CONNECTIVE TISSUE ACTIVATION

XXVII. The Behavior of Skin Fibroblasts from Patients with Scleroderma

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Four normal (NF) and 4 scleroderma skin fibroblast (SF) strains were compared with respect to 1) basal ^{14}C-glucosamine and ^{35}SO$_4$-labeled glycosaminoglycan (GAG) synthesis, 2) responsiveness to autacoid mediators, and 3) performance following maximal stimulation. Under basal conditions, SF synthesized and secreted 2–3 times more radioactive hyaluronic acid than the NF ($P < 0.001$); molecular volume by gel chromatography was similar and suggested a high molecular weight product. SF were essentially as responsive to normal lymphoid and platelet factors as were NF. No consistent qualitative or quantitative differences in sulfated GAG synthesis were noted between the 2 groups of cells. Incubation of NF and SF with a false "core protein" such as p-nitrophenyl-β-D-xyloside suggested that synthesis of the core protein was rate limiting; SF and NF were equally facile in SO$_4$-GAG chain synthesis in the presence of a β-xyloside. SF appear to retain in vitro a partially activated state for many generations, at least with respect to hyaluronic acid synthesis.

Scleroderma or progressive systemic sclerosis (PSS) is a multisystem disease characterized by prolif-

erative vascular lesions, chronic inflammatory infiltrations, and in late stages, excessive deposition of connective tissue in many organs (1,2). The skin of PSS patients is the most common site of involvement and the most common cause of complaint. Although the etiology remains obscure, it seems clear that abnormal accumulation of some components of the extracellular matrix is an important pathophysiologic feature.

Recent studies have shown that involved scleroderma skin has an increased dry weight associated with an increased collagen content (3), increased amounts of type I procollagen in the deeper dermis and subcutaneous tissue (4), and abnormal distribution of fibronectin (5). The mechanisms of such abnormalities have been studied by using skin fibroblasts from affected individuals and measuring their capacity to synthesize some of these proteins in vitro. Thus, it is known that the scleroderma skin fibroblast (SF) cultures exhibit an exaggerated collagen synthesis rate when compared with normal skin fibroblasts (NF) (6–8).

Data from several laboratories concerning glycosaminoglycan (GAG) synthesis in PSS have yielded conflicting results. In 1958, Denko and Stoughton (9) presented evidence indicating that sclerodermatous skin of 3 PSS patients had higher ^{35}SO$_4$ uptake than uninvolved skin. Later, Uitto et al showed that grossly unaffected skin from 9 scleroderma patients had twice the total GAG content found in 9 controls, mostly due to an increase in chondroitin 4/6 sulfate (10). In contrast, studies from 2 different laboratories (11,12) have found increased GAG content in scleroderma skin to be primarily due to marked increase in dermatan sulfate associated with either high or decreased

hyaluronic acid (HA) content. Unfortunately, none of these studies measured the cellularity or DNA content of the specimens, and technical aspects of GAG fractionation schemes may explain the disagreement in results with respect to specific GAGs. It is also possible that the variable data result from different stages in the disease process.

More recent reports of GAG synthesis profiles in the 7 PSS dermal cell strains reported thus far have furnished apparently different results (13,14). Bashey et al (13) reported ^{3}H-glucosamine incorporation into GAG by 4 NF and 4 SF cultures. Although total uptake of labeled glucosamine and total secretion of ^{3}H-GAG appeared somewhat higher in confluent scleroderma fibroblast cultures, these authors concluded that "no differences were noted in ^{3}H-glucosamine incorporation or in GAG biosynthesis between normal and scleroderma cultured fibroblasts." Buckingham et al (14) found that dermal fibroblast cultures derived from the lower portions of biopsy samples from 3 patients with early PSS synthesized "increased amounts of all major glycosaminoglycans" when studied with ^{3}H-glucosamine.

