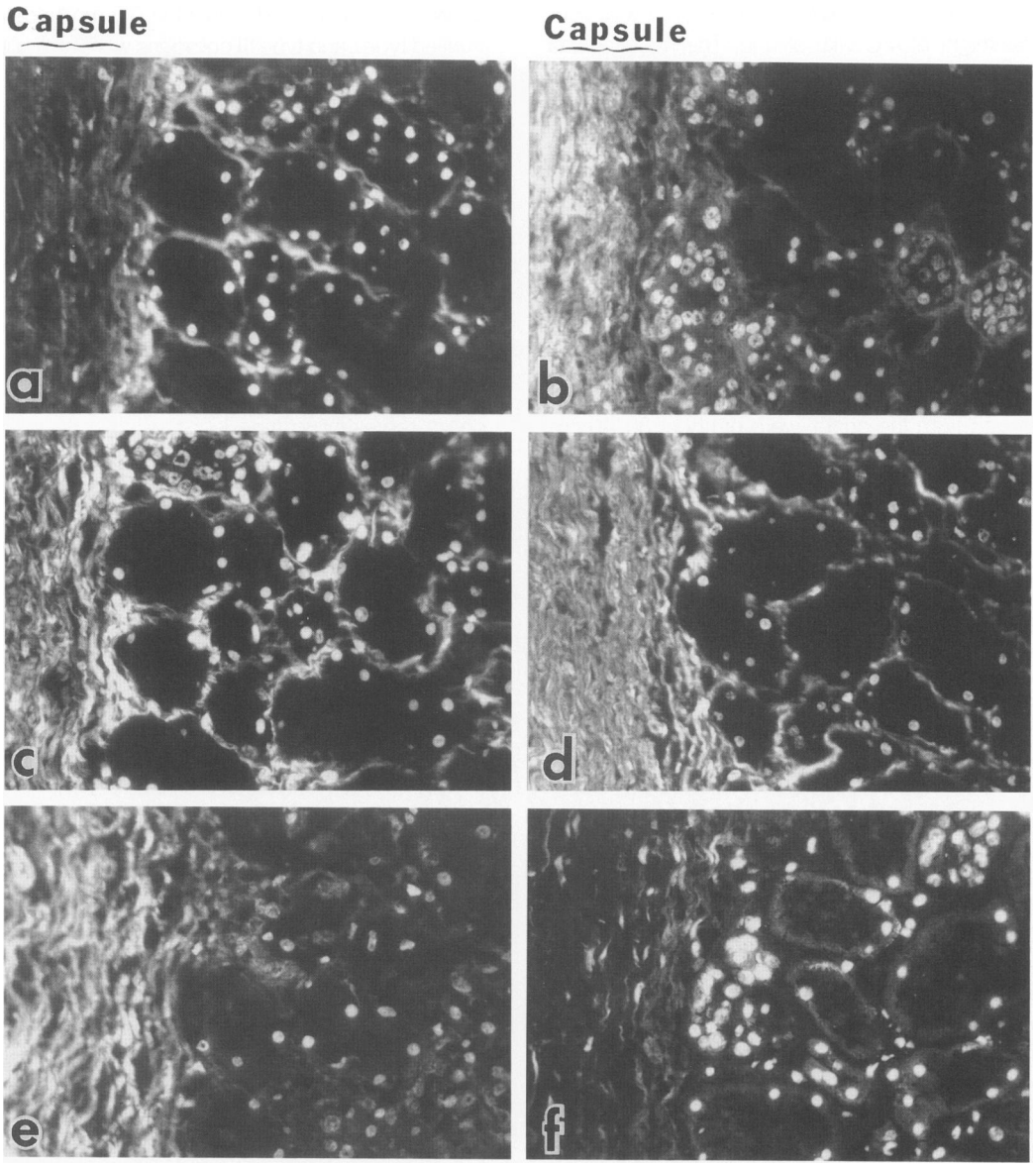

Figure 2. *Indirect immunofluorescence microscopic examination of kidney visceral capsule and adjacent cortex.* **a:** *Aminopropeptide of type I procollagen is only present in the cortex.* **b:** *Type I collagen is only present in the capsule.* **c:** *Aminopropeptide of type III procollagen is present in the capsule and cortex.* **d:** *Type III collagen is present in the capsule and cortex.* **e:** *Decorin is only present in the capsule.* **f:** *Control with nonspecific IgG shows no fluorescence of capsule and cortex. Nuclei of cells stained with propidium iodide, ×560.*

