The Effect of Ascorbic Acid on the Nature and Production of Collagen and Elastin by Rat Smooth-Muscle Cells

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1. The effects of various concentrations of ascorbic acid on the quality and quantity of the insoluble extracellular matrices produced by two strains of cultured rat smooth-muscle cells were studied. 2. Ascorbic acid was necessary for the appearance of insoluble collagen in the extracellular matrix. 3. Secretion of soluble collagen continued in the absence of ascorbic acid, but this soluble collagen was markedly underhydroxylated. 4. The amount of insoluble collagen present in the matrix was directly related to the ascorbic acid concentration. 5. The insoluble collagen that appeared in the matrix under conditions where ascorbic acid was limiting was no more than 7% underhydroxylated. 6. In contrast, the amount of insoluble elastin produced was inversely proportional to the ascorbic acid concentration. 7. The elastin produced in the absence of ascorbic acid had the expected amino acid composition, but hydroxyproline was absent. 8. The hydroxyproline content of elastin was also directly dependent on the ascorbic acid concentration. 9. Ascorbic acid had variable effects on the quantity of glycoprotein(s) present in the matrix. 10. The appearance of insoluble collagen in the extracellular matrices produced by cultured human fibroblasts and calf endothelial cells was also completely dependent on the presence of ascorbic acid.

Different proportions of the extracellular matrix proteins are responsible for the physical and chemical properties of connective tissues. Major components of connective tissue are collagen (Grant & Prockop, 1972*a,b,c*), elastin (Gallop *et al.*, 1972), glycoproteins (Anderson, 1976; Sear *et al.*, 1977) and proteoglycans (Hascall & Heinegard, 1974).

Collagen and elastin are particularly difficult to study because of their highly cross-linked structures and their insolubilities (Gallop *et al.*, 1972). Recent advances in our understanding of the biosynthesis and structure of these proteins have been due to the isolation of soluble precursor molecules (Sykes & Partridge, 1972; Rucker & Tinker, 1977; Fessler & Fessler, 1978). Mesenchymal cells are easily cultured outside the animal and have been shown by many authors to produce extracellular matrices composed of connective-tissue proteins (Peterkofsky & Prather, 1974; Evans & Peterkofsky, 1976; Gay *et al.*, 1976; Kao *et al.*, 1977; Berman *et al.*, 1978).

We have reported the production of an extensive extracellular matrix containing glycoprotein(s), elas-

tin and collagen by smooth-muscle cells derived from neonatal rat hearts (Jones *et al.*, 1979). This extracellular matrix can be analysed by sequential enzymic digestion and is a useful model for investigating not only the biosynthesis of the connective-tissue proteins but also their degradation by other cells, such as macrophages (Jones & Scott-Burden, 1979) and tumour cells. Because the properties of connective tissue are known to be modulated by ascorbic acid, we have investigated the effects of this vitamin on the quality and quantity of the extracellular matrix produced by smooth-muscle cells in culture. Our results demonstrate the important role of ascorbic acid not only in collagen biosynthesis but also in the quality and quantity of insoluble elastin produced by the cells.

Materials and Methods

Production of an extracellular matrix by rat smooth-muscle cells

A low-passage strain (R22) and a high-passage strain (R9) of rat smooth-muscle cells, derived as

described previously (Jones *et al.*, 1979), were used for most of the experiments. Cells obtained from cryopreserved stock cultures were seeded at 2×10^5 cells/35mm plastic dish (Falcon Plastic, Oxnard, CA, U.S.A.) and cultured for 12 days in Eagle's minimal essential medium (Grand Island Biological, Grand Island, NY, U.S.A.) containing 2% (v/v) tryptose phosphate broth (Difco Laboratories, Detroit, MI, U.S.A.), 10% (v/v) heat-inactivated foetal calf serum (Irvine Scientific, Irvine, CA, U.S.A.) and penicillin/streptomycin (Grand Island Biological Co.). Medium was changed twice a week and L-[3,4- ^3H]proline (25–50 Ci/mmol, 0.33 $\mu\text{Ci/ml}$; New England Nuclear, Boston, MA, U.S.A.) was added on days 3, 7 and 10 to label the matrix proteins. L-Ascorbic acid (Sigma Chemical Co., St. Louis, MO, U.S.A.) was recrystallized from ethanol, stored at -20°C at a concentration of 5 mg/ml and added daily to the cultures at the various final concentrations described. Then 12 days after cells were seeded, medium was removed and cells lysed with aq. 0.25 M- NH_3 for 30 min at room temperature (Jones & Scott-Burden, 1979). An extensive extracellular matrix remained anchored to the bottom of culture dishes and was washed with a stream of water followed by 70% (v/v) ethanol, and then dried at 37°C .