Previous studies from this laboratory identified small molecular weight proteins present in lymphoid tissue (15), platelets (16,17), and neutrophils (18) with the capacity to elicit multiple metabolic responses from human connective tissue cells in vitro. These included increased formation of GAG, increased glucose uptake and lactate formation, increased DNA synthesis, enhanced prostaglandin production (particularly the E series) (19), increased activity of HA synthetase (20), and induction of increased synthesis of plasminogen activator (21). Connective tissue activation has been defined as the sum of these activities, and the stimulatory agents have been termed "connective tissue activating peptides" (CTAPs).

In view of the uncertainty concerning GAG synthesis by PSS fibroblasts, we undertook this study to examine basal cellular performance, responsiveness to normal autacoid mediators, and performance under maximal stimulation, in an attempt to enhance our understanding of the physiologic performance of "PSS fibroblasts" confronted with different regulatory settings. In the same experiments, we defined several superficial characteristics of proteoglycans (PGs) and hyaluronic acid synthesized in vitro by normal and PSS dermal fibroblasts.

PATIENTS AND METHODS

Skin fibroblast cultures were derived from 4 normal volunteers, and after informed consent, from the affected skin of 4 patients with an established diagnosis of progressive systemic sclerosis (Table 1). Details of culture methods have been reported previously (22). Briefly, NF and SF were developed from explants and grown as monolayer cultures in medium CMRL 1066 (Gibco, Grand Island, NY), supplemented with 10% fetal calf serum (FCS; Reheis Chemical Co., Kankakee, IL), 10% heat inactivated human serum (normal donors), L-glutamine (Sigma, St. Louis, MO), penicillin (Pfizer, New York, NY), streptomycin (Pfizer), erythromycin (Dista Laboratories, Indianapolis, IN), 0.02M HEPES (Sigma), and sodium bicarbonate. Complete medium changes were carried out 3 times a week and trypsin (Worthington Biochemicals, Freehold, NJ) dispersal was performed as required for propagation or study. The cells used in this study had been transferred 3 to 11 times.

Isotope incorporation studies. As previously described in detail (23), cells were plated in microtiter wells (Costar), 2.0 × 10^4 cells per well, in assay medium consisting of 100 μl of Eagle's synthetic media (ESM; Gibco), supplemented with 3% FCS, L-glutamine, antibiotics, HEPES, and Na$_2$CO$_3$. After 20–24 hours incubation at 35–37°C in a humidified chamber to allow cell attachment and spreading, an additional 100 μl of assay media containing uniformly labeled D(U-^{14}C)-glucosamine-HCl, (Amersham Corp., Arlington Heights, IL; specific activity 254 mCi/ml), 0.5 μCi/ml medium, or 50–100 μCi/ml ^{35}SO$_4$ (specific activity 20–40 Ci/mg; Amersham Co.) was added. Test additives consisted of 20 μl of CTAP-I$_b$ (180 μg/ml) from spleen, CTAP-P$_2$ (160 μg/ml), CTAP-III (180 μg/ml) from platelets, p-nitrophenyl-β-D-pyranoside (100 μg/ml) (Sigma), or the appropriate vehicle. These microcultures (4 wells/group) were then incubated under the same conditions for an additional 40–48 hours.

Measurement of ^{14}C-glucosamine or ^{35}SO$_4$ incorporation into GAG was quantitated as described (23). In brief, 100-μl aliquots of media were spotted on Whatman 3 MM chromatography paper, and the GAG fixed to the paper by immersing it in a 0.1% solution of cetylpyridinium chloride (CPC; Sigma). Unincorporated label was removed by sequential washes with 0.1N NaOH in the case of ^{14}C-labeled media or with 0.1% CPC in 0.3M NaCl for ^{35}SO$_4$-labeled media. The remaining media was pooled separately by group and stored at −20°C.

