Identification of Collagen Fibrils in Scleroderma Skin*

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Skin from early and late stages of scleroderma has been shown to contain large amounts of thin (30–40 nm diameter) collagen fibrils that may be present in bundles or intermingled with large diameter fibrils (90–120 nm). The nature of these fibrils is unknown. Skin biopsies were obtained from involved areas of nine patients with progressive systemic sclerosis (PSS), one case of generalized morphea, one case of morphea, and six normal controls. Intact skin was analyzed by immunoelectron microscopy (IEM), while extracts were subjected to sodium dodecyl polyacrylamide gel electrophoresis (SDS-PAGE), Western immunoblotting, radioimmunoassay (RIA), and enzyme-linked immunosorbent assay (ELISA). Fine fibrils 20–40 nm in diameter in the mid to lower dermis of scleroderma skin were labeled with antibodies directed against the aminopropeptide (AP) of type III procollagen. Antibodies directed against the AP of type I procollagen labelled fine fibrils in the lower dermis. Larger fibrils (80–120 nm) did not label. pNα_1 (III) was found to be present in both normal and scleroderma skin. Extracts of scleroderma skin contained 2.5 times the amount of pN (III) collagen and 3.0 times the amount of fibronectin as did extracts of normal skin. The data indicate that the increase in thin fibrils in scleroderma skin is most likely due to an increase in type III collagen, which retains the AP at its surface.
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One of the most striking features in systemic scleroderma is the massive deposition of collagen in various organ systems including skin, lung, gastro-intestinal tract, heart, etc. It is well established that fibroblasts derived from scleroderma skin synthesize more collagen as compared to normal controls [1–4]. This exaggerated collagen production is associated with fibroblasts derived from the lower dermis [2,4–6]. However, with multiple subpassages, this phenotypic expression does not appear to be retained [6]. Fibronectin is another matrix component that has been shown by immunofluorescence microscopy to be increased in scleroderma in the lower dermis [7-9]. This is consistent with its function as a matrix protein produced in large quantities by fibroblasts which serves to link them to their collagenous matrix [10,11].

It has been demonstrated that scleroderma skin in early and late stages of the disease contains increased amounts of thin collagen fibrils (30–40 nm in diameter) that are present either in bundles or intermingled with large diameter fibrils [12-15]. It is not known whether these thin collagen fibrils represent type I or type III collagen. Previous electron microscopy studies showed that type I collagen fibrils reach maturity at about 90–120 nm, while type III collagen reaches maturity at 30–40 nm [8,9]. Immunofluorescence microscopy with antibodies directed against type I and type III collagens and their corresponding aminopropeptides (AP) revealed the presence of type I and type III collagen throughout the dermis and subcutaneous tissue in scleroderma skin [8]. Indirect immunoelectron microscopy of scleroderma skin using specific collagen antibodies to type III collagen labeled fine fibrils 20–40 nm in diameter while antibodies to type I collagen labelled fibrils with diameters greater than 60 nm [16]. When the AP is retained by the collagen molecule, the collagen is termed pN collagen. It has been shown that the AP of type I and type III collagen is present at the surface of fine collagen fibrils up to 30–40 nm in diameter, but not in larger diameter fibrils in normal human skin [9].

The purpose of this paper was to determine whether the fine fibrils observed in the scleroderma dermis were pN collagen (type I and/or type III). As part of this study we also determined the concentration of pN type III collagen and fibronectin in extracts of scleroderma skin.

MATERIAL AND METHODS

Skin Biopsies Excision skin biopsies were performed under local anesthesia from the dorsum of the hand or external surface of the forearm of patients with early (less than 1 year), mid (1–2 years), or late (2–5 years) stages of systemic scleroderma (Table I). Controls consisted of normal skin specimens obtained from matched volunteers or from surgical specimens.

