Collagenase in Scleroderma

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ABSTRACT Collagenase activity was measured by direct assay in skins from 12 patients afflicted with systemic sclerosis. In seven of those cases where extensive involvement of the forearm and trunk skin existed, collagenase activity of the involved skin was minimal or absent. Moreover, in the same patient, regions of marked skin involvement (e.g., forearm) showed no collagenase activity, when clinically uninvolved areas (thigh) exhibited normal or nearly normal levels of enzyme activity. In other patients where clinical symptoms were systemic and not associated significantly with the skin, collagenase activity approximated normal levels. Measurements of collagenase activity and tensile strength in another condition (basal cell carcinoma) that includes changes in mechanical properties of skin that may be regarded as the opposite end of the spectrum from those of sclerodermatous skin support a general correlation between collagenase activity and tensile strength. These studies indicate that the major defect responsible for the hidebound skin lesions of scleroderma may be decreased collagenase activity.

INTRODUCTION

Although the general course of scleroderma of the skin passes through three stages of edema, induration, and then atrophy, the middle stage is most readily associated with the disease and attracts most attention. In the many notes and articles published on the subject of scleroderma little evidence can be found to suggest any chemical abnormalities of skin, or for that matter gross changes in the composition of connective tissue. The histological work of Fisher and Rodnan (1), as well as the biochemical evidence of Fleischmajer (2), support this point of view. Often in fact one is impressed that histological sections of skin show only minor and nonspecific changes. One clearly identifiable abnormality related to the physical properties of sclerodermatous skin is the increase in tensile strength (3). That observation might suggest more collagen in sclerodermatous skin. Harris and Sjoerdsma (4) detected a decrease in the acid-soluble collagen fraction, while Laitinen et al. and (5) measured variable amounts of neutral salt-soluble fraction, depending on the stage of the disease. Black et al. (6) measured the collagen content of sclerotic plaques from patients with systemic sclerosis and found that content per units surface area was not significantly different from normal controls. Rather, they believe the usual impression one gets of skin hardening and thickening in this disease is an illusion resulting from the increased tethering of skin to deeper structures. Fleischmajer et al. (7) described a replacement of subcutaneous tissue by connective tissue. Their findings therefore suggest a possible mechanism for the tethering of superficial skin to subcutaneous layers. On the other hand, if we believe with Fleischmajer and Perlish (8, 9) that a defect lies in the metabolism of the carbohydrate fraction of connective tissue ground substance, we are hard pressed to rationalize the substantial increase in tensile strength (3) of sclerodermatous skin. In point of fact, a crucial observation to account for in the cutaneous manifestations of this disease is this increase in tensile strength, and that can only be done by finding an altered state or quantity of cutaneous collagen. Of all fibrous macromolecules, collagen makes the overwhelming contribution to tensile strength in skin (10). Data on other components are peripheral and avoid the central issue, that something must be physically different about the network of collagen, because skin tensile strength is substantially raised.

Recently it has become possible to assay directly the activity of the enzyme collagenase by the method of Ryan and Woessner (11). Although in theory the absence of collagenase activity could account for much of what we observe in sclerodermatous skin, the past inability to follow reliably the activity of this enzyme

Dr. Brady is a Research Career Development Awardee of the National Cancer Institute, National Institutes of Health.

Received for publication 8 January 1975 and in revised form 23 June 1975.

has made evaluation of breakdown of the collagenous framework difficult. It is the purpose of this article to show that the apparent sclerosis in patients with scleroderma correlates quite well with the assayable levels of collagenase in the skin.

METHODS

Patients with cutaneous manifestations of scleroderma were identified both on clinical and histological grounds. All patients chosen for their cutaneous involvement had stiff skin with grossly obvious restriction of mobility of fingers and forearm. There was mild to moderate hair loss over the affected areas. Histological sections showed mildly decreased number of sweat glands in the biopsied areas but inflammatory changes were minimal. Occasional hyalinization of collagenous fibers was seen. Biopsies were taken from the centers of the lesions, usually from the dorsum of the forearm, unless noted otherwise.