Long-term culture experiments. Using duplicate plates, 5 × 10^4 cells/well were plated in 24-well plates (Costar), in 500 μl of growth medium/well. After 20–24 hours incubation at 35–37°C, an additional 500 μl of growth medium containing 200 μCi/ml of uniformly labeled ^{35}SO$_4$ was added to one of the plates; the same volume of cold medium was added to the duplicate plate and incubation was resumed. On day 3, 100 μl of labeled medium from each well was spotted on Whatman 3 MM chromatography paper and subjected to the CPC fixation-wash procedure described above. The remaining media was pooled separately by group and stored at −20°C. The cell sheet was rinsed 3 times with phosphate buffered saline (PBS) at room temperature, and the total protein content (24) and identification of GAGs were determined in the cell lysate. For this purpose, cells were lysed with 1.1 ml of either Lowry's Reagent C or Zwitterionic detergent (12 mg/ml) (Calbiochem-Behring Corp, San Diego, CA).

Media in the duplicate plate was changed on days 3

Table 1. Clinical data: progressive systemic sclerosis (PSS) patients and normal controls

Patients	Age	Sex/race	Site of biopsy*	Organ involvement†	Disease duration (years)
PSS					
GA	53	M/B	Forearm (V)	Skin, lung, esophagus, Raynaud's phenom- enon, heart	23
GG	51	F/W	Forearm (V)	Skin, esophagus, Raynaud's phenom- enon, joints	1
IC	54	F/W	Forearm (V)	Skin, lung, esophagus, Raynaud's phenom- enon	3
MM	35	F/W	Hand (D)	Skin, lung, esophagus, Raynaud's phenom- enon, heart	6
Normal controls					
1	20	M/B	Prepatellar		
2	73	F/W	Prepatellar		
3	62	F/W	Prepatellar		
4	36	F/W	Forearm (V)		

* V = volar; D = dorsum.

and 6. On day 8, 100 μCi/ml of $^{35}SO_4$ was added to the cultures with a complete medium change, and the cultures were harvested on day 10 and processed as described above. All cultures were confluent by day 6–7.

Enzymatic analysis of GAGs. Qualitative identification of GAGs was accomplished by incubating labeled media with hyaluronidase (ex. *Streptomyces hyalurolyticus*, Cal- biochem) in 0.15M sodium acetate pH 5, chondroitinase ABC, or AC (Miles Laboratories, Elkhart, IN) in 0.2M Tris pH 7.5 or the appropriate buffer for 3 hours at 37°C. The digested and control samples were subjected to the CPC fixation-wash procedure and GAG identity was determined on the basis of specific enzyme lability.

Characterization of proteoglycans. Tissue culture flasks (25 cm^2) were plated with 2×10^6 normal or scleroder- ma fibroblasts and nourished with 5 ml of growth media. After 20–24 hours incubation at 37°C, the initial medium was removed, the cell sheet rinsed twice with sterile PBS, 5 ml of assay medium containing $^{35}SO_4$ (100 μCi/ml) was added, and incubation was resumed for another 40–48 hours. Medium was collected and dialyzed for 72 hours at 4°C against 200 volumes of PBS with 2 changes over this period. The retentate was lyophilized, resuspended in PBS, and chroma- tographed over a calibrated 2.5×25 cm Sephacryl S-300 column (Pharmacia Fine Chemicals, Piscataway, NJ). One hundred microliters of each 3-ml fraction was spotted on chromatography paper, and the eluted radiolabeled material was measured by liquid scintillation counting. The remain- der of the column fractions were lyophilized, hydrated to working volumes with PBS, and subjected to enzymatic analysis as described above.