Preparation of Antibodies Type I and type III aminopropeptides were prepared from dermatosparactic calf skin type I and calf skin type III pN-collagens. Purified antibodies against the aminopropeptide (AP) of type I and type III procollagens were prepared as described by Nowack et al [17]. Antisera were obtained from rabbits and purified by precipitation, DEAE cellulose chromatography, and immunoabsorption on native or denatured procollagens. There was negligible cross-reactivity between the antibodies against the AP of

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Abbreviations:
AP: aminopropeptide
EDTA: ethylenediaminetetraacetic acid
ELISA: enzyme-linked immunosorbent assay
IEM: immunoelectron microscopy
NaN_3: sodium azide
pNα_1: alpha chain of collagen containing the aminopropeptide
proα_1: alpha chain of procollagen
PSS: progressive systemic sclerosis
RIA: radioimmunoassay
SDS-PAGE: sodium dodecyl sulfate-polyacrylamide gel electrophoresis

Table I. Clinical Profile

Case	Age	Sex	Duration	Biopsy Site	Clinical Picture	Skin Involvement	Diagnosis
1	37	F	5 years	Chest	Raynaud's arthralgia, dysphagia, constipation, myalgia	Face, chest, abdomen, arms, legs, ulcers	PSS
2	58	F	2 years	Forearm	Raynaud's, dyspnea, arthralgia	Face, hands, arms, erythema, telangiectasis	PSS
3	61	F	1½ years	Forearm	Raynaud's, Sjogren's	Face, chest, arms	PSS
4	32	F	3 years	Hand	Raynaud's, dyspnea	Face, hands, edema	PSS
5	79	F	1½ years	Forearm	Arthritis, Raynaud's, Sjogren's	Edema, ulcers, hands, feet, legs, pruritus	PSS
6	48	F	3 years	Hand	Raynaud's, dysphagia, dyspnea, arthralgia	Edema, hands, forearm	PSS
7	50	F	7 months	Forearm	Arthritis, Raynaud's, dyspnea	Edema, erythema, face, arms, hands, ulcers	PSS
8	48	F	9 months	Arm	Raynaud's, dyspnea, hypertension, urinary infection	Face, chest, abdomen, arms, legs	PSS
9	70	F	3 years	Forearm	Raynaud's, arthritis, hypertension, urinary infection, dyspnea, dysphagia	Face, hands, chest, back, abdomen	PSS
10	50	F	9 years	Thigh	Negative	Chest, abdomen, arms, legs	Generalized morphea
11	46	M	4 months	Waist Forearm	Negative	Forearm, waist	Morphea

type I and type III procollagen as demonstrated by hemaglutination and radioimmunoassays.

Purified antibodies against human plasma fibronectin were obtained from Cappel Laboratories (Malvern, PA). All of the purified antibodies were found to cross-react with their equivalent antigens in human skin using indirect immunofluorescence microscopy [8].

Immunoelectron Microscopy Labeling and fixation of specimens was carried out at 4°C as previously described [9]. Skin specimens were cut into slices (1 × 0.2–0.5 mm) and fixed in 1% glutaraldehyde in 0.16 M cacodylate buffer, pH 7.4, for 20 minutes. They were rinsed 3 times for 10 minutes with buffer and 3 times for 1 hour with 0.15 M Tris-HCl, pH 7.4. Fixed specimens were incubated with 50–80 μg purified antibody in 0.1 ml of phosphate-buffered saline with gentle shaking for 24 hours. After removal of antibody, the specimens were washed in several changes of 0.15 M Tris-HCl, pH 7.4 (16 hours) and incubated for 24 hours in 0.1 ml of ferritin-labelled goat anti-rabbit IgG diluted 1 : 5 (Cappel Laboratories, PA). The samples were then washed for 24 hours with 0.15 M Tris-HCl, pH 7.4, followed by three or four 10-minute washes with 0.16 M cacodylate buffer, pH 7.4. Sections were fixed in 1% OsO_4 for 90 minutes, dehydrated (graded alcohol, 50–100%), and embedded in Epon. Sixty to 80 nm thick sections were prepared with a Porter-Blume ultramicrotome. Sections were stained with 1% aqueous phosphotungstic acid/1% uranyl acetate/Reynold's lead citrate and examined in an electron microscope.

Extraction of Collagen From Biopsy Specimens Skin specimens were minced, using two scalpels, and pulverized in 0.125 M Tris-HCl buffer, pH 6.8, containing 2% sodium dodecyl sulfate (SDS) in the presence of proteinase inhibitors (phenylmethylsulfonyl fluoride, 3 mg/L), p-chloromercuribenzoate (3 mg/L), 0.01 M EDTA, and 0.5 mM iodoacetamide using a mortar and pestle. Samples were heated in a boiling water bath (100°C) for 5 minutes, centrifuged at 12,000 *g* for 30 minutes at room temperature and the supernatants used for Western immunoblotting, radioimmunoassay, and enzyme-linked immunosorbent assay (ELISA) procedures for the detection of pN type I and III collagen and quantification of pN type III and fibronectin.