Skin samples were acquired either by scalpel excision or by dermal punch (5 or 6 mm) and immediately put on ice. A specimen weighing 100–175 mg wet wt was then finely minced at 4°C with a razor blade, after scraping off the fatty underlayer, and suspended in a Tris buffer at pH 7 containing 50 mM calcium chloride with a Ten-Broeck homogenizer. Collagenase activity was assayed by the procedure of Ryan and Woessner (11). In brief, their method relies on the fact that collagenase has a strong affinity for native collagen, and the enzyme can be separated from whole tissue homogenate by low-speed centrifugation and decanting of the supernatant solution, which contains serum collagenase inhibitors, to leave behind a fibrous pellet with bound collagenase. After resuspension of the pellet in a Tris buffer at pH 7 containing 50 mM calcium chloride and incubation at 35°C for 36 h, bound collagenase releases soluble collagen fragments into solution. These fragments are separated from the undigested fiber by centrifugation. Hydroxyproline content of the hydrolyzed supernatant solution is then determined by the method of Woessner (12).

The solubilization of hydroxyproline-containing material is not sufficient demonstration of collagenase activity, for trypsin also releases hydroxyproline-containing material into solution. It must be shown that fragments smaller than an α-chain are produced and that more is produced than is generated by the action of nonspecific proteases such as trypsin. Therefore the supernate from the collagenase digestions were dialyzed through cellulose membranes and hydroxyproline determinations were done on the dialyzate.

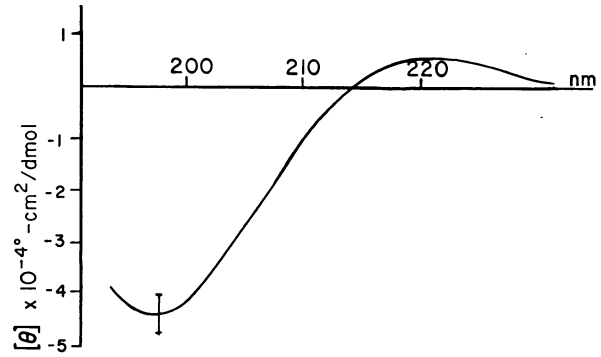

FIGURE 1 CD of collagen extracted in 0.1 M acetic acid from sclerodermatous skin. Normal skin collagen showed the same spectrum. Spectral noise is indicated by bar at 198 nm minimum.

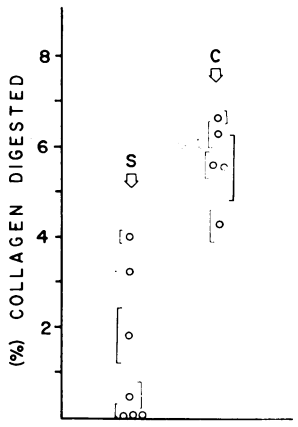

FIGURE 2 Collagenase activities of severely sclerodermatous (S) and nonsclerotic control (C) skin specimens. Control skin was acquired from men in the same age bracket as the male patients (46–62 yr) and site-matched. Collagenase activities are determined as percent of collagen solubilized in a 36-h incubation at 35°C. These activities are in excess of the trypsin blanks (cf. Table I). Individual points are the means of duplicate determinations whose ranges are indicated by the adjacent brackets. < means two coincident values.

Measurements of tensile strength were made by suspending one end of a 6-mm skin core from a stationary frame; then the other end, clamped to a loading pan, was loaded at a rate of 20 g/min until rupture of the specimen.

Skin from random, consecutive patients with basal cell epitheliomas were also studied, because the local invasion of this neoplasm seems to correlate with the extent of destruction of the surrounding connective tissue.

Lesions of human basal cell epitheliomas were either curetted, punched, or excised. In some instances excisions with extra-wide margins were made, so that the normal skin periphery could be separated and assayed as controls for collagen content and collagenase activity independently of the basal cell core. In other cases, control skin specimens were obtained from the patient's back.

