Changes in the Components of Extracellular Matrix and in Growth Properties of Cultured Aortic Smooth Muscle Cells upon Ascorbate Feeding

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ABSTRACT   Culture conditions can modify the composition of the extracellular matrix of cultured calf aorta smooth muscle cells. In the absence of ascorbate the major components of the matrix are microfibrillar proteins; deposition of collagen occurs upon ascorbate supplementation and, with increased time of exposure of cells to ascorbate, collagen becomes the dominant protein of the extracellular matrix (>80%). Collagen accumulation follows a sigmoidal time-course, suggesting that it is a cooperative phenomenon. Covalent crosslinks are not required for collagen accumulation in the matrix. Microfibrillar proteins and increased amounts of proteoglycans and fibronectin accumulate concurrently with collagen but elastin deposition was not observed either with or without ascorbate feeding. Addition of ascorbate leads to a general stimulation of incorporation of [^{14}C]proline into cellular protein and to changes in cell growth parameters and morphology: cell-doubling time decreases from 62 to 47 h and plating efficiency increases approximately fourfold. We conclude that the composition of the extracellular matrix assembled by cultured cells is subject to experimental manipulation and that changes in endogenously deposited matrix may have significant effects on cellular functions.

The role of the substrata in cell attachment, proliferation, and differentiation has recently received much attention (14, 18). Artificial collagen substrates and those of more complex composition approximating the extracellular matrix of the cell in vivo are being widely used in studies of growth and differentiation of a number of diploid cell types. For example, improved survival and maintenance of differentiated function were observed when rat hepatocytes were cultured on "biomatrix" or collagen gels than when cultured on plastic (26), and growth factors were not required when bovine vascular smooth muscle cells were maintained on the extracellular matrix assembled by corneal endothelial cells (13). Liotta et al. reported that collagen was required for cell attachment, spreading, and proliferation, and that artificial collagen substrates could substitute for the endogenously deposited collagen (22). These observations support the idea that the substrate, either provided to the cell and/or the extracellular matrix that the cell elaborates, is crucially important in determining cell behavior and differentiative fate.

The role of the endogenously produced extracellular matrix of cultured cells in regulating cellular properties such as growth and metabolism has received little attention. Recent studies on rat heart smooth muscle cells showed that the matrix composition can be altered by ascorbic acid; in the absence of ascorbate, elastin was the major component whereas in its presence glycoprotein and collagen were deposited (9, 30). However, in this system, ascorbate had a variable effect on cell growth.

In a previous study, we presented evidence that the major components of the extracellular matrix of the calf aorta smooth muscle cells cultured in the absence of ascorbate are microfibrillar proteins and that in the presence of ascorbate insoluble collagen is the major component of the matrix. Here we extend these observations to show that the deposition of collagen in the matrix is a cooperative phenomenon and that increased amounts of fibronectin and proteoglycans are deposited concurrent with the insoluble collagen. We describe changes in various cell parameters following addition of ascorbate (e.g.,

doubling time, plating efficiency, incorporation of radioactive proline, and morphology) and propose that these changes may be modulated by the endogenously produced matrix.

MATERIALS AND METHODS

Materials

L-[^{14}C]Proline and [^{35}S]Na$_2$SO$_4$ were purchased from Amersham Corp. (Arlington Heights, Ill.). Tissue culture supplies (media, fetal calf serum, nonessential amino acids, antibiotics, and trypsin-EDTA) were obtained from Gibco Laboratories (Grand Island Biological Co., Grand Island, N.Y.). β-Amino propionitrile (βAPN) was obtained from Calbiochem-Behring Corp. (American Hoescht Corp., San Diego, Calif.).

Cell Culture

The preparation of explants from the medial layer of calf thoracic aorta and the subculturing of the cells were performed as previously described (8). Briefly, freshly excised thoracic aortas of 3-month-old calves were brought to the laboratory and explants were prepared in Dulbecco's modified Eagle's medium supplemented to contain 1% nonessential amino acids, 10% fetal calf serum, 0.04 M Tris-acetate, pH 7.5, and 100 U of penicillin with 0.25 μg fungizone/ml (complete medium). When confluent, cells were subcultured at a 1:4 ratio. Cells between 3 and 27 population-doubling levels were used. Cells were usually grown in 150-cm^2 flasks in complete medium without ascorbate, and the medium was changed every 3 d. The protocol for additions of ascorbate, βAPN, and the radioactive precursors was as follows: cells, 1–2 wk past confluence, were exposed to media containing ascorbic acid (50 μg/ml) for times indicated in the text. In some experiments, ascorbic acid was added at seeding. Freshly prepared ascorbic acid was added daily to the flasks. [^{14}C]Proline (0.125 μCi/ml), [^{35}S]Na$_2$SO$_4$, (0.5 μCi/ml), or βAPN (75 μg/ml) was added, as indicated, to control and ascorbate-supplemented cells.

Attachment Efficiency

Smooth muscle cells were seeded at 10^3–10^5 cells/60-mm dish with and without added ascorbate (50 μg/ml) or βAPN (75 μg/ml). After 1 d, plates were rinsed with phosphate-buffered saline (PBS), and the cells were detached by trypsinization and counted in a Coulter Counter (Coulter Electronics Inc., Hialeah, Fla.).

Plating Efficiency

Smooth muscle cells were seeded at 100 cells/60-mm dish in the presence or absence of ascorbate (50 μg/ml) or βAPN (75 μg/ml) and maintained for 3 wk. Cells were fixed in methanol, stained with Van Gieson's, and the number of clones was counted.

Doubling Time and Saturation Density

Cells were seeded at 10^4/60-mm dish with and without ascorbate (50 μg/ml) or βAPN (75 μg/ml). At various time-points, triplicate plates were rinsed with PBS, trypsinized, and cells were counted in a Coulter Counter (Coulter Electronics Inc.).

Extraction of the Cell Layers

Culture media were decanted, and the cell layers rinsed twice with PBS containing 2 mM phenylmethylsulfonyl fluoride (PMSF) and 0.02% NaN$_3$. The cells were removed from the flask with a rubber policeman and centrifuged at 100 g for 10 min at 0–4°C.