SDS Polyacrylamide Gel Electrophoresis (PAGE) The PAGE procedure is based on that originally described by Laemmli [18]. Extracts were electrophoresed on polyacrylamide gels consisting of a 4.5% stacking gel and a 5.5% running gel at 5 mamp overnight. The stacking gel buffer and running gel buffers were 0.5 M Tris-HCl, pH 6.8, and 1.5 M Tris-HCl, pH 8.8, respectively. The tank buffer was 0.025 M Tris-HCl, pH 8.3, 0.19 M glycine, 0.1% SDS. All samples were reduced with β-mercaptoethanol before electrophoresis.

Western Blot The electrophoretic transfer of proteins from the polyacrylamide gel to nitrocellulose was based on the procedure of Towbin et al [19]. Protein bands were transferred from PAGE gels to nitrocellulose (0.45 μm pore size) at 30 V for 15 hours at room temperature. The nitrocellulose sheets were soaked in wash buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA, 0.05% Nonidet P-40, 0.02% NaN_3, 0.025% gelatin) at room temperature overnight, and treated for 6 hours with antibody directed against the aminopropeptide of proα_1(I) chains or proα_1 (III) chains (20 μg IgG/ml of the above buffer). Following treatment with antibody, the sheet was washed and incubated with ^{125}I-protein A (Amersham) for 1 hour at room temperature. After washing the nitrocellulose was exposed to x-ray film at −20°C with intensifying screens.

Radioimmunoassay (RIA) for pN III Collagen An RIA-procollagen-III-peptide kit (Behringwerke AG, Marburg, Federal Republic of Germany) for the radioimmunological determination of procollagen III peptide was used to determine the quantity of pN (III) present in the extracts.

ELISA for Fibronectin The ELISA procedure used to determine the quantity of fibronectin present in the extracts was based on that described by Rennard et al [20]. Ninety well flat bottom and U-shaped plates Linbro/Titertek (Flow Labs) were used to establish standard curves and for the assay of fibronectin in the skin extracts. Fibronectin and fibronectin antiserum were obtained from Cappel Laboratories. Plates were developed using anti-rabbit IgG (whole molecule) alkaline phosphatase conjugate (Sigma Chemical Co., St. Louis, MO) and Sigma 104 phosphatase substrate tablets. Absorbance was read at 405 nm using a Bio-Tek EIA Reader.

Fibril Diameter Distribution A morphometric analysis was performed by counting 1,012 fibrils in electron micrographs of specimens labelled for pNα_1 (III) and 939 fibrils in electron micrographs of specimens labelled for pNα_1 (I). A fibril was considered labeled if five or more ferritin cores were present along fibrils and the aggregates were arranged at about 60-nm intervals.

RESULTS

Immunoelectron Microscopy Using antibodies directed against the amino terminal extension of type I procollagen, fibrils 30–40 nm (Fig 1) and 30 nm (Fig 2*A*) in diameter were partially labeled in the lower dermis of scleroderma skin. Thick fibrils 80 nm in diameter or greater did not label with this antibody. In addition, antibodies directed against the aminopropeptide of type III procollagen labelled (Fig 2*B*) only the fine fibrils (30 nm in diameter) of the lower dermis while the thick fibrils remained unlabeled.

Figure 1. Systemic scleroderma. Note fine collagen fibrils, 30–40 nm which are partially labeled with antibody against the aminopropeptide of type I procollagen. Note that thick, 80-nm fibrils do not label. Uranyl acetate–lead citrate. ×60,000. *Bar* = 100 nm.