Comparisons of optical activity of collagen from normal and sclerodermatous skin were made by measurements of circular dichroism (CD) [1] on a Cary 60 spectropolarimeter with CD attachment (Cary Instruments, Monrovia, Calif.). Collagen for these studies was obtained by homogenization of skin either in 0.45 M NaCl for extraction of neutral salt collagen or in 0.1 M acetic acid for extraction of acid-soluble collagen.

RESULTS

In the initial attempts to explore the possibility of an abnormal collagen present in sclerodermatous skin, the

[1] *Abbreviations used in this paper:* BCC, basal cell carcinoma; CD, circular dichroism.

optical activity of the neutral salt-soluble and acetic-acid-soluble fractions were measured. Those CD spectra showed that the structure of soluble collagen from sclerodermatous skin (Fig. 1) was indistinguishable from normal skin collagen and agreed with the data of Blout et al. (13) for native calf skin collagen.

Once convinced from the above studies and amino acid composition (which paralleled Piez [14] and Fleischmajer and Fishman [15]) that the sclerodermatous collagen was typical collagen, we next questioned the possibility that degradation was reduced by assaying collagenase activity. Fig. 2 plots the range of collagenase activities seen in seven sclerodermatous skins with marked involvement of the forearms. Biopsies were taken from the forearm. All patients were white men. These seven specimens are compared ($P < 0.001$) to five control forearm skins from normal volunteers of the same sex and age bracket (46–62 yr of age). Activities are plotted as percent of total collagen digested in a 36-h period in excess of the trypsin blanks. Table I itemizes the amount of trypsin-released collagen as well as the amount of dialyzable peptide for the seven sclerodermatous specimens and the five normal skins.

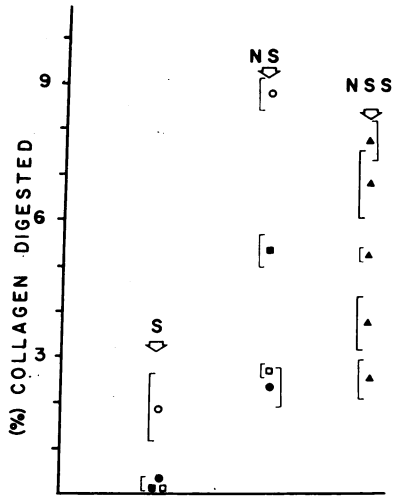

FIGURE 3 Collagenase activities of sclerotic (S) and nonsclerotic (NS) areas of skin in the same patient ($P < 0.05$). The column marked NSS represents activities measured in forearm skin from male patients diagnosed as having systemic scleroderma on the basis of pulmonary or esophageal disease but having no skin manifestations. Patients ranged in ages from 49 to 62 yr. ■, sclerotic forearm and nonsclerotic hip; □, asymmetrically involved back; ●, sclerotic forearm and uninvolved shoulder on same side; ○, dorsum of a sclerotic hand and uninvolved flank on same side. These activities are in excess of the trypsin blanks. Individual points are the means of duplicate determinations whose ranges are indicated by the adjacent brackets.

TABLE I
Trypsin and Collagenase Activities

Enzyme tested		Collagen digested*	HO-pro dialyzate‡
		%	*% collagen/ sample*
Collagenase			
Scleroderma sample	1	4	1.2 (30)
	2	3.2	1.0 (31)
	3	1.8	0.5 (28)
	4	0.5	Trace
	5	Trace	Trace
	6	Trace	Trace
	7	Trace	Trace
0.01% trypsin added§			
Scleroderma sample	1	6.2	2.4 (39)
	2	6.3	1.9 (30)
	3	4.4	1.6 (36)
	4	3.0	1.1 (37)
	5	2.5	1.0 (40)
	6	2.8	1.0 (36)
	7	3.7	1.4 (38)
Collagenase			
Control	1	6.7	2.0 (30)
	2	6.4	2.0 (31)
	3	5.6	1.6 (29)
	4	5.5	1.7 (31)
	5	4.3	1.5 (35)
0.01% trypsin added§			
Control	1	9.8	4.0 (41)
	2	9.8	3.8 (39)
	3	8.4	3.3 (39)
	4	8.8	3.7 (42)
	5	7.8	3.2 (41)