In selected experiments, cell layers were either trypsinized or treated with trypsin-collagenase to obtain a single-cell suspension. Medium was decanted and cell layers were treated with 0.05% trypsin/0.7 mM EDTA (3 ml/150 cm^2 flask) for 10 min at 37°C. Cell layers from flasks supplemented with ascorbate were initially treated with trypsin in a similar manner. When this treatment had no effect, 0.5% Type I collagenase (3 ml/150 cm^2 flask; Worthington Biochemical Corp., Freehold, N. J.) was added for 10 min at 37°C. The single cells were then centrifuged and the pellets collected. Viability was assessed by trypan blue exclusion.

Cell pellets were delipidated at room temperature by extraction with 2 vol of butanol: diisopropyl ether (40:60, vol/vol) as described by Cham and Knowles (6), centrifuged at 750 g for 10 min, and the aqueous phase containing the delipidated pellet was lyophilized. The lyophilized pellets were extracted (10 mg dry wt/ml buffer) with 1.0 M NaCl, 0.05 M Tris (pH 7.5), 2 mM PMSF, and 0.02% NaN$_3$ (NaCl) for 5 h at 0–4°C, and then centrifuged at 12,000 g for 30

min. The supernatant fraction was decanted and the pellet washed twice with water. The pellets were then extracted in sequence with 0.5 N acetic acid, 2 mM PMSF, 0.02% NaN$_3$ (HAc) for 15 h at 0–4°C; 4.0 M guanidine HCl, 0.05 M Tris (pH 7.5), 2 mM PMSF, 0.02% NaN$_3$ (GCl) for 5 h at 0–4°C; 1% SDS in 0.05 M Tris (pH 7.5), 0.33 M mercaptoethanol (ME), 2 mM PMSF, 0.02% NaN$_3$ (SDS/ME) under nitrogen at room temperature for 15 h. The supernatant fraction at each step was decanted and pellets were washed as described. The suspension with SDS/ME was centrifuged at 27,000 g for 1 h at room temperature and the final pellet washed several times with water and lyophilized (SDS/ME pellet). The various extracts were dialyzed overnight vs. H$_2$O, lyophilized, and reconstituted in distilled water containing 2 mM PMSF and 0.02% NaN$_3$. The HAc extract was reconstituted in 0.5 N acetic acid containing 2 mM PMSF and 0.02% NaN$_3$.

Characterization and Quantification of the Insoluble Cell-layer Components

The SDS/ME pellet proteins were subjected to amino acid analysis, and hydroxyproline was used to estimate the collagen content. The total content of amino acids was used to quantify the protein. The SDS/ME insoluble proteins were treated with CNBr as described (3), and the CNBr fragments were visualized by electrophoresis on polyacrylamide gels. Collagen types I and III were prepared from the medial layer of calf thoracic aorta by limited pepsin digestion followed by differential salt precipitation (27) for use as standards. For comparison with the cells, the intact tissue was subjected to an identical extraction scheme and the SDS/ME insoluble proteins were subjected to CNBr cleavage and analysis on polyacrylamide gels as described for the cells.

To establish the presence of mature elastin or the insoluble microfibrillar proteins, the SDS/ME insoluble proteins were treated with 0.1 N NaOH for 45 min at 98°C (20) and the amino acid composition of the soluble and insoluble fractions was determined. Protein contents were quantified from amino acid composition.

Analysis of the Culture Media

Spent media from cells grown in the presence of [^{14}C]proline with and without ascorbate were centrifuged at 100,000 g for 30 min. Supernatants were dialyzed overnight against several changes of H$_2$O and then lyophilized. The samples were reconstituted in H$_2$O to 1/10 of original volume and delipidated with 2 vol of butanol:diisopropyl ether (40:60, vol/vol) for 30 min. The suspensions were centrifuged at 750 g for 10 min and the aqueous layer was lyophilized. The reconstituted delipidated media were then chromatographed on Biogel A 0.5 M (1.6 × 30 cm) eluted with 0.1 M pyridine acetate, pH 5.0. The protein-containing fractions eluting in the void volume were pooled, dialyzed, and lyophilized. This fraction was then used for SDS polyacrylamide gel analysis.

The amounts of [^{14}C]hydroxyproline and [^{14}C]proline incorporated into medium proteins were determined as described (2). Briefly, an aliquot (15 ml) of pooled media from one or more feedings was treated with 3 vol of absolute ethanol, and the precipitate was hydrolyzed in 6 N HCl for 24 h at 110°C. The hydrolysate was chromatographed on a DC-6A resin. Fractions of effluent in the hydroxyproline and proline regions were collected and counted. Recovery was measured by addition of [^{3}H]hydroxyproline, as internal standard, before hydrolysis (2).

The SDS/ME pellets from corresponding cell layers were hydrolyzed and the radioactivity in [^{14}C]hydroxyproline and [^{14}C]proline was quantified as described above.

PAGE and Fluorescence Autoradiography

The procedure of Laemmli (19) was used as described in the legends to the figures. Proteins were stained with Coomassie Brilliant Blue. For fluorography, gels were soaked for 1 h in Enhance (New England Nuclear, Boston, Mass.) and for 1 h in distilled water, dried on a Bio-Rad gel drier (Bio-Rad Laboratories, Richmond, Calif.), placed against x-ray film, and exposed at −70°C for appropriate lengths of time.

Indirect Immunofluorescence and Electron Microscopy

Smooth muscle cells were plated onto sterile glass cover slips; at 2 wk past confluence, ascorbic acid (50 μg/ml) was added daily for an additional 10 d. Control and ascorbate-treated cells were rinsed twice with PBS, then fixed with 2% paraformaldehyde and examined for immunofluorescence as described by Kahn and Shin (17). Antiserum to fibronectin was a gift from Dr. Kay Fields (Albert Einstein College of Medicine); antiserum to basement membrane heparan

sulfate was a gift from Dr. George Martin (National Institute of Dental Research); and antiserum to type I collagen was a gift from Dr. Sam Seifter (Albert Einstein College of Medicine).