Characterization of HA molecular weight. NF and SF (1.2×10^6) were plated in 75 cm^2 plastic flasks and supported with assay medium. After approximately 20 hours incuba- tion at 37°C to allow attachment and spreading of cells, 1.0 μCi/ml of ^{14}C-glucosamine was introduced, and incubation

resumed. After 40 hours incubation with labeled precursor, media were harvested and an aliquot from each sample was dialyzed overnight against approximately 200 volumes of 0.02M acetate buffer pH 5 at 4°C. The retentate was centri- fuged at 30,000g, 4°C, and the precipitate, which contained ^{14}C-HA, was resuspended to working volumes with 0.5M NaAc, pH 6.9. Samples of dialyzed and nondialyzed labeled medium were chromatographed on a calibrated Sepharose 4B column $(27 \times 2.5$ cm) (Pharmacia Fine Chemicals, Uppsala, Sweden) and eluted with 0.5M NaAc pH 6.9. Two hundred microliters of each 2-ml fraction were spotted on chromatography paper and the CPC precipitable material was determined by scintillation counting. The remainder of the void volume fractions were lyophilized, hydrated to working volumes with PBS, and the GAG was identified enzymatically. Immediately after the media were harvested, the cell mats were rinsed 3 times with PBS, lysed with 5 ml of Zwitterionic detergent for 2 hours at 37°C, and the qualitative identification of GAG was determined by specific enzyme lability of CPC precipitable material.

Statistical analysis. Statistical analysis was performed using the Student-Fisher t-test. Bar graphs were constructed to display the mean $\pm$ 1 standard deviation of the data.

RESULTS

Hyaluronic acid formation. Normal and PSS fibroblasts were incubated in the presence of ^{14}C-glucosamine with and without the influence of three CTAP mediators; the results are depicted in Figure 1. It is clear that under basal conditions, scleroderma skin fibroblasts synthesize and secrete 2- or 3-fold more ^{14}C-hyaluronic acid than the normal control fibroblasts ($P < 0.001$). In the mediator-treated cultures, very
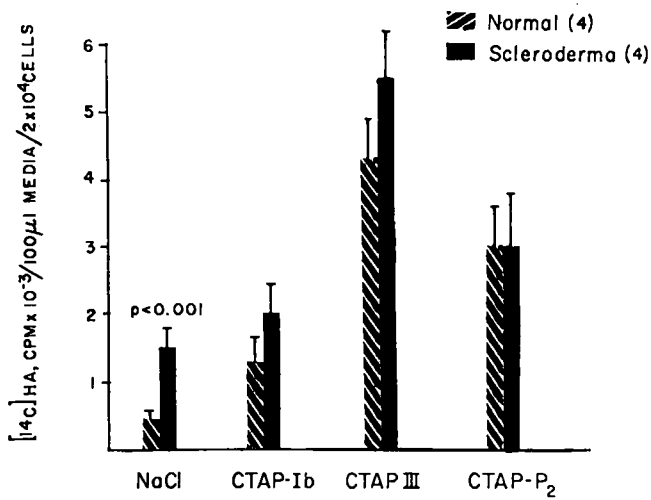
Figure 1. Stimulation of hyaluronic acid (HA) synthesis. The concentrations of agonists used were: connective tissue activating peptide I_b (CTAP-I_b), 180 μg/ml; CTAP-III, 180 μg/ml; CTAP-P_2, 160 μg/ml. All 3 mediators stimulated ^{14}C-HA synthesis compared with the appropriate (normal or scleroderma skin fibroblast) controls (NaCl-treated cultures).
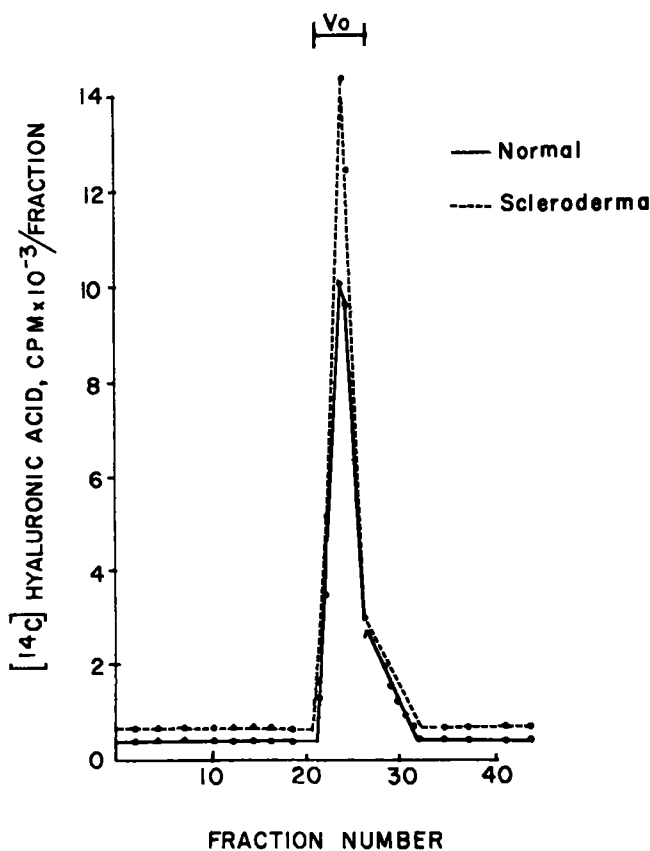