Cell growth

Because cells were trapped in the extensive extracellular matrix which was resistant to trypsin digestion, this enzyme could not be used to dissociate the cells from the matrix. A solution of 0.25% (w/v) Viokase (Viobin Corp., Monticello, IL, U.S.A.) and 0.05% (w/v) collagenase (Worthington, type CLS) was therefore used to dissolve the matrix and liberate the cells for counting. The cultures were incubated with Viokase/collagenase solution for 60–90 min at 37°C ; the released cells were then aspirated with a Pasteur pipette and the number of cells was determined in a Coulter counter (Coulter Electronics, Hialeah, FL, U.S.A.).

Analysis of the matrix

The composition of extracellular matrix labelled with [^3H]proline was analysed by sequential enzyme digestion (Jones *et al.*, 1979), with bovine pancreatic trypsin (Sigma Chemical Co., type III) pre-treated with elastin to adsorb contaminating elastase, followed by porcine pancreatic elastase (Sigma Chemical Co., type III) and finally bacterial collagenase (Worthington, type CLSPA). Enzymes were stored at -80°C at a concentration of 200 $\mu\text{g/ml}$ in 0.1 M-Tris/0.01 M- CaCl_2 buffer adjusted to pH 7.6 with HCl. Stock solutions were diluted to a final concentration of 10 $\mu\text{g/ml}$ in 0.1 M-Tris/HCl/0.01 M- CaCl_2 buffer, pH 7.6, for assay, and incubations were done for 3 h at 37°C in a

humid environment. Highly purified bacterial collagenase free of contaminating proteinases (Form III; Advance Biofactures Corp., Lynbrook, NY, U.S.A.) were used in some experiments. The radioactivity solubilized by each enzyme after 3 h was determined in a 100 μl sample in 5 ml of Biofluor (New England Nuclear) and counted in a Beckman liquid-scintillation spectrometer. Dishes were washed three times with water between each enzyme treatment. After the final treatment, the residual radioactivity was determined after overnight solubilization in 2 M- NaOH at 37°C .

Detection of soluble collagen in culture medium

Cells grown in 75 cm^2 plastic flasks for 11 days were treated with [^3H]proline (1 $\mu\text{Ci/ml}$) 24 h before being lysed with aq. 0.25 M- NH_3 . Culture medium was collected before NH_3 treatment, heated for 10 min at 100°C to destroy any proteolytic enzymes and exhaustively dialysed against first 1 M- NaCl followed by distilled water. The dialysis residue was adjusted to a volume of 10 ml with Tris/HCl buffer, pH 7.6, and CaCl_2 added to a final concentration of 0.01 M-Tris and 0.01 M- CaCl_2 .

A 5 ml sample of the residue was incubated at 37°C for 4 h in the presence of 5 units of highly purified bacterial collagenase/ml (Form III; Advance Biofactures Corp.), and then dialysed against 30 ml of distilled water at 4°C for 24 h. The total radioactivity present in the diffusate was determined in a 100 μl sample, and the remainder of the diffusate evaporated *in vacuo*, redissolved in 5 ml of 6 M-HCl, and hydrolysed by autoclaving overnight at 120°C . The hydrolysate was then evaporated *in vacuo* and dissolved in 2 ml of distilled water to be assayed for hydroxyproline content.

Protein determinations

The quantities of proteins solubilized from the matrix by each enzyme were determined by the method of Lowry *et al.* (1951). For these experiments phosphate-buffered saline [NaCl (8 g/litre), KCl (0.2 g/litre), $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$ (2.16 g/litre) and KH_2PO_4 (0.2 g/litre)], pH 7.6, containing 1 mM- CaCl_2 was used instead of the 0.1 M-Tris/HCl/0.01 M- CaCl_2 buffer, which interfered with the colorimetric reaction. The matrix was treated sequentially with trypsin, elastase and collagenase (10 $\mu\text{g/ml}$) in 1 ml of buffer. Samples (500 μl) of the supernatants obtained after 3 h incubation at 37°C with each enzyme were assayed. A blank containing the enzyme alone at 10 $\mu\text{g/ml}$ was run in each case. Bovine serum albumin incubated for 24 h at 37°C with 10 μg of trypsin/ml served as a standard for the trypsin release, and bovine ligamentum nuchae elastin and rat tail collagen incubated with their respective degradative enzymes under the same conditions were used as standards for the elastase and collagenase release.