For electron microscopy, cells were grown in 25-cm^2 flasks and fixed and processed as follows: cell layers were fixed in 3% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4 for 1 h, followed by 1% osmium in 0.1 M phosphate buffer, pH 7.2, for 1 h. To demonstrate elastin, cultures were fixed in 2% osmium tetroxide in 0.1 M phosphate buffer, pH 7.2, + potassium ferrocyanide (10 mg/ml) for 18 h (12). Fixation, processing, and embedment in Epon-Araldite for electron microscopy were carried out in the 25-cm^2 flasks in which the cells were grown. After polymerization (3 d, 50°C), the Epon-Araldite layer was separated from the flask, cut into 2-mm squares, mounted on blocks with Epon, and repolymerized overnight in an oven set at 80°C. Thin sections were cut on an LKB ultramicrotome (LKB Instruments, Inc., Rockville, Md.) and examined, with and without counterstaining with uranyl acetate and lead citrate, in either a Zeiss 109R or Siemens 101 electron microscope.

Analytical Procedures

Proteins were hydrolyzed in constant boiling HCl under vacuum at 107°C for 24 h. Amino acids were analyzed on a JLC-6AH amino acid analyzer adapted to a Durrum DC-6A column (0.9 × 30 cm). Protein in the soluble fractions was determined according to Lowry et al. (23) using bovine serum albumin as standard.

Radioactivity was quantified by liquid scintillation using Liquiscint on a Packard Tri-Carb 3380. The radioactivity in the SDS/ME insoluble pellet was determined in an aliquot of the hydrolysate used for amino acid analysis.

RESULTS

The Major Components of the Extracellular Matrix

Intracellular proteins and extracellular matrix proteins can be distinguished by comparing intact cell layers and single-cell suspension. The proteins extracted with NaCl, HAc, GCl, and SDS/ME from both control and ascorbate-treated cells were predominantly intracellular. This localization was evidenced by closely similar gel patterns of soluble proteins of intact cell layers and single-cell suspensions when visualized either by Coomassie Brilliant Blue stain or by fluorography of [^{14}C] proline-labeled protein (gels not shown); in addition, the soluble fractions of trypsinized cell layers were ~10% less radio-active and contained ~2% less total protein than the untrypsinized controls. However, certain extracellular components are present in the extracts, e.g. the soluble subunit of microfibrillar proteins (29), proteoglycans (see below), and small amounts of soluble collagen (in the ascorbate-treated cells).

The insoluble proteins in the SDS/ME pellets are predominantly extracellular (>90%); the pellets from the trypsinized control or ascorbate-supplemented cells (viability 78% in control cells and 81% in ascorbate-treated cells) contained <10% of the protein found in pellets from the identically treated intact cell layers. In a representative experiment, values in control cells (in units of mg protein/150-cm^2 flask) in the SDS/ME pellet were 0.31 for the intact cell layer and 0.03 for the single-cell suspension, whereas in ascorbate-treated cells the values were 1.35 for intact cell layer and 0.07 for single-cell suspension. Comparable losses of protein were noted in pellets from trypsin-collagenase-treated viable single cells.

When cells were grown without ascorbate, the amino acid composition of the insoluble pellet was typical of insoluble microfibrillar proteins and indicated that collagen was absent; with time past confluence, the weight of the pellet increased but the composition remained unchanged (Table I, [29]). After the addition of ascorbate, hydroxyproline and hydroxylysine appeared in significant quantities as collagen became a component of the pellet. The amino acid composition and the

increased protein content of the pellet suggested that collagen and microfibrillar proteins are deposited concurrently. After 9–12 d of ascorbate supplementation, the amino acid composition became typical of collagen (~100 hydroxyproline, 300 glycine, 100 alanine), and collagen was then the major protein of the pellet.

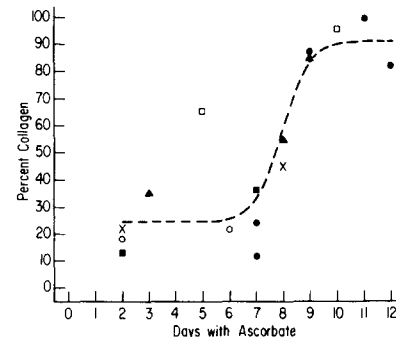The quantity of collagen can be estimated from the content

FIGURE 1 Time-course of collagen deposition as a function of ascorbate feeding. Collagen is expressed as a percentage of total protein in the SDS/ME pellet. Data fit a theoretical sigmoid curve defined by the equation: percent collagen = 33.2 tanh (t − 8) + 57.6, where t = days with ascorbate. Analysis of variance on the regression showed that the fit was highly significant (F (2, 13) = 27.4, P < 0.001). The symbols represent the following cell cultures: △, generation 20, fed ascorbate 2 wk past confluence; □, generation 16, fed 2 wk past confluence; X, generation 8, fed 13 d past confluence; O, generation 27, 7 d past confluence; ■, generation 27, 12 d past confluence; ●, single time-points, generations 6–27, fed 7–14 d past confluence; the dashed line is the theoretical sigmoid curve.