Collagenase activities of the sclerodermatous skin samples ranged from the lowest limit of normal, as seen in one specimen, to nonmeasurable amounts in three specimens. The activities shown with 0.01% trypsin added comprise the effect of collagenase plus trypsin digestion. The number in parentheses is the percent of column one that passed through the membrane. Ryan and Woessner (11) reported that 30% of collagen could pass through cellulose dialysis membranes. Our values with trypsin added are generally a little higher than that and can be attributed to more vigorous homogenization of tissue, as well as to trypsin digestion of fragments not otherwise dialyzable.

* Total collagen content ranged from 75 to 140 mg/specimen.

‡ Percent of total hydroxyproline-containing material that passes through a cellulose dialysis membrane.

§ Incubations with trypsin were carried out for 8 h.

Fig. 3 depicts the variation in collagenase activities in an individual patient between sclerotic and nonsclerotic areas. The four paired samples are among the seven patients in Fig. 2 with enzyme activities deter-

mined in duplicate. If decreased collagenase activity is a cause of increased tensile strength in sclerotic skin, it is important conversely to know that abnormally increased collagenase activity contributes in the opposite extreme to disintegration of skin. The ulceration of basal cell carcinoma (BCC) is an appropriate contrasting example, because it invades and replaces dermis and it has been shown indirectly that collagenase activity in BCC is probably elevated (16). Measurements of collagenase activity in these lesions are plotted in Fig. 4. As in the previous figures, the percent of total collagen digested over a 36-h period is displayed on the ordinate. Control specimens were taken from noncarcinomatous skin either on the periphery of the BCC lesion or at a remote site. Some lesions have strikingly high levels of collagenase activity, and were associated with active invasive tumors.

Table II shows the rupture loads for the sclerodermatous and normal skins of Table I. Of the BCC specimens in Fig. 4, only the lower seven could be used for tensile strength measurements; the other two were too friable.

The enzyme activities expressed in Fig. 4 for BCC lesions may be gross underestimates of the true activity. For the collagen content of these neoplasms is usually less and sometimes much less than that of the surrounding skin. If collagenous substrate is not at saturating levels, the true activities are underestimated. However, a corrected value would only magnify the differences in the two groups in Fig. 4 ($P < 0.001$).

DISCUSSION

The reasoning behind the attention to collagenase was based on its ability to explain the increased tensile strength of sclerodermatous skin. Our measurements of tensile strength (Table II) agree with those of Rasmussen et al. (3). If collagenase is deficient, one expects decreased turnover of collagen with more time for cross-links to form, resulting in less acid-extractable collagen (4). Consequently, with a higher fraction of mature (nonextractable) collagen, one anticipates an increased tensile strength.

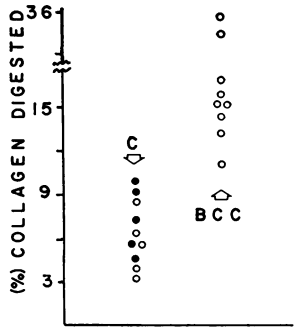

FIGURE 4 Collagenase activities of BCC. Control skins (C) were taken both from the skin peripheral to the lesions (●) and skin from remote sites (○). Each lesion came from a different patient. These activities are in excess of the trypsin blanks.

TABLE II
Tensile Strength

		Rupture load
		g
Scleroderma specimen	1	150
	2	180
	3	170
	4	150
	5	190
	6	160
	7	170
Control specimen	1	110
	2	130
	3	150
	4	150
	5	140
BCC specimen	1	10
	2	40
	3	40
	4	10
	5	20
	6	20
	7	30

As Fig. 2 indicates, seven patients with conspicuous cutaneous manifestations of scleroderma, to the point that their forearm skin was hidebound and woody, showed little or no collagenase activity. Outside the sclerotic areas collagenase activity was comparable to levels in normal skin (Fig. 3). These differences are particularly impressive in view of the findings by Eisen (17), who detected no differences between skin from the trunk and that from the extremities in normal subjects. And age of the subjects did not influence total collagenolytic activity. The typically elevated tensile strength of the sclerotic areas parallels closely this diminished enzyme activity. In matching these depressed levels of collagenase activity further to a contrasting condition, i.e., basal cell carcinoma, where skin tensile strength is greatly diminished (Table II), as evidenced by the comparative ease with which these lesions can be curetted, it suggests that over a broad range collagenase activity parallels tensile strength.