Purification of Insoluble Collagen from Kidney Capsule and Cortex

The visceral capsule was removed from adult human kidney, cut into small pieces, and placed in acetone for 3 days (three changes daily) followed by 50/50 acetone-ether (0.5 hr) and ether (2 hr) to defat the tissue. Dry and defatted tissue (100 mg) was homogenized in 6 mol/l guanidine-HCl (pH 7.4) that contained the protease inhibitors phenylmethylsulfonyl fluoride (3 mg/l), p-chloromercuribenzoate (3 mg/l), and iodoacetamide (5×10^{-4} mol/l) using a Virtis (model 60 K) homogenizer at 24,000 rpm for 10 minutes. After an additional 5 ml of extraction medium was added, the extraction was continued overnight at 4°C with stirring. The material was centrifuged (10,000 rpm for 30 min), and the pellet was washed three times with distilled water and lyophilized. The cortex was homogenized in the same extraction medium using a Potter-Elvejhem homogenizer and protein processed as with the capsule. Aliquots of the capsule and cortex pellets were removed before lyophilization and processed for electron microscopic examination to judge the purity. The capsule revealed a rather homogeneous population of cross-striated collagen fibrils of 60–70 nm diameter, which frequently had a tendency to form larger structures consisting of several fibrils. The collagen fibrils derived from the cortex were of about 30–40 nm diameter and showed no tendency to coalesce (Figure 1).

Cyanogen Bromide Digestion and Electrophoresis

Lyophilized kidney capsule and cortex prepared as described earlier (10 mg) were added to 1 ml 70% formic acid containing 1g CNBr. The digestion was allowed to proceed overnight at room temperature. At the end of the reaction, approximately 200 ml of water was added and the samples were lyophilized. After lyophilization, the CNBr peptides were dissolved in 0.5 mol/l acetic acid. Purified type I and type III collagens were also subjected to CNBr digestion to serve as standards for analysis by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). CNBr peptides were analyzed by SDS-PAGE using a 11–23% acrylamide gradient gel in a system described by Laemmli.[17] Samples were added to Laemmli buffer containing 5% 2-mercaptoethanol and heated in a boiling water bath for 2 minutes before electrophoresis. Peptides were stained with Coomassie blue and bands were scanned using a Beckman DU8 spectrophotometer (Beckman Instruments, Inc., Irvine, CA) equipped with a gel scan cassette. The area under peaks representing type I or type III collagen-specific

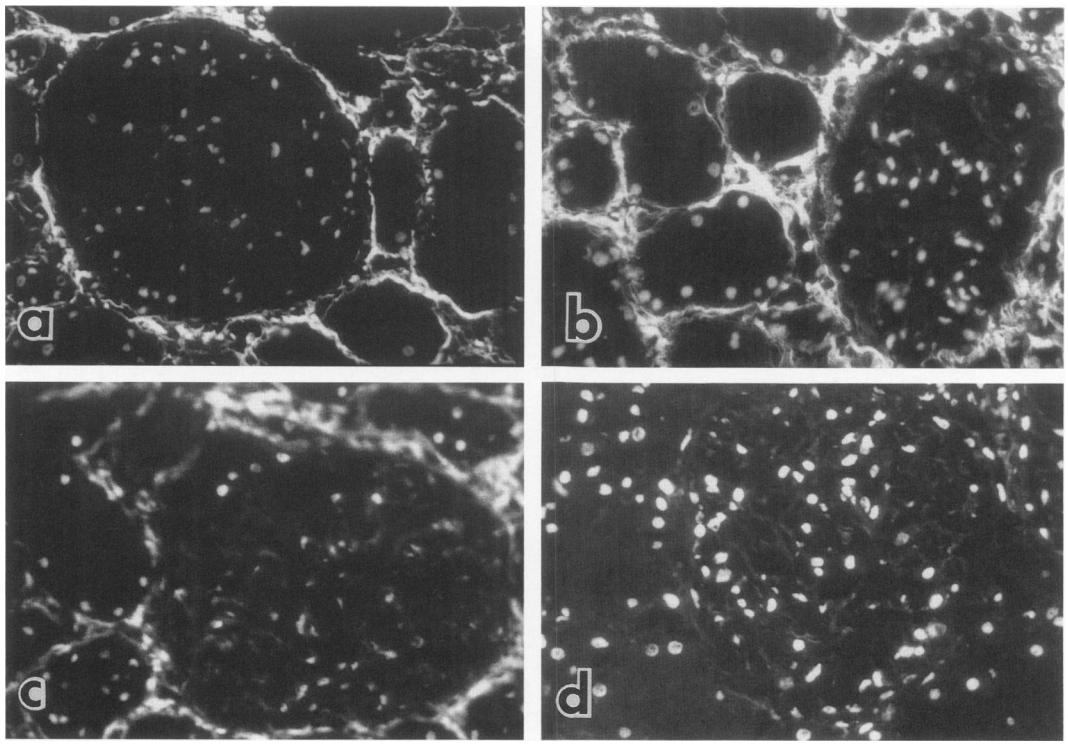

Figure 3. *Indirect immunofluorescence microscopic examination of kidney cortex;* **(a)** *type III collagen, and* **(b)** *the aminopropeptide of type III procollagen are present around glomeruli and adjacent tubules;* **(c)** *aminopropeptide of type I procollagen is demonstrated around a glomerulus;* **(d)** *decorin is absent, ×560.*