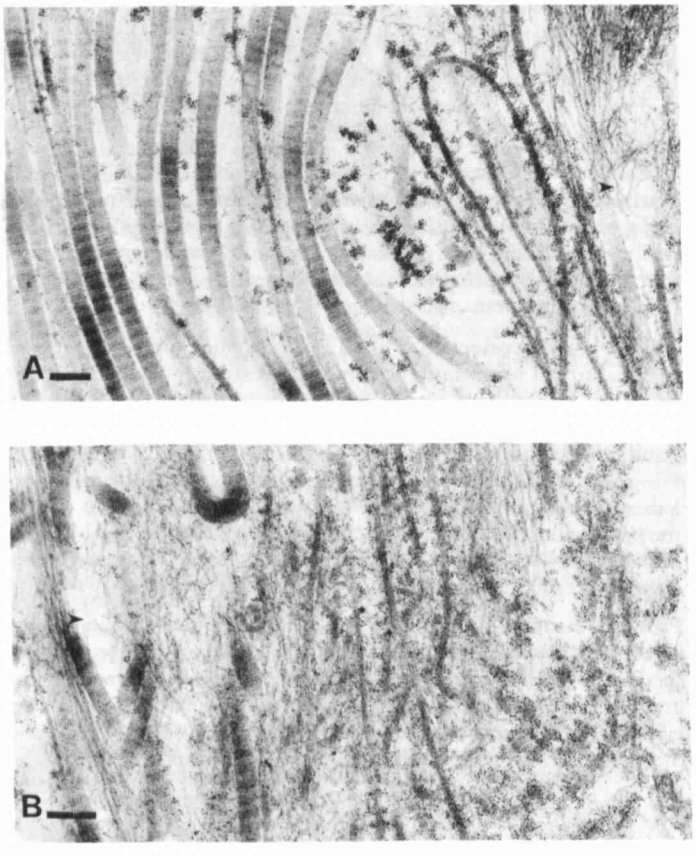


Figure 2. Generalized morphea. *A*, Lower dermis showing a few fine collagen fibrils, about 30 nm, which are partially labeled with antibody against the aminopropeptide of type I procollagen. *B*, Lower dermis showing fine collagen fibrils, about 30 nm, which are labeled with antibody against the aminopropeptide of type III procollagen. Note extremely thin fibrils (arrowheads, *A* and *B*) that did not label with either antibody. These may represent another type of collagen not yet well characterized or noncollagenous filamentous material. ×38,345. *Bar* = 200 nm.

Very thin fibrils (Fig 2*A*,*B*) did not label in the presence of either antibody. These very thin fibrils may represent another type of collagen not yet well characterized or noncollagenous filamentous material.

In the mid to lower dermis, bundles of type III collagen fibrils retaining the aminopropeptide were detected in areas in which fibrils with diameters from 10 to 40 nm were abundant (Fig 3). Periodic labelling (60–65 nm) was observed along fibrils ranging in diameter from 40 to 50 nm (Fig 4*A*). Treatment of skin samples with normal IgG followed by antibody-ferritin conjugate produced no staining (Fig 4*B*), which verifies the specificity of the uptake of label.

Fibrils that showed labelling with ferritin were measured and plotted in histogram form. The antibody directed against the extension aminopropeptide of type I procollagen was found to be associated with fibril diameters of 30 to 40 nm, while the antibody to the aminopropeptide of type III procollagen labelled fibrils with diameters in the range of 10 to 40 nm (Fig 5). Of the total number of type III fibrils labelled, 45% had diameters of about 10 to 19 nm. Fibrils with diameters greater than 60 nm were not labelled.

Western Blotting Western blot analysis of normal and scleroderma skin extracts revealed only the presence of pNα_1 (III) (Fig 6).

Determination of Procollagen III Peptide and Fibronectin Concentration in Normal and Scleroderma Skin Extracts A radioimmunoassay was performed on extracts of normal and scleroderma skin to determine the content of soluble type III procollagen. The amount of procollagen III peptide present in the extracts of normal and scleroderma skin was 10.2 ± 3.1 ng/mg wet weight and 26.5 ± 7.1 ng/mg wet weight, respectively (Table II).

An ELISA was performed on the extracts to determine the content of soluble fibronectin in the skin. The content of extractable fibronectin was approximately 3 times greater in scleroderma skin when compared to normal controls. The values were 159.4 ± 36.9 for scleroderma skin and 49.1 ± 8.8 ng/mg wet weight for normal skin, respectively (Table III).

The concentration of pN type III and fibronectin could not be determined in the residue because of the limited amount of biopsy skin available for analysis.