FRACTION NUMBER

Figure 2. Gel filtration on a Sepharose 4B column of labeled hyaluronic acid from skin culture media showed that the macromolecules eluted in the void volume (Vo).
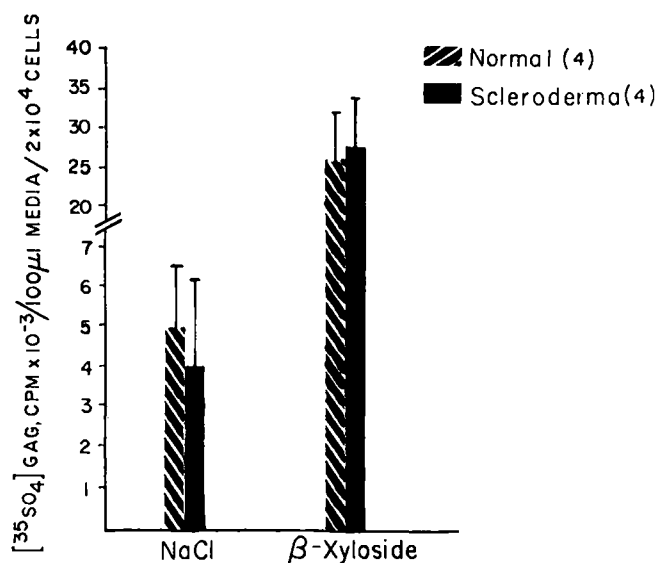
high concentrations were used in an attempt to promote a maximal response. The platelet factors (CTAP-III and CTAP-P_2) stimulated ^{14}C-HA synthesis to similar absolute levels, while both types of fibroblasts were relatively resistant to CTAP-I_b from human spleen. Thus, it is clear that SF propagated in vitro retain the capacity to be "activated" by autacoid mediators to levels comparable with normal cultures.

The molecular weights of ^{14}C-HA secreted by normal and scleroderma skin fibroblasts were also compared. ^{14}C-glucosamine labeled HA was chromatographed on a Sepharose 4B column; results of a typical experiment are shown in Figure 2. As can be seen, normal and scleroderma ^{14}C-HA eluted in the void volume of the calibrated column. We interpret the data to mean only that both NF and SF synthesize large HA molecules, not that they necessarily have identical molecular weights. These secreted macromolecules were entirely hydrolyzed by fungal hyaluronidase.

Synthesis of sulfated GAG. Normal and scleroderma skin fibroblast cultures were compared with regard to total synthesis and secretion of ^{35}S-GAGs. As shown in Figure 3, in short-term assays (i.e., approximately 72 hours) both NF and SF incorporated and secreted variable amounts of CPC-precipitable ^{35}S-macromolecules with no clear-cut difference in total synthesis under nonstimulatory conditions. In

Figure 3. ^{35}S-glycosaminoglycan (GAG) synthesis was modified by introduction of a β-xyloside, leading to marked and similar increase in GAG chain synthesis in normal and scleroderma skin fibroblast cultures.