peptides was integrated and compared. To further identify the aforementioned samples as collagen, amino acid analysis was performed. Lyophilized CNBr samples were hydrolyzed for 24 hours in constant boiling HCl at 107°C under vacuum. Amino acid residues were derivatized with 9 fluorenyl methyl chloroformate[18] and resolved by reverse phase high performance liquid chromatography. The fluorescence was monitored using a Kratos FS 970 spectrophotometer.[19] The amino acid analysis indicated that the CNBr peptides studied by SDS-PAGE consisted primarily of collagen (Capsule, (res/1000 res) Pro 106, OH Pro 112, Gly 364; Cortex (res/1000 res) Pro 89, OH Pro 102, Gly 310).

Results

Immunofluorescence Microscopic Examination

The capsule stained well with antibodies directed against type I and type III collagens and decorin (Figure 2b, d, e). The aminopropeptide of type III procollagen was present in the capsule but not the aminopropeptide of type I procollagen (Figure 2a, c). The reticulin fibers around the glomeruli, tubules, and capillaries stained for type III collagen and its corresponding aminopropeptide (Figure 3a, b). On the other hand, staining for type I collagen and its extension N-propeptide was light (Figure 2a, b). However, there were isolated areas where reticulin fibrils around glomeruli retained the aminopropeptide for type I collagen (Figure 3c). Decorin was consistently absent in all areas of the cortex (Figure 3d).

Immunoelectron Microscopic Examination

A fibril was regarded as labeled when gold particles were present at the fibril surface for at least four consecutive periods of 67 nm. Fibrils were also regarded as positively labeled when gold particles were present in a spiral-like configuration around the fibril.

Double-labeling experiments were carried out using the capsule and cortex with antibodies to type I and type III collagens. Histograms were constructed to determine the range in fibril diameter of those fibrils labeling for type I collagen; those that labeled simultaneously for both type I and type III collagens and those that labeled only for type III collagen (Figure 4). In the capsule collagen fibrils were tightly packed and although their diameter ranged from 25 to 115 nm, about 90% were in the range of 60–80 nm. Type I collagen labeling was noted in thin collagen fibrils about 25–40 nm, whereas double labeling for type I and type III collagens was present in fibrils ranging in diameter from 55 to 65 nm. Single labeling for type III collagen was seen in fibrils ranging from 60 to 80 nm

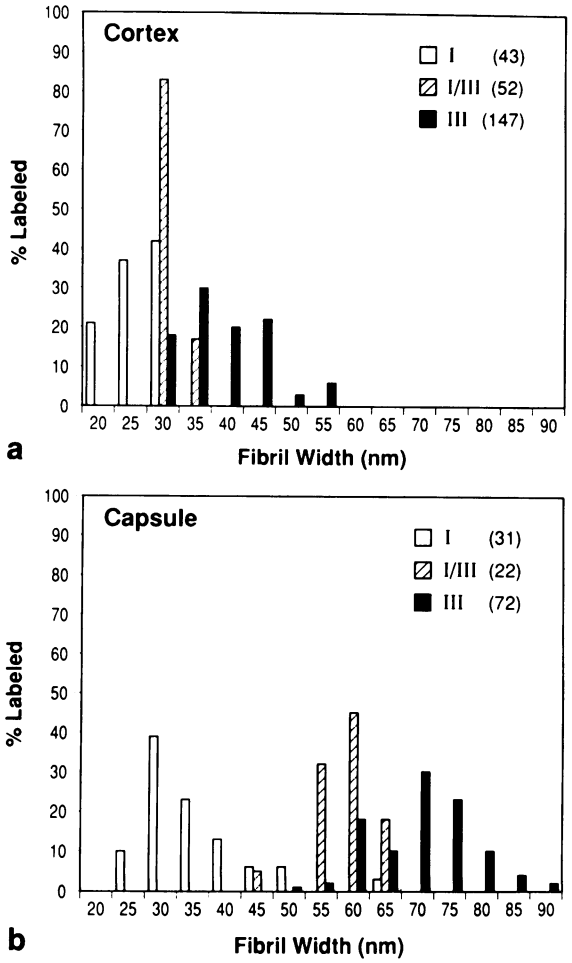

Figure 4. *Histogram of type I and type III collagen fibril thickness in* (a) *kidney cortex and* (b) *capsule. Specimens were exposed simultaneously to antibodies directed against type I and type III collagens. ( ) represents the number of fibrils observed in each category (I, I/III, III). The percent labeled is based on 100% for each category.*