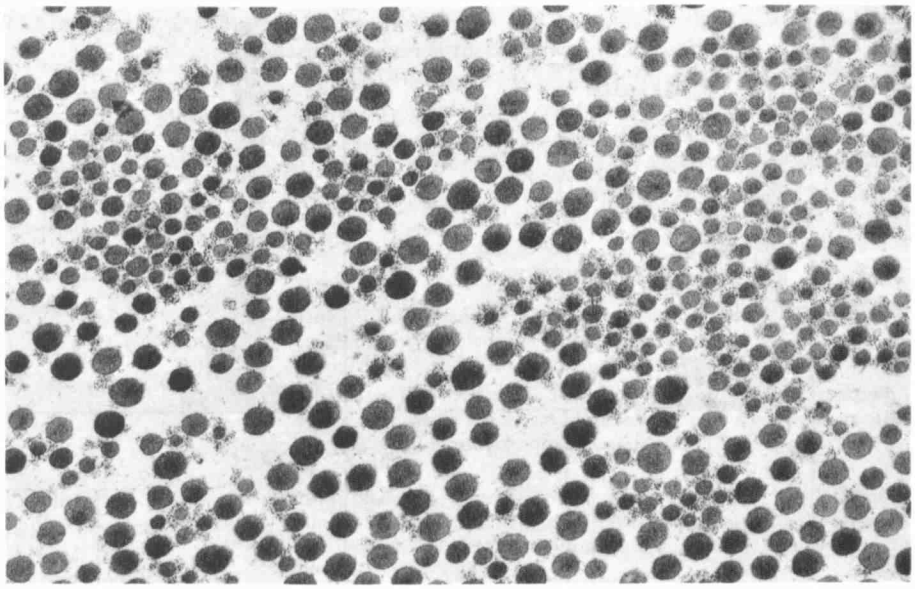


Figure 3. Systemic scleroderma. Cross-sections of collagen showing clusters of small fibrils labelled with an antibody against the aminopropeptide of type III procollagen. Uranyl acetate-lead citrate. ×38,345. *Bar* = 200 nm.

DISCUSSION

The classical concept in collagen fibrillogenesis is that fibroblasts synthesize collagen in a precursor form known as procollagen, with extension propeptides at the amino and carboxyl ends of the molecule. Following cleavage of the extension propeptides by specific proteases, the collagen molecules polymerize in the extracellular matrix to form fibrils.

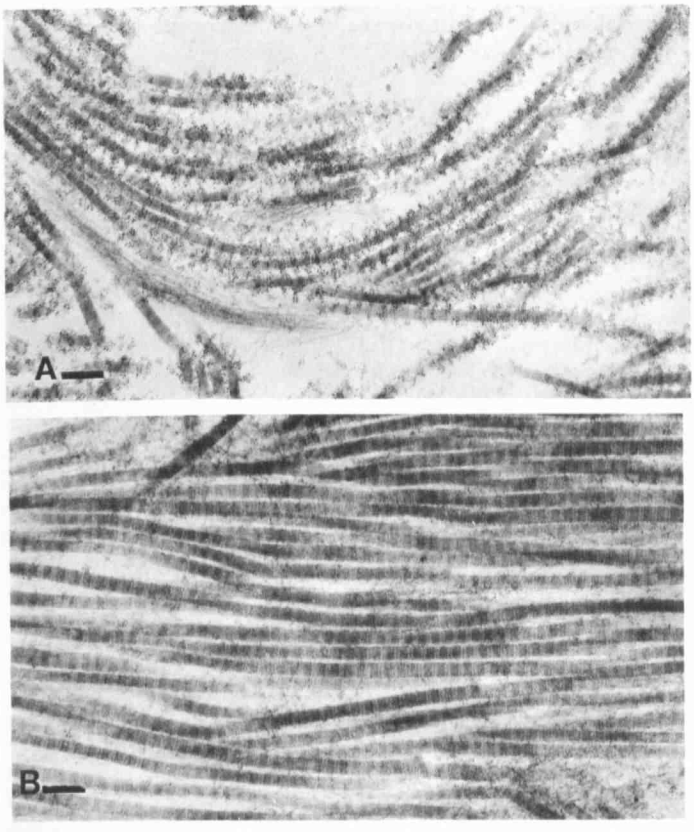


Figure 4. Systemic scleroderma. *A*, Note periodic labeling of fibrils, about 40–50 nm, which are labeled with antibody against the aminopropeptide of type III procollagen. *B*, Control labeled with nonimmunized rabbit IgG. Uranyl acetate–lead citrate. ×38,345. *Bar* = 200 nm.