Table 2. $^{35}SO_4$-glycosaminoglycan formation by normal and progressive systemic sclerosis (PSS) fibroblast cultures*

Cell source	Short-term (3 days)		Long-term (10 days)	
	Media	Cells	Media	Cells
1 (normal)	7.14 ± 0.35	2.80 ± 0.14	3.73 ± 0.23	3.37 ± 0.03
2 (normal)	7.58 ± 0.33	2.58 ± 0.07	5.64 ± 0.36	4.13 ± 0.24
3 (normal)	5.51 ± 0.22	3.60 ± 0.18	3.18 ± 0.11	3.55 ± 0.44
GA (PSS)	5.92 ± 0.32	3.43 ± 0.54	4.73 ± 0.08	3.07 ± 0.54
GG (PSS)	10.81 ± 0.51	3.38 ± 0.16	7.58 ± 0.62	2.05 ± 0.24
IC (PSS)	6.71 ± 0.64	2.75 ± 0.29	4.96 ± 0.25	3.30 ± 0.50

* Cultures were treated as described in the Patients and Methods section, and harvested at days 3 and 10 after being exposed to $^{35}SO_4$ for the final 48 hours. Numbers represent $^{35}SO_4$-glycosaminoglycan counts per minute × 10^4 per mg cell protein, mean ± SD of 6 cultures.

contrast, when a β-xyloside such as p-nitrophenyl-β-D-xyloside was introduced into the cultures, higher, but similar, rates of carbohydrate chain biosynthesis were noted ($P < 0.4$), suggesting that this is not a rate limiting factor in these cultures. Aberrations in core protein synthesis or xylosyltransferase activity have not been ruled out.

In view of these results, experiments were performed in which "short-term" cultures (72 hours) and "prolonged" cultures (240 hours) were compared with respect to the quantitative and qualitative aspects of ^{35}S-GAG synthesis. Duplicate plates were nourished with regular growth medium to obtain maximal serum-dependent stimulation and cultures were terminated at either day 3 or 10, after having been exposed to $^{35}SO_4$ for the final 48 hours. The results of these experiments (Table 2) show clearly that under both short- and long-term assay conditions, no clear-cut quantitative or qualitative differences in synthesis of S-GAG were noted between the PSS and control cells ($P < 0.5$), regardless of whether one examined younger (less confluent) or older (denser) cultures.

Radiolabeled macromolecules released into the culture media were subjected to hydrolysis by fungal hyaluronidase and chondroitinases ABC and AC. Ta-

ble 3 summarizes the results of the analyses of labeled GAG isolated from NF and SF cultures. Most of the ^{14}C-glucosamine labeled GAG was hydrolyzed by the fungal hyaluronidase, in agreement with a previous report (13). In contrast, when $^{35}SO_4$ was used as a precursor, 60–70% was incorporated into chondroitinase AC–sensitive GAG and released into the culture medium; 20–25% of the incorporated radioactivity was found in cell-associated dermatan sulfate. It is interesting to note that overall, there were no qualitative differences in the labeled macromolecules synthesized by the PSS and control fibroblasts ($P < 0.1$) regardless of the isotopic precursor utilized and culture duration. As can be seen, approximately 30% of the incorporated radiosulfate was not hydrolyzed by the chondroitinases; this may include heparan-sulfate as well as sulfated lipids and glycoproteins.