(Figure 5). However, in terms of absolute counts, 90% of the collagen fibrils in the capsule only labeled with type III collagen, suggesting that in most fibrils the epitopes for type I collagen were masked. The aminopropeptide of type III procollagen could be demonstrated at the site of fibrils ranging in diameter from 30 to 60 nm, whereas beyond those diameters it was apparently cleaved or masked (Figure 6a). On the other hand, collagen fibrils in the capsule did not retain the aminopropeptide of type I procollagen (Figure 6b). Most collagen fibrils in the capsule labeled with antibodies directed against decorin in a periodic or spiral manner (Figure 6c).

In the cortex, collagen fibrils ranged in diameter from 20 to 55 nm although about 90% of the fibrils counted were in the range of 30 to 45 nm. Thin fibrils 20–25 nm in diameter only labeled with type I collagen antibodies (Figure 7a). Those in the range of 30–35 nm showed double labeling for type I and type III collagen (Figure

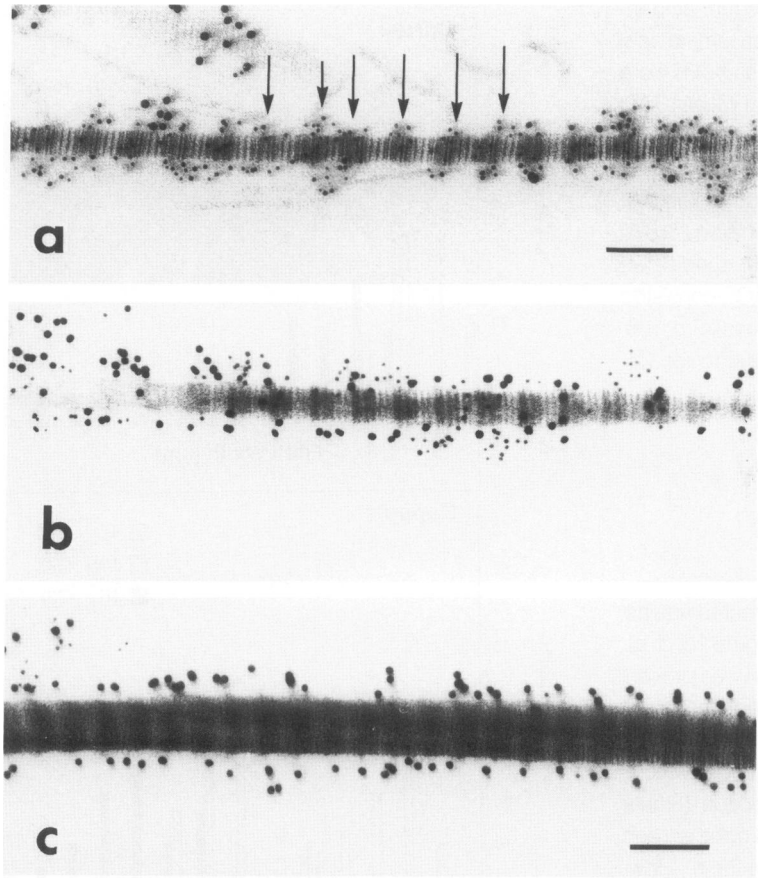

Figure 5. *Double-immunolabeling of kidney capsule collagen fibrils with antibodies against type I (5 mm gold) and type III (10 nm gold) collagens;* **(a)** *a 25-nm collagen fibril showing periodic labeling (arrows) mainly for type I collagen,* **(b)** *a 45-nm collagen fibril showing simultaneous labeling for type I and type III collagen,* **(c)** *a 75-nm collagen fibril showing mainly labeling for type III collagen. Bar = 100 nm.*

7b). Most fibrils however labeled only with type III collagen antibodies and showed a wide range in diameter between 30 to 55 nm (Figure 7c). Few fibrils showed at their surface the aminopropeptide of type I procollagen but those that did label showed a fibril diameter from 20 to 30 nm (Figure 8b). The aminopropeptide of type III procollagen labeled most fibrils usually in the range of 25–35 nm in diameter (Figure 8a). Reticulin fibrils in the cortex did not label with decorin antibodies (Figure 8c).