Hydroxyproline determination

Hydroxyproline contents of the peptides sensitive to elastase and collagenase were determined by the method of Juva & Prockop (1966). Samples (1 ml) were made 6 M with conc. HCl, hydrolysed by autoclaving overnight at 120°C, evaporated to dryness *in vacuo* and the residues dissolved in 2 ml of distilled water. The total radioactivity was determined from a 100 µl sample, and a 1 ml sample was used for hydroxyproline determination.

Amino acid analysis of elastin

Unlabelled extracellular matrices, produced by cells growing in 75 cm² plastic flasks, were first treated with trypsin (20 µg/ml) for 5 h at 37°C. After being washed with water, the residual matrices were incubated in the presence of porcine pancreatic elastase (Sigma, type III) at a concentration of 1 µg/ml in 0.1 M-Tris/HCl/0.01 M-CaCl₂ buffer, pH 7.6, overnight at 37°C. The supernatant was then frozen at -80°C, freeze-dried, and hydrolysed in 6 M-HCl at 109°C. Hydrolysates were analysed in a Durrum D-500 amino acid analyser equipped with an L-6 lithium column.

Production of an extracellular matrix by human skin fibroblasts and bovine endothelial cells

Fibroblasts from human skin and endothelial cells from bovine pulmonary artery (Jones, 1979) obtained from stock cultures were seeded at 2 × 10⁵ cells/35 mm culture dishes and grown in Eagle's minimal essential medium containing 2% tryptose phosphate broth, 10% heat-inactivated foetal calf serum and penicillin/streptomycin. L-[3,4-³H]Proline (0.33 µCi/ml) was added to the medium to label the matrix. After 21 days the cells were lysed and the matrix was prepared as described above. Bovine endothelial cells were grown in dishes containing an unlabelled extracellular matrix previously produced by rat smooth-muscle cells.

Results

Ascorbic acid modulation of cell growth and matrix composition

Ascorbic acid deprivation had different effects on the final cell densities reached in the low-passage (R22) and high-passage (R9) smooth-muscle cell strains used. Whereas no effect was observed with the R22 cells, the final cell density was 50% higher with R9 cells grown in the absence of vitamin C. Ascorbic acid did not, however, influence the rate of growth of sparse R9 cultures.

The extracellular matrix produced by rat smooth-muscle cells may be analysed by sequential enzymic digestion with trypsin, elastase and collagenase after removal of the cells by aq. NH₃ lysis (Jones *et al.*, 1979). Trypsin digests glycoprotein(s) components and un-cross-linked elastin and collagen precursors from the matrix; elastase removes elastin, and the residual collagen is sensitive to collagenase digestion. Table 1 shows the effects of ascorbic acid on the composition of the matrices synthesized by the two strains of rat smooth-muscle cells. The R9 strain produced a matrix in the presence of ascorbic acid in which 18% of the total radioactivity was released by trypsin, 27% by elastase and 54% by collagenase.

Table 2. Collagenase-sensitive material present in the matrix produced by the R22 strain in the presence and absence of ascorbic acid

Cells were grown for 12 days with or without the daily addition of ascorbic acid in the presence of [³H]proline. Extracellular matrices were prepared by NH₃ lysis. Highly purified collagenase was incubated for 48 h on native matrices and on matrices pretreated with trypsin and elastase.

Ascorbic acid addition (µg/ml per day)	Total radioactivity released by collagenase (c.p.m.)	
	Native matrix	Matrix pretreatment with trypsin and elastase
50	54 000	39 000
0	2500	240

Table 1. Composition of matrices produced by smooth-muscle cells in the presence and absence of ascorbic acid
Cells were grown with or without the daily addition of ascorbic acid. The insoluble matrices were labelled by addition of [³H]proline (0.33 µCi/ml) to the culture medium. After 12 days, cells were harvested with aq. NH₃, and the extracellular matrix compositions analysed by determining the amount of [³H]proline solubilized by trypsin, elastase and finally collagenase. Residual radioactivity was determined after NaOH solubilization.