The abolition of collagenase activity may not be the only enzymatic abnormality of skin in scleroderma, but

one probably need not indict any additional structural defects to account for the mechanical properties of sclerodermatous skin. Why collagenase activity is depressed is another question. The depression must result from (*a*) less enzyme, either as amount synthesized or as decreased cell population producing the enzyme; (*b*) inhibited enzyme; (*c*) blocked substrate; or (*d*) failure of enzyme to bind homogenized substrate, a variant of (*c*). The last of these possibilities has no experimental evidence to recommend it; collagen derived from homogenized sclerodermatous skin is spectroscopically indistinguishable from normal skin collagen and its melting (shrinkage) temperature is unchanged (3). These data would argue against abnormal intramolecular cross-links that block collagenase access to its substrate.

The work of Fries et al. (18) does not help to separate these possibilities. They grafted sclerodermatous skin to uninvolved areas and grafted normal skin to a sclerodermatous bed. The normal skin became sclerodermatous, and the originally sclerodermatous donor graft remained sclerodermatous at the normal receptor site. An argument for (*c*) can be made to explain their results. Since collagenase would be expected to diffuse into a region from adjacent tissue and to exchange with other collagenase whether enzyme-bound inhibitor was present or not, one expects that any inhibited collagenase in the sclerodermatous graft could exchange with uninhibited collagenase from the nearby normal skin. Consequently, remodeling of the sclerodermatous graft would eventually occur. Since collagenase cleaves tropocollagen initially at a unique site (19), only if collagenase-binding sites on the tropocollagen network of the sclerodermatous graft were blocked, e.g., by antibody or by maturation, could one allow that remodeling might fail to occur in the new skin bed. On the other hand, (*a*) and (*b*) fully explain the failure of the sclerodermatous graft to change its texture. If skin tensile strength rises, while the concentration of collagen does not, there ought to be an increase in the amount or stability of cross-links. If the concentration of collagenase drops, turnover is reduced and more time is allowed for cross-links to develop. Once extensive and stable cross-links exist, grafting of this collagen to a new site will not affect its digestibility, even if active collagenase becomes available. Thus we also expect to find with Harris and Sjoerdsma (4) less acid-extractable collagen. Although Herbert et al. (20) in their studies on scleroderma say that diseased skin collagen matures more rapidly than that from normal skin, it may be more to the point that the absence of collagenase simply permits cross-linking to proceed unimpeded by the competing process of breakdown. Still it should be emphasized that in the face of a normal melting temperature for collagen from sclerodermatous skin —this has been verified also by Rasmussen et al (3)— the argument for a collagen substrate inaccessible to enzyme because of cross-links is quite weak.

One special point of difficulty is raised by the studies reported here. Since the steady state in vivo is a balance between synthesis and degradation, and since collagenase activity is diminished, why isn't the accumulation of collagen more impressive? It appears necessary also to suggest decreased collagen synthesis in sclerodermatous skin to account for the normal density of collagen. However, in vitro culture of fibroblasts from sclerodermatous skin by LeRoy (21) exhibited increased collagen synthesis over normal skin fibroblasts. Whether this in vitro state occurs in vivo or represents the release and rebound from a suppressed state will have to await cytokinetic measurements of both states along with accurate estimates of corresponding cell populations.

ACKNOWLEDGMENTS

The author would like to express his gratitude to Drs. Robert J. Boucek, J. F. Woessner, Jr., and Harvey Blank for their helpful discussions and criticisms, and also to Dr. H. Menn for the two BCC lesions with very high collagenase activities.

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