Cyanogen Bromide Peptides

Sixty two percent of the cortex and 84% of the capsule preparation was solubilized after cyanogen bromide digestion. Amino acid analysis of the insoluble fraction from the cortex demonstrated that it was primarily type IV collagen. CNBr soluble peptides representing type I and type III collagens were resolved using SDS-PAGE and 11–23% gradient gels (Figure 9). Peptide bands alpha 1 (I) CB8 and alpha 1(III) CB8 were used to determine the relative amounts of type I and type III collagens present.[20] The locations of alpha 1 (I) CB8 and alpha 1 (III) CB8 are depicted in a typical densitometric scan of a CNBr digest of type I collagen and a mixture of type I and type III collagens (Figure 10a). A typical densitometric scan of a CNBr digest of adult human kidney capsule and cortex is shown in Figure 10b. It can readily be seen that the capsule is composed primarily of type I collagen, alpha 1 (I) CB8 compared with alpha 1 (III) CB8, whereas in the cortex the relative concentrations of these two peaks are reversed (Table I).

Discussion

The nature and biologic significance of reticulin remains poorly understood. Reticular fibrils were recognized by Kuffer in 1876[21] but their actual tissue distribution remained unknown until Maresch in 1905[22] applied the silver impregnation method of staining. Since then, histologists and pathologists recognized the presence of reticulin fibrils during embryonic development, wound healing, and in various disorders including myeloproliferative disorders accompanied by myelofibrosis (polycytemia vera, chronic granulocytic leukemia, idiopathic myelofibrosis),[23] liver cirrhosis,[2] Hodgkin's[5] and non-Hodgkin's lymphomas,[24] and scleroderma.[25,26] The compound responsible for the argyrophilia of reticular fibers remains unknown. Pras and Glynn[27] isolated from

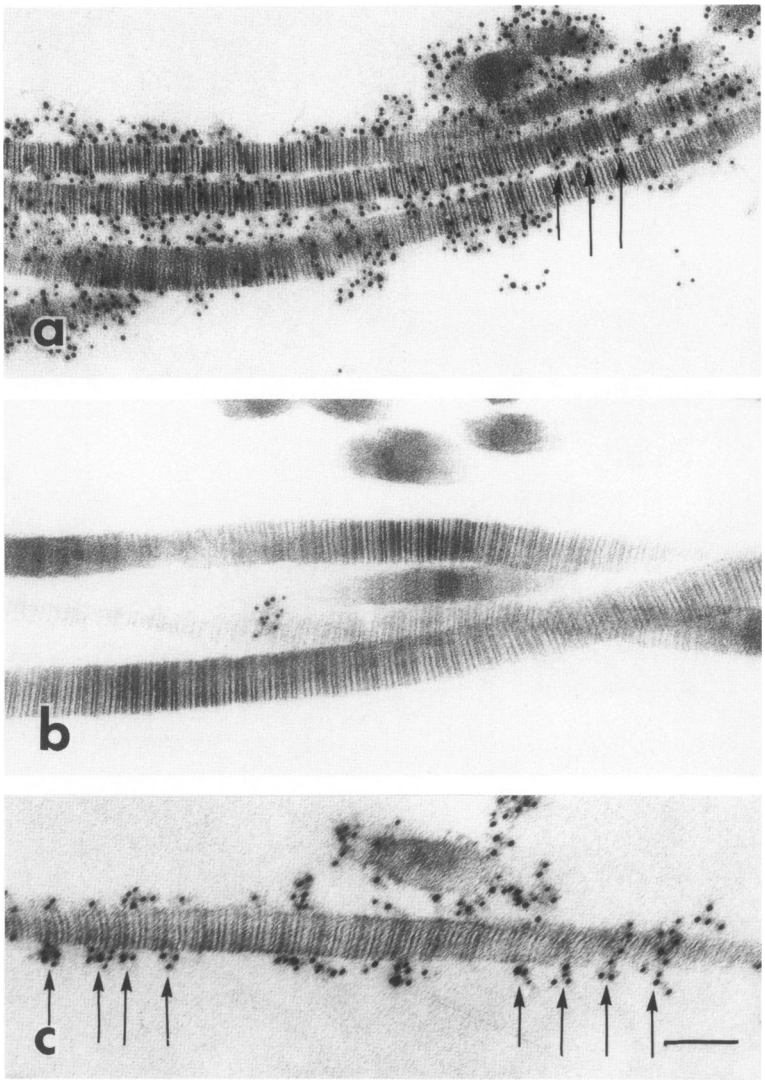

Figure 6. *Single gold-immunolabeling of kidney capsule.* **a**: *Note intense labeling with antibodies directed against the aminopropeptide of type III procollagen. Some labeling with periodicity is present (arrows).* **b**: *No labeling is present when antibodies against the aminopropeptide of type I procollagen are used.* **c**: *Labeling with decorin antibodies showing some periodicity. Bar = 100 nm.*