Cell strain	Ascorbic acid addition (µg/ml per day)	Total matrix radioactivity (c.p.m./dish)	Percentage of total radioactivity released by:			Residual radioactivity (%)
			Trypsin	Elastase	Collagenase	
R9	50	435 000	18.0	27.0	54.0	1.0
	0	284 000	29.0	67.7	1.3	2.0
R22	50	243 000	36.8	31.0	31.0	1.2
	0	339 000	20.0	79.0	0.3	0.7

However, in the absence of ascorbic acid, 29% of the proline radioactivity was released by trypsin, 67.7% by elastase but only 1.3% by collagenase. Similar changes in the amounts of elastase- and collagenase-sensitive [^3H]proline were also observed with the R22 strain grown in the presence and absence of ascorbic acid (Table 1). Results were more variable for the trypsin-sensitive [^3H]proline, showing an increase with R9 strain and a decrease with R22 strain cells in ascorbate-free conditions.

However, it was possible that in the absence of ascorbic acid the cells produced an abnormal form of collagen, which was digested by trypsin and/or elastase during the sequential enzyme treatment used for the compositional analysis. These possibilities were therefore tested by incubating native matrices that were not pretreated with trypsin and elastase with a highly purified bacterial collagenase (Table 2). The amount of [^3H]proline released from a native matrix produced by R22 strain cells in the absence

Table 3. *Effect of ascorbic acid on soluble-collagen production*

The supernatant medium from rat smooth-muscle cultures incubated for 24 h in the presence of [^3H]proline (1 $\mu\text{Ci}/\text{ml}$) was exhaustively dialysed. The non-diffusible fraction was then incubated with collagenase for 4 h and dialysed again. The amount of [^3H]proline and hydroxyproline in collagenase-sensitive macromolecules were determined on the diffusate.

Cell strain	Ascorbic acid addition ($\mu\text{g}/\text{ml}$ per day)	Total matrix radioactivity (c.p.m./flask)	Hydroxyproline (c.p.m./flask)	Proline (c.p.m./flask)	Proline/hydroxyproline ratio
R9	50	61 000	23 000	38 000	1.6
	0	519 000	9 400	510 000	54.0
R22	50	98 000	34 000	64 000	1.8
	0	110 000	3 300	107 000	32.0

Fig. 1. *Effect of ascorbic acid on the composition of the extracellular matrix*

R9 cells (a) and R22 cells (b) were grown for 12 days with the daily addition of various ascorbic acid concentrations. After cells were lysed with aq. NH_3 , matrices were analysed by sequential enzymic digestion, and the amount of protein solubilized by trypsin (O), elastase (□) and collagenase (Δ) was determined by the method of Lowry *et al.* (1951).

of ascorbic acid was only slightly higher than that solubilized from an identical matrix pretreated with trypsin and elastase. This confirmed the absence of any insoluble collagen in a matrix synthesized without ascorbic acid. However, more [^3H]proline was released by collagenase from the native matrix produced in the presence of ascorbic acid than from the pretreated matrix. This result suggests that some denatured or immature collagen is removed by the trypsin and elastase pretreatments used during the compositional analysis.

Soluble collagen in the culture medium

Culture medium from smooth-muscle cells grown in the presence and absence of ascorbic acid was analysed for collagenase-sensitive proteins (Table 3). In the absence of ascorbate the R9 cells secreted into the medium 8.5 times more soluble radioactive peptides susceptible to collagenase than in the presence of 50 μg of ascorbate/ml. The R22 cells secreted similar amounts of collagenase-sensitive peptides both in the presence and absence of ascorbic acid. The soluble collagen produced in the absence of ascorbic acid was markedly underhydroxylated, as reflected by the high proline/hydroxyproline ratio with both cell strains. Therefore ascorbic acid deprivation did not block collagen synthesis, but con-

siderably affected the extent of proline hydroxylation and completely prevented the appearance of insoluble collagen in the extracellular matrix.