Initial studies, performed with normal adult skin, showed that the AP was not free in the ground substance, but was actually incorporated into collagen fibrils [9]. The AP of type I collagen was present at the surface of fine collagen fibrils up to 30 to 40 nm in diameter, but not in large diameter fibrils. The AP of type III collagen was present in fibrils 30 to 40 nm in diameter and was periodically labelled at 60 nm [9]. The incorporation of the AP into thin collagen fibrils suggested the presence of pN collagen (procollagen minus the carboxyl propeptide). This was confirmed using embryonic chick skin [21]. It appears that pN-collagen rather than collagen is the unit for early polymerization of type I collagen, up to about 30 to 40 nm, at which time the propeptide is cleaved. pN-collagen is also involved in type III collagen fibril formation, but it appears that when the fibril reaches maturity at 30 to 40 nm, the AP is retained at the surface [21].

There is a possibility that the fibrils observed in scleroderma skin represent a mixture of type I collagen and pN type III collagen, ie, a hybrid. Such hybridization has recently been demonstrated in normal connective tissue [22]. Using a monoclonal antibody to the triple-helical domain of type III collagen, it was demonstrated that in skin, tendon, and amnion type III collagen was present on all banded collagen fibrils. Therefore, we may hypothesize that

Table II. pN Type III Collagen Content in Extracts of Normal and Scleroderma Skin

Scleroderma		Normal	
Case	ng/mg wet wt	Specimen	ng/mg wet wt
1	14.6	1	23.9
2	31.4	2	5.9
3	69.8	3	2.6
4	7.8	4	7.7
5	13.7	5	7.4
6	72.0	6	13.6
7	32.0		
8	6.7		
9	13.5		
10	21.5		
11	8.8		
Mean ± SEM[a]	26.5 ± 7.1		10.2 ± 3.1
$P < 0.1$[b]			

[a] Standard error of the mean.
[b] Level of significance; both tails of the *t* distribution considered.