reticulin a noncollagenous protein that was water soluble, argyrophilic, and isotropic. Puchtler and Waldrop[28] showed that silver staining of reticulin could be abolished by treatment for 2 hours at 60°C with distilled water, thus suggesting that the collagen fibril, per se, was not responsible for the argyrophilia. Silver affinity alone cannot be used as a specific criteria to identify reticulin. It appears that tissues with active collagen synthesis such as seen during embryogenesis, woundhealing, and in certain fibrotic diseases, initially produce collagen fibrils that are thin and stain with silver. However, as this mechanism develops further those fibrils that continue to grow to reach diameters of 50 or more nm progressively lose their affinity for silver whereas those that do not grow beyond 40 nm in diameter retain the argyrophilia permanently. It is likely that only those collagen fibrils that fulfill the last criteria are actually reticulin. Most argyrophilic collagen fibrils in wounds do not contain the noncollagenous component of reticular fibers described by Pras et al.[29] Reticular fibers also codistribute with other components of the connective tissue including fibronectin[4,30] and basement membrane substances such as type IV collagen and laminin.[31–33] Our study showed that reticulin in the kidney cortex represents a hybrid of type I and type III collagens. Fibrils in an early stage of development, up to 25–30 nm, consist of type I collagen which retains its aminopropeptide. This finding correlates well with previous observations in chick skin and bone and in human skin suggesting that the aminopropeptide of type I procollagen may have a function during the initiation of fibrillogenesis.[34–36] Larger reticular fibrils, ranging from 30–35 nm in diameter, label simultaneously for type I and type III collagens. However, beyond 35 nm up to 55 nm all reticular fibrils only label for type III collagen. Furthermore, another striking feature is the retention of the aminopropeptide of type III procollagen at the surface of the fibril with a characteristic 67 nm periodicity. It is likely that previous studies with adult human skin, showing 40 nm

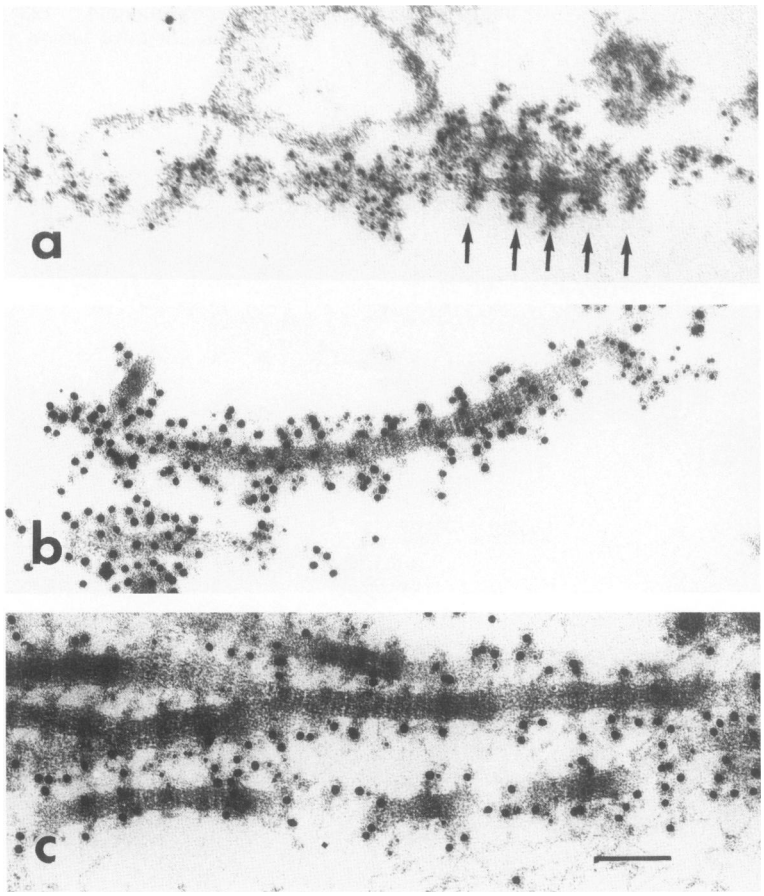

Figure 7. *Double-immunolabeling of kidney cortex collagen fibrils with antibodies against type I (5 nm gold) and type III (10 nm gold) collagens.* **a**: *a 25-nm collagen fibril showing periodic labeling (arrows) for type I collagen.* **b**: *a 32-nm collagen fibril showing labeling for type I and type III collagens.* **c**: *Several 40 nm collagen fibrils labeling mainly for type III collagen. Bar = 100 nm.*