TABLE I

Amino Acid Composition of SDS/ME Insoluble Components from Cells Supplemented with Ascorbate for Increasing Periods of Time

	Without ascor-bate*	Ascorbate-supplemented		
		2 d	8 d	12 d
	residues/1,000 residues			
Lysine	45.8	42.7	55.9	24.6
Histidine	19.0	16.9	16.4	6.7
Arginine	56.6	58.2	55.7	25.4
Aspartic Acid	121.1	112.3	86.4	61.6
Threonine	83.0	73.5	61.6	17.1
Serine	89.5	78.2	74.2	31.3
Glutamic Acid	123.0	119.2	114.5	86.4
Proline	80.7	80.1	81.9	106.6
Glycine	121.8	135.8	162.0	327.7
Alanine	62.8	66.9	73.2	99.5
Valine	45.7	47.9	41.8	29.8
Isoleucine	43.5	46.0	28.2	18.7
Leucine	57.9	57.6	56.5	32.8
Tyrosine	19.2	16.3	18.9	9.4
Phenylalanine	30.4	28.9	21.7	16.6
Hydroxypro-line	nd	19.5	44.4	90.8
Hydroxylysine	nd	nd	6.7	15.0
mg Protein/150 cm^2 flask	0.25, 0.31, 0.72‡	0.30	1.35	3.36
% Collagen	nd	20.0	45.0	90.0

The means of duplicate analyses are given. Results are not corrected for losses during hydrolysis. nd, not detectable.
* This composition remained unchanged in parallel control cultures maintained without ascorbate for the days indicated.
‡ At 2, 8, and 12 d.

of hydroxyproline. Fig. 1 shows the amount of collagen in the SDS/ME insoluble pellet (expressed as a percentage of total protein in the pellet) as a function of time of exposure to ascorbate. Deposition of collagen follows a sigmoid curve; the relative amount of collagen changes little (12–20%) for 6 d and then increases rapidly to 80–90%. Pellets from cells seeded in the presence of ascorbate contained ~90% collagen after 7 d of feeding (experiments now in progress). Neither the time past confluence, when ascorbate feeding was initiated (7–14 d), nor the cell generation doubling (6–27) affected the pattern of collagen deposition. Cells derived from different donor calf aortas behaved similarly. After long-term feeding (9–12 d) with ascorbate, cell layers detached and contracted to <1% of the original surface area. In these cell layers, collagen was found to comprise >80% of the pellet proteins. In contrast, parallel cultures of control cells (without ascorbate) remained adherent.

Treatment of the SDS/ME pellets from control or ascorbate-supplemented cells with 0.1 N NaOH at 98°C for 45 min resulted in an insoluble material with an amino acid composition typical of the microfibrillar proteins but not of elastin. This finding was in agreement with results reported previously (29) for the control cells and indicated that the deposition of

collagen in the ascorbate-fed cells was not accompanied by the deposition of elastin.

Electron microscope examination of the matrix of cells grown without ascorbate showed that microfibrils were the only extracellular material present; when cells were grown with ascorbate, both collagen and microfibrils were present (Fig. 2). The microfibrils were ~10 nm in width and their appearance was identical in cell layers grown with or without ascorbate. Elastin was not observed under either culture condition.

Properties of the Deposited Collagen

The CNBr peptides of the SDS/ME pellet of ascorbate-fed cells (Fig. 3) suggest that collagen type I is the predominant species present. This is evidenced by the coincidence of peptide bands with the standard type I collagen and the absence of lower molecular weight peptides. CNBr treatment of SDS/ME pellet from control cells resulted in peptide fragments that moved with the front under identical conditions of electrophoresis (not shown). Indirect immunofluorescence using antiserum to type I collagen confirmed that collagen type I was a constituent of the cell layer in ascorbate-fed cells (Fig. 4 B).

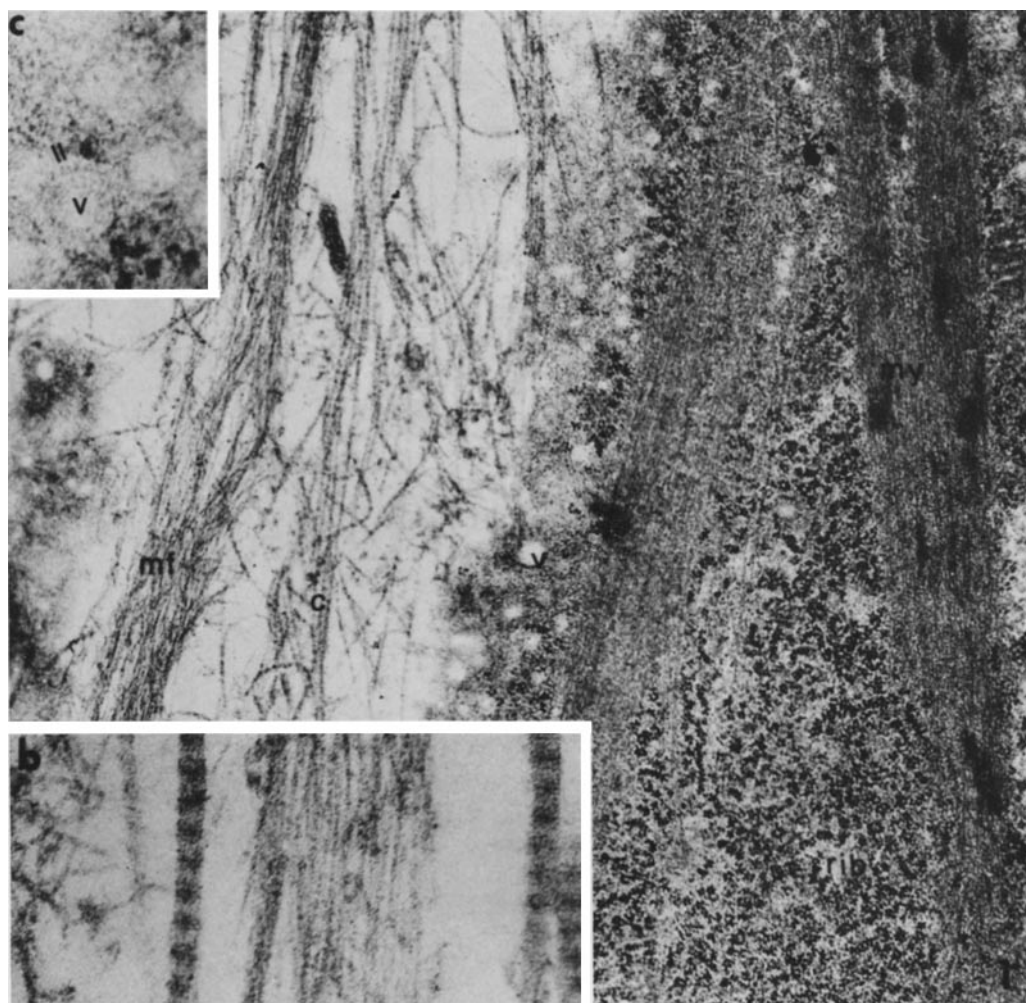To determine whether collagen crosslinking played a role in