Proteoglycan formation. Nonstimulated confluent cultures of control and PSS fibroblasts were incubated with $^{35}SO_4$ for 40 hours and the labeled macromolecules secreted into the media were subjected to gel filtration over Sephacryl S-300; the results are shown in Figure 4. In this experiment, scleroderma fibroblasts synthesized and secreted more $^{35}SO_4$-GAG than controls; these macromolecules eluted in the void

Table 3. Qualitative comparison of glycosaminoglycan (GAG) synthesis in fibroblast cultures (percentage of total GAG hydrolyzed)*

GAG and enzyme	Media		Cells		P†
	Normal	Scleroderma	Normal	Scleroderma	
^{14}C-GAG + S. Hyaluronidase	76 ± 9	79 ± 7	71 ± 21	75 ± 25	NS
^{35}S-GAG + chondroitinase AC‡	60 ± 3	72 ± 9	42 ± 20	41 ± 19	NS
^{35}S-GAG + chondroitinase ABC‡	0	0	25 ± 9	22 ± 9	NS

* Numbers represent mean percentage hydrolyses of total label ± 1 SD.
† NS = not significant.
‡ Results from short-term cultures and long-term cultures were found to be the same.
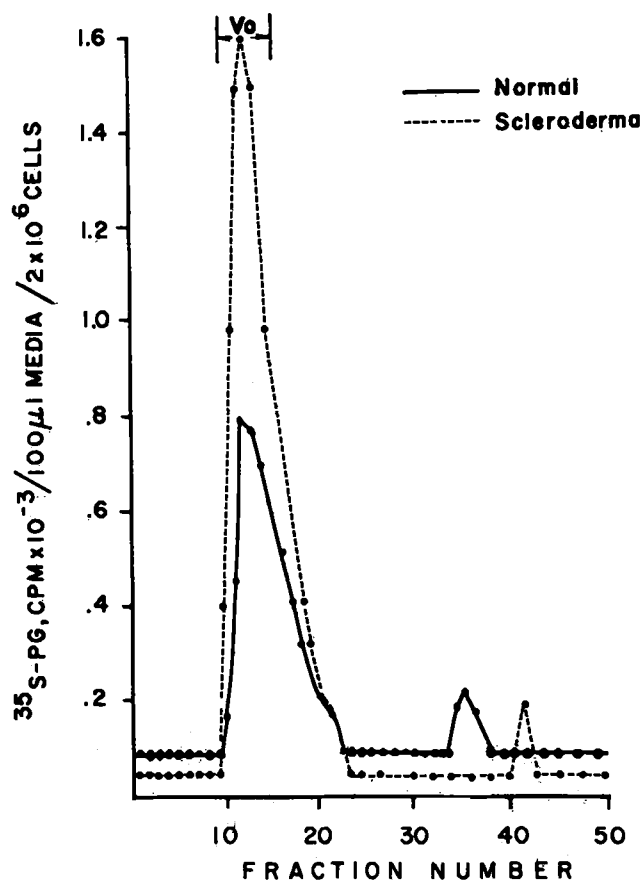
Figure 4. Gel filtration of ^{35}S-proteoglycan (PG) over a Sephacryl S-300 column. Virtually all radiolabeled material appeared in the void volume (Vo), indicating that its size was >0.75 × 10^6 daltons, a size clearly compatible with PG monomer and too large for glycosaminoglycan "chains."

volume of the column, suggesting a molecular weight of >0.75 × 10^6 daltons. Enzymatic analysis showed that most of it was digestible by chondroitinase AC. Virtually no free GAG chain was detected.

DISCUSSION

In vitro culture of connective tissue cells has been useful in the study of human disorders such as heritable connective tissue diseases (25,26), pretibial myxedema (27), and rheumatoid arthritis (28) by permitting measurement of biosynthetic rates and/or response to hormones, mediators, and drugs. Fibroblasts derived from affected scleroderma skin have been studied, and there is now convincing documentation of overproduction of collagen and fibronectin by these cells growing in monolayer cultures (5–8). The present work extends these observations to include another component of the extracellular matrix, particularly high molecular weight hyaluronic acid. Four scleroderma and 4 normal skin strains were studied with regard to their ability to synthesize and secrete GAG into the culture medium; when the ^{35}SO$_4$ precursor was used, no consistent differences in synthesis were seen. This may reflect the assay conditions in these experiments or heterogeneity in the cell populations with respect to sulfated GAG synthesis and/or differences in activity of clinical disease at the time of biopsy (Table 2).