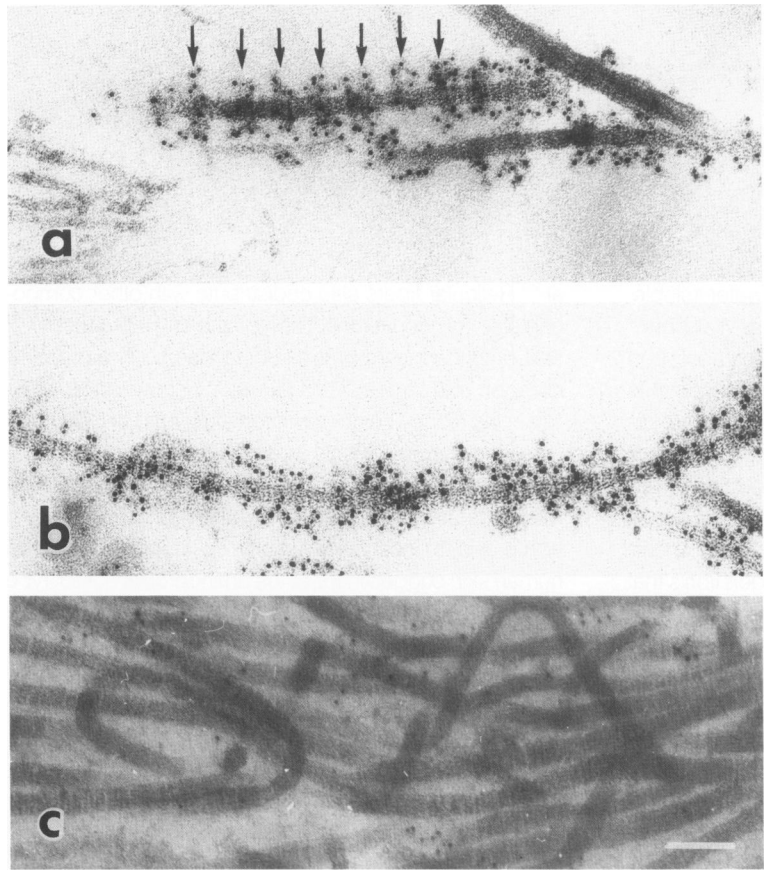

Figure 8. *Single gold-immunolabeling of kidney cortex.* **a**: *Labeling with antibodies against the aminopropeptide of type III procollagen showing periodicity (arrows).* **b**: *Labeling with antibodies against the aminopropeptide of type I procollagen without discernable periodicity.* **c**: *Labeling with antibodies against decorin is negative. Bar = 100 nm.*

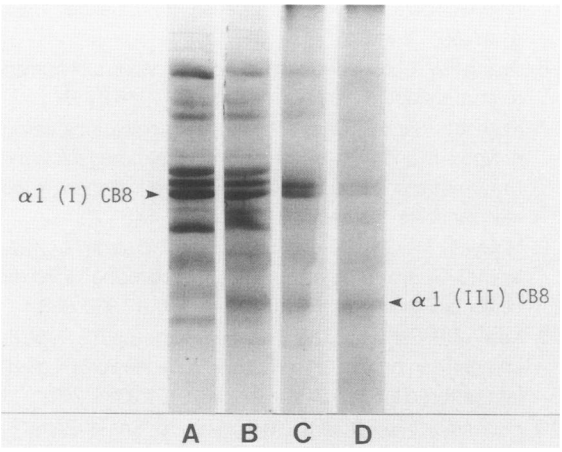

Figure 9. *SDS-polyacrylamide gel electrophoresis of CNBr digests of pure type I, type III collagens and adult human kidney capsule and cortex. CNBr peptides were separated on a 11–23% Phorcast gel (Amersham) and stained with Coomasie Blue. Lane A, type I collagen; Lane B, a mixture of type I and type III collagen; Lane C, kidney capsule; Lane D, kidney cortex. See Material and Methods for preparation of the extracts. Alpha 1(I) and alpha 1(III) CB8 bands were selected as markers for type I and type III collagens. These bands were used for determining type I/type III ratios presented in Table 1.*

fibrils with the aminopropeptide of type III procollagen at their surface also identified reticular fibrils.[37] Larger collagen fibrils up to 60 nm in diameter may also show the aminopropeptide of type III procollagen but the labeling is usually sporadic and often lacks periodicity. The presence of type III collagen and its aminopropeptide at the surface of reticular fibers opens interesting questions about its possible function. We have previously suggested that pN-type III collagen may play a role in regulating the growth of fine fibers into larger diameter fibers.[10,35] It appears that the aminopropeptide of type III procollagen is present at the surface of type I collagen fibers when full growth has been obtained.[10] It is also reasonable to speculate that pN-type III collagen could be associated, or may interact, with other components of the extracellular matrix like glycoproteins and proteoglycans. In this regard, we could not demonstrate decorin associated with reticular fibrils although it was present in the collagen of the capsule. In a previous study, we showed that decorin interacts with fibrillar collagens in fetal and adult human skin.[38] On the other hand, reticulin may interact with proteoglycans like heparan sulfate as was previously suggested.[39] Recently type V collagen has been reported to be associated with reticular fibrils in monkey liver.[40]