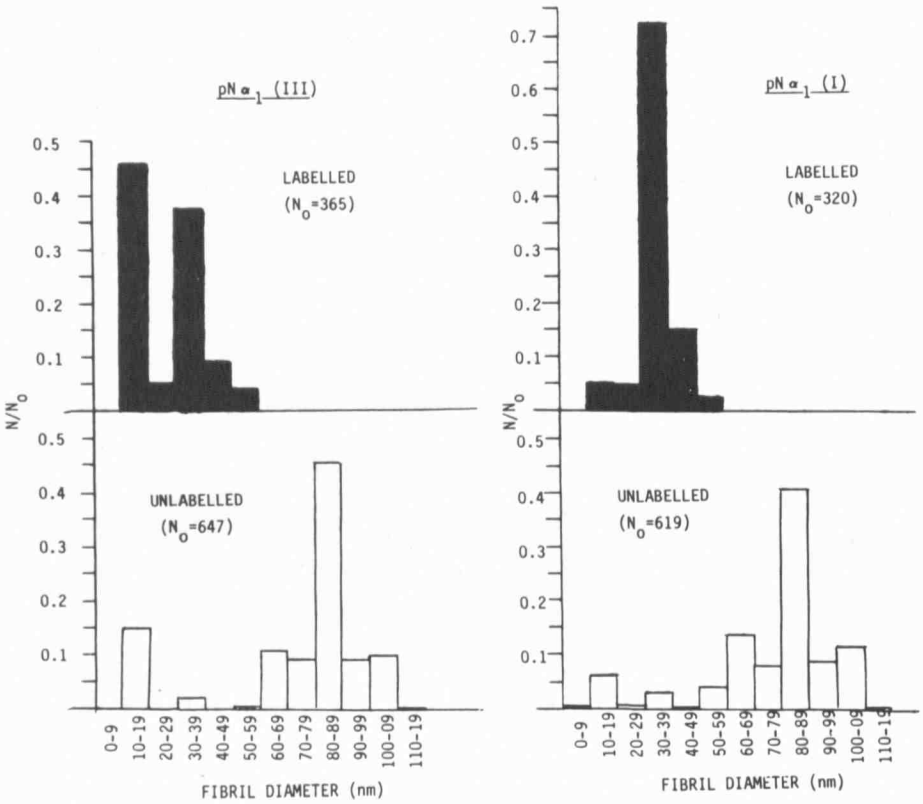
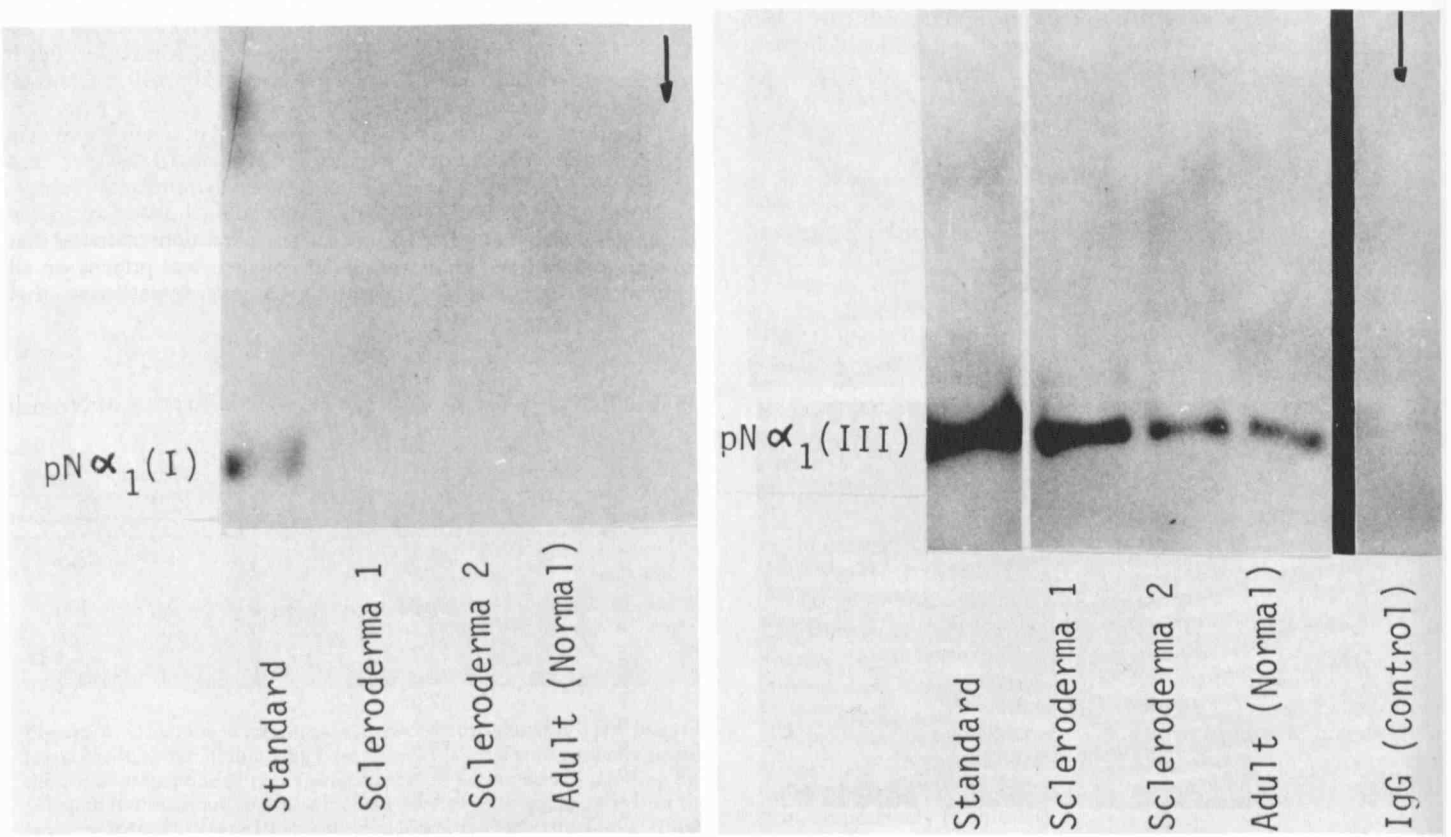