The experiments with p-nitrophenyl-β-D-xyloside, which presumably acts as a "false core protein" by serving as an attachment site for carbohydrate chains, shed some light on this issue. When NF and SF were incubated in the presence of a β-xyloside (Figure 3), a similar rate of synthesis was noted, suggesting that sulfated carbohydrate chain formation was not a rate limiting factor in these cultures. Most of the radiosulfate-labeled material in the media and cell layer was chondroitin 4/6 sulfate. Dermatan sulfate was only found in association with the cell sheet. The present data showing sulfated GAG from normal skin fibroblasts to be in the form of proteoglycan monomer are in agreement with a previous report (29), a finding now extended to include synthesis of proteoglycan monomer by scleroderma skin fibroblast cultures.

It appears that the skin fibroblasts from patients with PSS, under basal in vitro conditions, form 2–3 times more hyaluronic acid than normal control fibroblasts ($P < 0.001$). It is tempting to speculate that this quantitative metabolic peculiarity may underlie the edema often seen in the early "wet" phase of scleroderma.

Although abnormal accumulation of radiolabeled hyaluronate may result from increased synthesis and/or decreased rate of degradation, it is important to note that these studies most likely reflect the former, since the radiolabeled hyaluronic acid released into the media in a 48-hour incubation is a reflection of the de novo synthesis of this polymer; the plasma hyaluronidases are known to be inactive at neutral or alkaline pH. Further, it is known that the intracellular hyaluronidase (also active at low pH) is present primarily in lysosomes, and the quantity of HA reaching this organelle in short-term experiments is unlikely to interfere with the present results. It is not possible, however, to completely rule out a role for decreased rate of breakdown. The chromatographic profile of the hyaluronic acid secreted by normal cells is in accor-

dance with the work of others (30); the parallel data from PSS cultures should be interpreted within the limitations of the gel filtration method.

Other reports describe increased synthesis of HA by cells isolated from human patients. It has been shown that fibroblasts derived from the skin of patients with Marfan's syndrome (25), Hurler's syndrome (31), osteogenesis imperfecta (26), and from rheumatoid synovial membrane (22,28) accumulate excessive amounts of hyaluronate when compared with normal cells of similar origin. Several factors are known to influence the rate of HA production by mammalian cells in vitro, including insulin, cortisol and Mg^{++} (32), CTAPs, prostaglandins and cAMP (19), bacterial endotoxins (33), viral infection (34), and viral transformation (35). In addition, culture conditions such as temperature, pH, serum concentration, and cell density are important (32). At least with cAMP and a few of the CTAPs, it appears that they exert their effect by increasing the activity of the hyaluronic acid synthetase complex (20). Serum components, insulin, and cortisol are also said to regulate HA synthetase activity (32). Thus, it may be useful to study the HA synthetase activity of PSS connective tissue cells in attempting to explain the present data.

In the present study, we show that, with respect to HA and PG synthesis, the SF respond to lymphoid and platelet derived factors in a normal fashion; this suggests that the enzymatic mechanisms known to mediate these metabolic responses are intact. The synthesis of HA appears to be activated in vivo by cause(s) yet unknown which lead to the persistence of a hypermetabolic state similar to that described for rheumatoid synovial fibroblasts (22,28).

Suitable explanation(s) as to why PSS fibroblasts accumulate more connective tissue components when isolated from their in vivo environment must take into account the fact that these cells manifest this "abnormal" behavior even after several generations in vitro. In the absence of strong evidence suggesting a viral or genetic defect in PSS (1), the models of in vivo fibroblast selection (36) and in vivo activation (22,28) are both compatible with the existing data.

ACKNOWLEDGMENTS

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