FIGURE 2   Cultured calf aorta smooth muscle cells grown in the presence of ascorbate. Fixation, glutaraldehyde-cacodylate; post-fixation, osmium-phosphate (a). The cultured cells retained features of aorta smooth muscle cells. This photograph, of a culture sectioned parallel to the plane of growth, shows the massive arrays of myofilaments (my) and subplasmalemmal vesicles (v). Ribosomes (rib) are abundant. Two types of extracellular fibers are present: collagen (c) with cross striations evident at low magnification and bundles of microfibrils (mf). The periodicity of collagen fibers (b) and the coating on the subplasmalemmal vesicles (parallel lines) (c) are better seen at higher magnifications. a, × 23,000; b, × 67,000. c, × 66,000.
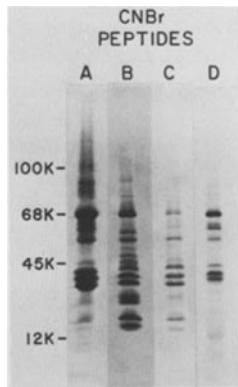
FIGURE 3 SDS PAGE pattern (10% gel) of peptides derived from cyanogen bromide treatment. Type I (lane A) and type III (lane B) collagens were purified from intact calf aorta medial layer and treated with cyanogen bromide. The SDS/ME pellet from intact medial layer (lane C) and calf aorta smooth muscle cells grown in

collagen deposition within the extracellular matrix, βAPN was added to ascorbate-supplemented cultures, and incorporation of [^{14}C]proline into proteins of the cell extracts and the insoluble pellet was assessed. The amounts of label in the NaCl and HAc extracts from cultures treated with ascorbate and βAPN were increased approximately two- and sevenfold, respectively, whereas the insoluble pellet proteins were 40% less radioactive. As shown in Fig. 5 the increase in [^{14}C]proline in the extracts is due to the presence of solubilized collagens; α_1- and α_2-chains are prominent in the NaCl and acetic acid extracts. Treatment of the acetic acid extract with CNBr resulted in peptide patterns of SDS polyacrylamide gels typical of type I collagen (gels not shown).

the presence of ascorbate and [^{14}C]proline (lane D) were treated with cyanogen bromide. Patterns shown in lanes A, B, and C were stained for protein with Coomassie Brilliant Blue. Pattern shown in lane D is a fluorogram of radioactive peptides.
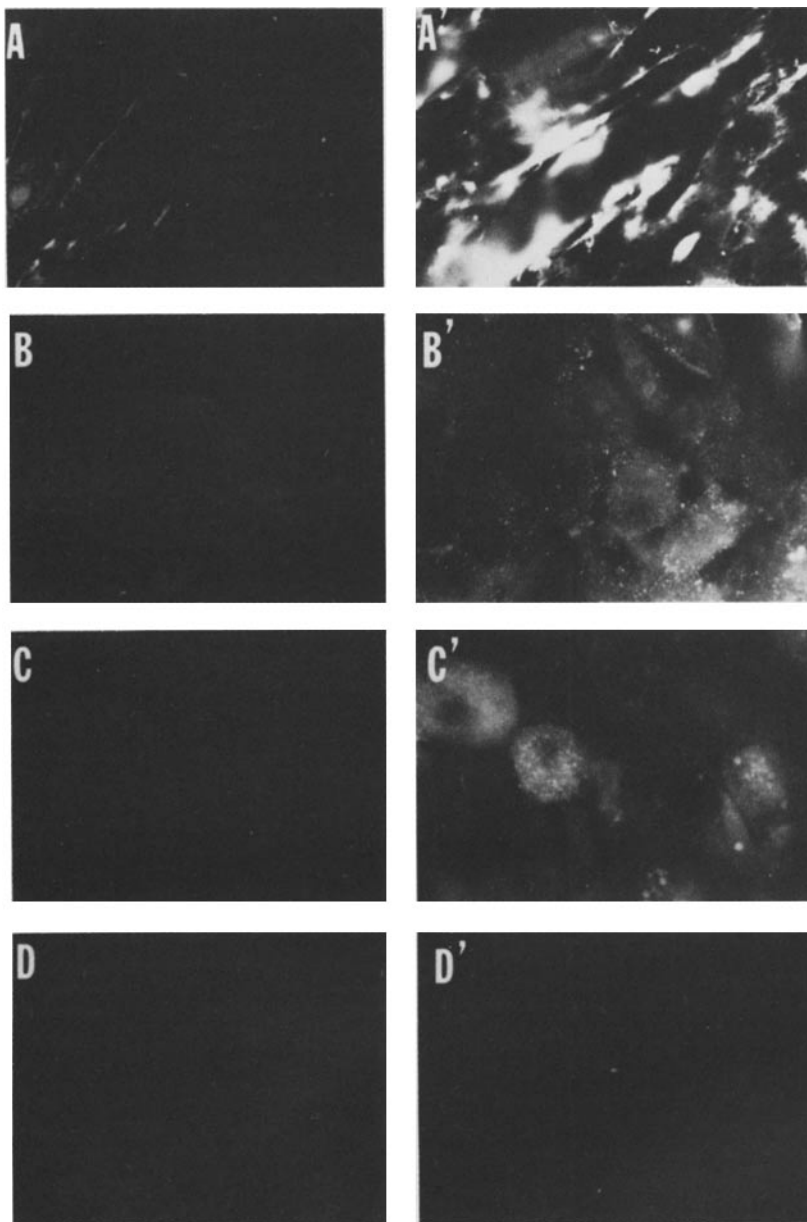