The current study suggests that type III collagen is a major component of reticular fibers. At present, we do not know whether type III collagen is restricted to the periphery or is present throughout the structure of the fibril. In a

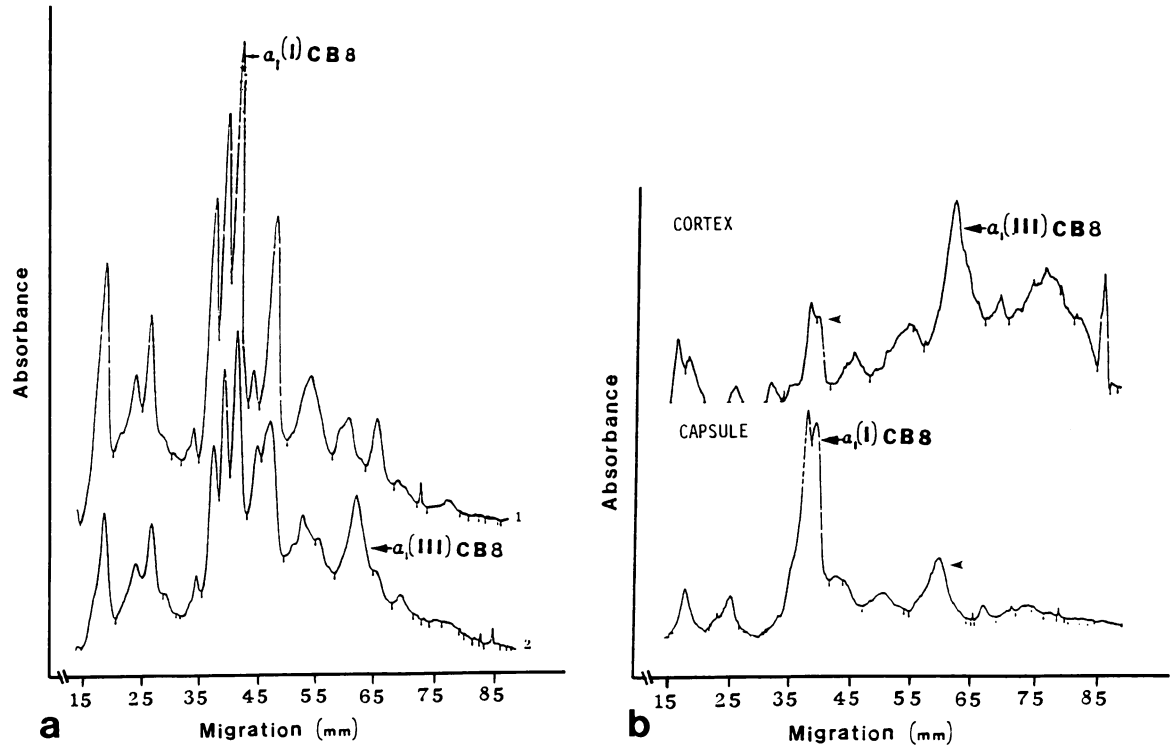

Figure 10. *Densitometric scans of an SDS-polyacrylamide gel separation of CNBr peptides derived from human kidney capsule and cortex.* **a**: *Scan of pure type I collagen CNBr peptides (upper scan) and a mixture of type I and type III collagen CNBr peptides (lower scan).* **b**: *Scan of CNBr peptides from kidney cortex (upper scan) and kidney capsule (lower scan). Note relative amounts of alpha 1(III) CB8 to alpha 1(I) CB8 in kidney cortex and capsule. See Materials and Methods for preparation of CNBr peptides.*

Table 1. *Percent Type I and Type III Collagens Present in Cortex (Reticulin) and Parietal Capsule*

	Human Kidney		
	(I)	%	(III)
Capsule	76		24
Cortex	18		82

previous study, adult human skin was treated with 8 mol/l urea to disrupt the microfibrils followed by double-labeling with type I and type III antibodies. That study showed that dermal fibrils consist mostly of type I collagen whereas type III collagen was restricted to the periphery of the fibril.[12] A similar study was performed with the kidney cortex but 8 mol/l urea treatment for 18 hours did not disrupt the reticular fibrils. Since the ratio of type III/type I in reticulin is high (Table I), type I collagen is probably restricted to the core whereas the rest of the fibril consists mostly of type III collagen with its amino-propeptide at the periphery. On the other hand, regular dermal collagen, which is composed of about 85% type I and 15% type III collagens, consists mostly of type I collagen with type III collagen restricted to the periphery. Thus, the ratio of type III/type I collagen probably varies in different fibrillar collagens depending on their specific function.

Acknowledgments

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