Figure 5. Histogram correlating fibril diameter with the aminopropeptide of type I and III collagen. Note that most of the fibrils that are labelled with antibodies directed against the pro α_1 (III) aminopropeptide had diameters ranging from 10 to 40 nm, while those labelled with antibodies directed against the pro α_1(I) aminopropeptide had diameters ranging from 30 to 40 nm. Most fibrils above 60 nm were unlabelled for both antibodies. N_0 = total number of fibrils counted; N = number of fibrils counted.



a b

Figure 6. Western blot analysis of scleroderma and normal skin extracts using antibodies directed against (A) the pro α_1(I) and (B) pro α_1(III) aminopropeptides. The results are typical of all scleroderma and normal extracts studied. Note presence of pN α_1(III) in all samples and absence of pN α_1(I).

Table III. Fibronectin Content in Extracts of Normal and Scleroderma Skin

Scleroderma		Normal	
Case	ng/mg wet wt	Case	ng/mg wet wt
1	91.7	1	67.7
2	122.9	2	45.0
3	302.5	3	31.2
4	149.3	4	30.1
11	130.8	5	71.3
Mean ± SEM	159.4 ± 36.9		49.1 ± 8.8
$P < 0.01$[a]			

[a] Level of significance; both tails of the *t* distribution considered.

growth of type I collagen fibrils may be regulated by its interaction with type III procollagen and therefore type III collagen becomes incorporated into the rapidly growing fibrils [22,23].

Immunofluorescence microscopy of normal skin has revealed the presence of the AP of type I collagen only at the epidermo-dermal junction [8,24,25] and around the hair follicles, while, in scleroderma skin, marked fluorescence has been reported to be present around small blood vessels and the mid to lower levels of the dermis and upper subcutaneous tissue [8]. The AP of type III procollagen was diffusely present throughout the dermis and increased in the lower dermis [8].

Using immunoelectron microscopy with antibodies directed against the AP of type I and type III procollagens, we have demonstrated that the AP of type I procollagen is restricted to fine fibrils having diameters of about 30 nm and located in the lower dermis of scleroderma skin. The presence of these fine fibrils suggests areas of active collagen synthesis. This agrees with "in vitro" fibroblast studies that show accelerated collagen synthesis by fibroblasts isolated from the lower dermis [2,4,5] and immunofluorescence microscopy studies [8].

We have also been able to establish that the fluorescence observed in scleroderma skin using antibodies directed against the AP of type III procollagen [8] is restricted to fine fibrils (10–30 nm in diameter) in the mid and lower scleroderma dermis. Since we were unable to detect pN I collagen in extracts of scleroderma skin using immunoblotting techniques, but could detect pN III, the data strongly suggest that most of the fine fibrils observed by others [13–15] represent pN III collagen.

We were unable to detect procollagen type III in our skin extracts by Western immunoblotting, indicating that our measurement of procollagen III peptide in our RIA was really a measure of pN III. We found a 2.5-fold increase in pN III content in the scleroderma skin extracts compared to controls. This agrees with in vitro studies with scleroderma fibroblasts [3,4]. The variability of extractable pN type III collagen values we observed with respect to the control values might be a reflection of the state of the disease at the time of the biopsy, ie, active versus inactive.

Fibronectin and collagen appear to be codistributed in tissues and cell culture [26]. Ultrastructural analysis has revealed a close association of fibronectin with various collagenous fibrils [27]. Fibronectin has been reported to be increased in the lower dermis of scleroderma skin by indirect immunofluorescence [7,8]. Fibronectin is synthesized by fibroblasts and functions as a link between cells and their collagen matrix [10,11]. There appears to be a concomitant increase in fibronectin synthesis with type III procollagen synthesis by scleroderma fibroblasts [4]. Therefore, one would expect fibronectin levels to be elevated in the scleroderma dermis as we have reported. It is interesting to note that extracts of scleroderma dermis from patient No. 3 had the greatest pN III content and also the greatest content of fibronectin.

In conclusion, it appears that the fine collagen fibrils found in clusters in the mid dermis and portions of the lower dermis in scleroderma contain at their surface pN III collagen.

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