FIGURE 4 Indirect immunofluorescence of calf aorta smooth muscle cells grown with (A'-D') and without (A-D) ascorbate. A and A' are antifibronectin. B and B' are antitype I collagen. C and C' are antiheparan sulfate. D and D' are preimmune sera. X 200.
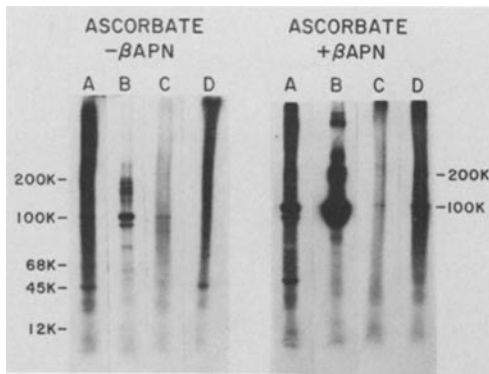
FIGURE 5 Fluorogram of SDS PAGE pattern (3–15% gradient gel) of extracts from calf aorta smooth muscle cells labeled with [^{14}C] proline in the presence of ascorbate with (+) or without (−) β-amino propionitrile (βAPN). A, 1.0 M NaCl; B, 0.5 N acetic acid; C, 4.0 M guanidine-HCl; and D, 1% SDS/0.33 M ME. Smooth muscle cells (PDL 6) were seeded at 2.5 × 10^6 cells/150 cm^2 flask and grown to confluence. At 7 d past confluence, ascorbate (50 μg/ml) and [^{14}C]proline (0.125 μCi/ml) were added to the media. β-amino propionitrile (75 μg/ml) was included in the experimental flasks. The ascorbate was supplemented daily and the [^{14}C]proline and β-amino propionitrile were added at each feeding (every 3 d). After 1 wk, the cell layers were removed by scraping and extracted as described in the text. Equivalent amounts of protein (100 μg) were loaded in each lane.

Hydroxyproline and hydroxylysine were not detected upon amino acid analysis of the SDS/ME pellet from the ascorbate-βAPN fed cells, suggesting the absence of collagen. The composition of the pellet remained typical of the microfibrillar proteins.

Electron microscopy showed that collagen with a normal banding pattern and microfibrils were present in the matrix of βAPN-treated cells. We conclude from these experiments that, in the presence of ascorbate and βAPN, collagen was deposited in the extracellular matrix but was not covalently cross-linked and therefore could be solubilized during the extraction.

Efficiency of Collagen Deposition

Calf aorta smooth muscle cells, whether grown with or without ascorbate, are able to synthesize collagen as evidenced by the presence of pro-α- and α-chains of collagen in fluorograms of gel patterns of labeled medium proteins (gels not shown). The levels of [^{14}C]hydroxyproline in the medium proteins of control cells confirmed that smooth muscle cells can synthesize collagen in the absence of ascorbate (Table II); in agreement with the data in Table I, the cell layer of control cells contained low amounts of [^{14}C]hydroxyproline and therefore only trace amounts of collagen. This indicates that the collagen secreted by the cell in the absence of ascorbate is not deposited as a component of the extracellular matrix.

As expected, levels of [^{14}C]hydroxyproline in the spent media and the cell layers of the ascorbate-fed cells were increased (Table II) and the pro-α- and α-bands in fluorograms of medium proteins were more intense. (The specific effect of ascorbate on collagen synthesis was not assessed.) The ratio of [^{14}C]hydroxyproline to [^{14}C]proline in the SDS/ME pellet of 0.50 is consistent with results obtained by quantitative determination of total hydroxyproline which indicated that in this experiment the SDS/ME pellet consisted of ~45% collagen. (The soluble extracts from the cell layer after ascorbate feeding contained insignificant amounts of hydroxyproline as com-

pared to the SDS/ME pellet.)

Efficiency of collagen deposition can be estimated by comparing the hydroxyproline found in the medium and the cell layer. In cells supplemented with ascorbate, only ~10% of collagen (or [^{14}C]hydroxyproline) was deposited in the cell layer, whereas in cells grown without ascorbate, ~0.5% of total hydroxyproline was associated with the cell layer.

Accumulation of Proteoglycans and Fibronectin in the Extracellular Matrix

Supplementation with ascorbate leads to a two- to threefold increase in incorporation of [^{35}S]Na$_2$SO$_4$ into the NaCl, acetic acid, and GCl extracts and the insoluble pellet proteins; the most pronounced increase (approximately thirteenfold) occurs in the SDS/ME soluble fraction. The labeled components are shown in Fig. 6. Especially heavy labeling of lower molecular weight species in the presence of ascorbate should be noted.

TABLE II

[^{14}C]Hydroxyproline in Collagen in the Media and the Insoluble Proteins of the Cell Layer

	Hydroxy-proline	Proline	Ratio of hydroxy-proline to pro-line
	Total dpm × 10^5	Total dpm × 10^5	
Control			
Spent Media	7.63	51.0	0.15
SDS/ME Insoluble Proteins	0.04	0.6	0.07
Ascorbate-fed			
Spent Media	34.30	98.0	0.35
SDS/ME insoluble	4.17	8.3	0.50

Smooth muscle cells (PDL 8) were seeded at 2.5 × 10^6 cells/150 cm^2 flask 2 wk past confluence; [^{14}C]proline (0.125 μCi/ml) and, in experimental flasks, ascorbate (50 μg/ml) were added to the media. Cells were fed every 3 d with complete media containing [^{14}C]proline. Those flasks receiving ascorbate were supplemented with ascorbate daily. After 8 d, the cell layers were removed by scraping and extracted as described in the text. The radioactive-spent media from the three feedings were combined.
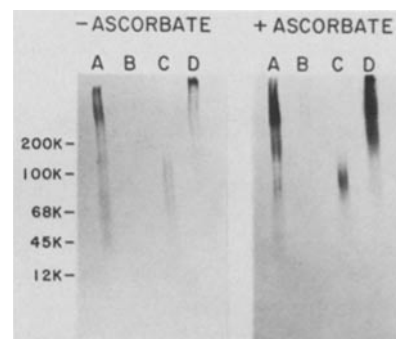

FIGURE 6 Fluorogram of SDS PAGE pattern (3–15% gradient gel) of extracts from calf aorta smooth muscle cells labeled with [^{35}S] Na$_2$SO$_4$ in the presence (+) or absence (−) of ascorbate. A, 1.0 M NaCl; B, 0.5 N acetic acid, C, 4.0 M guanidine-HCl; and D, 1% SDS/ 0.33 M ME. Smooth muscle cells (PDL 18) were seeded at 2.5 × 10^6 cells/150 cm^2 flask and grown to confluence. At 1 wk past confluence, ascorbate (50 μg/ml) and [^{35}S]Na$_2$SO$_4$ (0.5 μCi/ml) were added to the media. Cells were fed every 3 d with complete media containing [^{35}S]Na$_2$SO$_4$ and were given ascorbate daily. After 1 wk, the cell layers were removed by scraping and extracted as described in the text. Ascorbate was not added to control flasks. Equivalent amounts of protein (100 μg) were loaded in each lane.

The newly synthesized cell layer-associated proteoglycans have not yet been characterized.

When control and ascorbate-treated cell layers were reacted with antiserum to heparan sulfate the staining patterns of the control cells showed background fluorescence similar to that obtained with preimmune serum; in contrast, the ascorbate-fed cells showed a diffuse staining pattern of increased intensity (Fig. 4 C). These results confirm that ascorbate supplementation leads to deposition of a sulfated proteoglycan.

The immunofluorescence obtained with fibronectin antiserum is localized to discrete fibers in the control cell layers, as observed previously (29); in ascorbate-fed cells the intensity and number of fibers are increased (Fig. 4 A).

Changes in Cell Properties upon Ascorbate Feeding

When cells were supplemented with ascorbic acid the incorporation of [^{14}C]proline into all the fractions was stimulated. Data for a typical experiment are shown in Table III. The increased radioactivity in the pellet proteins can be explained by accumulation of insoluble collagen (rich in proline and hydroxyproline). The SDS polyacrylamide gel patterns of the extracts indicate that ascorbate stimulates [^{14}C]proline incorporation into many proteins rather than a particular class of proteins.

In the presence of ascorbate, cells show a more rapid growth and increased plating efficiency. The doubling time is reduced from 61.7 ± 3.5 (SD) h in control cells to 47.0 ± 3.6 h in ascorbate-fed cells (data are for three independent experiments) and the plating efficiency increased from 1.7 ± 1.4 (n = 7) to 7.6 ± 1.3 (n = 7). The gross morphology of the cells is also affected by ascorbate; control cells are elongated, whereas cells exposed to ascorbate are polygonal (Fig. 7). The saturation density (~7 × 10^6 cells/60-mm dish for an inoculum of 10^4 cells) is not affected by ascorbate. βAPN does not modify the effect of ascorbate on these cellular properties.

DISCUSSION

These studies have shown that ascorbate modulates the composition of the extracellular matrix of cultured aorta smooth muscle cells. In the absence of ascorbate the major components of the matrix are microfibrillar proteins (29) as concluded from chemical studies and their appearance as extracellular microfibrils. Similar structures have been observed in association with developing elastin or collagen (7, 28). Burke and Ross (5) and Fisher-Dzoga et al. (10) also demonstrated the presence of these fibrils in the extracellular matrix of aorta smooth muscle cells grown in culture.

Collagen becomes the predominant component of the extracellular matrix following ascorbate feeding. In addition, there is a concurrent increase in the amounts of proteoglycans and fibronectin deposited. The cross-linking of collagen is not essential for the deposition of the matrix components since addition of β amino propionitrile, an inhibitor of lysyl oxidase (31), has no effect. Ultrastructural studies confirm that collagen with a normal banding pattern is present in the cell layer in the presence of ascorbate and βAPN. Interestingly, Van Den Hooff et al. (32) observed that skin of chick embryos administered βAPN contains collagen fibrils of normal periodicity.

The deposition of collagen from the medium onto the cell layer is regulated by several processes, among which are rate of collagen synthesis, extent of hydroxylation, and rate of

Table III

Effect of Ascorbate Supplementation on the Incorporation of [^{14}C]Proline into Proteins in Fractions from Smooth Muscle Cells

	Radioactivity	
	− Ascorbate	+ Ascorbate
	(dpm × 10^3)/mg protein	
Soluble Extracts		
1.0 M NaCl/0.05 M Tris-HCl, pH 7.5	69.4	128.4
0.5 M Acetic Acid	36.7	66.6
4.0 M Guanidine-HCl/0.05 M Tris-HCl, pH 7.5	40.8	86.2
1% SDS/0.33 M Mercaptoethanol	43.7	115.6
Insoluble Pellet*	57.6	254.3

Smooth muscle cells (PDL 8) were seeded at 2.5 × 10^6 cells/150 cm^2 flask. At 2 wk past confluence, ascorbate (50 µg/ml) and [^{14}C]proline (0.125 µCi/ml) were added to the media. Cells were fed every 3 d with complete media containing [^{14}C]proline and were supplemented with ascorbate daily. After 8 d, the cell layers were removed by scraping and extracted as described in the text. Ascorbate was not added to control flasks.

* The quantity of proteins in the insoluble pellet increased from 0.3 mg/150 cm^2 flask in the control cells to 1.35 mg/150 cm^2 flask from ascorbate-treated cells; the pellet contained 45% collagen.

conversion of procollagen to collagen (21). Our data show that additional processes must be involved because a relatively long time is required (in postconfluent cultures fed ascorbate) before collagen becomes the dominant component of the matrix and, even when deposition is maximum, only a very small amount (~10%) of total collagen synthesized by the cell is associated with the matrix (also observed in other cell systems [4, 9, 21]). Deposition of collagen in the cell layer follows a sigmoidal time-course, suggesting that collagen accumulation is a cooperative phenomenon. The "order parameter" for this process is not known but it may be the length of collagen filaments in the matrix (11).

Proteoglycans and fibronectin are increased in the matrix when ascorbate is used. The source of the fibronectin is not clear; part of it may be synthesized by the cells, but a significant amount may derive from the culture medium (fetal bovine serum contains a relatively high level of fibronectin [16]). This raises the interesting possibility that the extracellular matrix is constructed, in part, of components not necessarily synthesized by the cell in culture. It is well known that collagen, proteoglycans, and fibronectin bind strongly to each other (1, 24), and collagen may play a central role in coordinating the deposition of the other two matrix elements (25). Thus, the fact that only a small amount of fibronectin is present in the matrix when ascorbate is absent, even though there is a significant quantity of fibronectin in the serum, suggests that collagen may provide sites necessary for fibronectin binding. Similarly, proteoglycans may bind strongly only when collagen is present. It is interesting to note that proteoglycans and collagen are deposited concurrently in chrondrocytes fed ascorbate (15).

Whereas deposition (and possibly synthesis) of certain components of the matrix, collagen, proteoglycans, and fibronectin, is increased by ascorbate, the amount of microfibrillar protein is variable, suggesting that synthesis and deposition of this matrix constituent may be regulated by a different set of control mechanisms. The complete absence of elastin in the extracellular matrix is especially significant in view of the intimate association between elastin and microfibrillar proteins observed in situ (28). Elaboration of the matrix appears to be
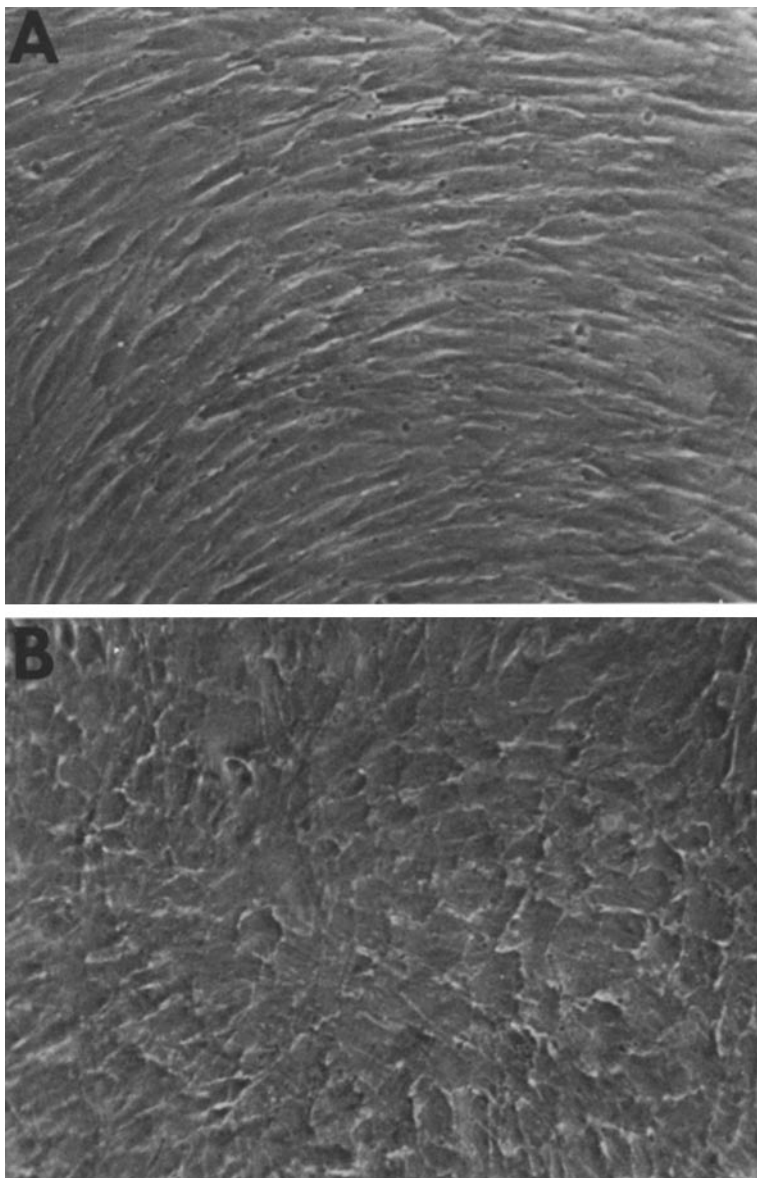
FIGURE 7   Phase-contrast micrograph of calf aorta smooth muscle cells. (A) Smooth muscle cells cultured without ascorbate. (B) Smooth muscle cells cultured with ascorbate. × 100.

regulated by several different processes that can function independently.

Caution must be used in interpreting the data on radioactive labeling of proteins (both intra- and extracellular) and proteoglycans. We have demonstrated that incorporation of radioactive precursors is generally increased when ascorbate is present in the culture medium. These results are most simply explained by postulating an increase in biosynthesis, and the significant increase in the rate of cell division supports the idea that general metabolic activity is elevated in the presence of ascorbate. However, other explanations for the increase in incorporation of radioactivity are possible, e.g., changes in size of precursor pools or in rates of turnover. We are pursuing these questions further and, in particular, we are investigating how the rates of synthesis of the matrix components, collagen, proteoglycans, fibronectin, microfibrillar protein, and also elastin, are affected by ascorbate.

Addition of ascorbate leads to changes in various cellular properties including growth rate, plating efficiency, shape and contractility (as evidenced by contraction of the cell layers after prolonged exposure to ascorbate). Whereas ascorbate may have direct effects on cell metabolism, these changes may also be due to interactions between cells and components of extracellular matrix whose deposition depends on ascorbate. The importance of these interactions is strongly supported by recent evidence showing that artificial substrates have profound effects on cell behavior (14, 26).

In contrast to varying the composition of artificial substrates, modulating the endogenously deposited matrix—by manipulating culture conditions—may provide a finer degree of control of cell behavior.

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