Effect of various concentrations of ascorbic acid

The effect of varying the concentration of ascorbic acid in the culture medium on the composition and quantity of the extracellular matrix was also examined (Fig. 1). The composition of the matrix produced by both cell strains was modulated by the concentration of ascorbic acid, particularly in the range of 0–5 $\mu\text{g}/\text{ml}$ per day. There was a marked increase in the total collagen, and a decrease in the total elastin and glycoprotein(s) contents of the R9 strain matrix as the ascorbic acid concentration increased (Fig. 1a). However, as mentioned above, ascorbic acid caused a 50% decrease in the final cell number reached with the R9 strain, so that the effects on collagen deposition were more pronounced on a per-cell basis, and less significant for the other two proteins. There was a large decrease in elastin deposition and increase in collagen production by the R22 cells with increasing ascorbic acid concentration, whereas little change occurred in the glycoprotein content of the matrix (Fig. 1b). Since ascorbic acid had no effect on the growth of

Fig. 2. *Effect of ascorbic acid on proline/hydroxyproline ratios in elastin and collagen*

R9 cells (a) or R22 cells (b) were grown for 12 days with the daily addition of various ascorbic acid concentrations. After cells were lysed with aq. NH_3 , the matrices were sequentially digested by trypsin, elastase and collagenase. Proline/hydroxyproline ratios were determined on the material solubilized by elastase ($\square$) and collagenase ($\triangle$) by the method of Juva & Prockop (1966).

the R22 cells, these results demonstrate direct effects of the vitamin on the composition of the extracellular matrix.

The proline/hydroxyproline ratios in the material solubilized by elastase and collagenase from matrices produced in the presence of various concentrations of ascorbic acid were determined (Fig. 2). A proline/hydroxyproline ratio of 1.5:1 was found with both strains for the collagenase-sensitive fraction when a daily concentration of 50 µg of ascorbic acid/ml was used. A slight increase in the ratio was observed with lower concentrations, representing a maximum decrease in the hydroxyproline content of 4% for R9 cells and 7% for R22 cells. The collagen that appeared in the matrix therefore had a proline/hydroxyproline ratio in the range of 1.5:1–2:1, showing that only fully hydroxylated or slightly underhydroxylated molecules remained associated with insoluble matrix.

In contrast, large changes in the hydroxylation of proline in the elastase-sensitive fractions were observed (Fig. 2). A proline/hydroxyproline ratio of 9.4:1 for R9 cells, and 6.5:1 for R22 cells was found when cells were grown with 50 µg of ascorbic acid/ml per day. Ratios of 474:1 for R9 cells and 374:1 for R22 cells were obtained when cells were cultured in the absence of ascorbic acid, and the extent of proline hydroxylation was proportional to the vitamin C concentration. These results show that ascorbic acid not only regulates the elastin and collagen content of the matrix but also the hydroxyproline content of the insoluble elastin.

Fig. 3. Relationship between the number of days of exposure to ascorbic acid and the amount of collagen present in the matrix

Daily addition of ascorbic acid was started at different times after cell seeding. After 12 days, cells were lysed with aq. NH_3 , and matrices were analysed by sequential enzymic digestion. Two strains were tested: R9 (△) and R22 (▲).

Time response

The effect of varying the time of exposure of the smooth-muscle cells to vitamin C was investigated by starting the daily addition of the vitamin (50 µg/ml) at different times after the cells were seeded. A direct correlation was found between the amount of collagen present in the matrix and the number of days of exposure to ascorbic acid (Fig. 3). If ascorbic acid was added to the culture medium for only 2 days before cells were lysed, an insignificant amount of collagen was detected in the matrix. When cells were exposed to ascorbic acid for a period of 10 days, 35% of the [^3H]proline incorporated in the matrix produced by R9 cells and 17% in the matrix produced by R22 cells was sensitive to collagenase.

Amino acid analysis of elastin

Amino acid analysis of the material solubilized by pancreatic elastase from matrices produced by the R22 strain in the presence and absence of ascorbic acid are shown in Table 4. The amino acid compositions compared well with each other, especially with regard to glycine, alanine, proline and valine contents and also with published values for bovine ligamentum nuchae insoluble elastin (Rucker & Tinker, 1977). No hydroxyproline was detected in the elastin produced in the absence of ascorbic acid. This is the major difference between the two preparations.

Collagen production by other cell types

The effect of ascorbic acid on the composition of the extracellular matrices produced by two other cell

Table 4. Amino acid composition of elastin produced by R22 strain cells in the presence and absence of ascorbic acid

Amino acid analyses were performed on the material solubilized by elastase from matrices pretreated with trypsin. Values are compared with published values for insoluble elastin extracted from bovine ligamentum nuchae (Rucker & Tinker, 1977).

Amino acid	Composition (residues/1000 residues)		
	R22 with ascorbate	R22 without ascorbate	Bovine ligamentum nuchae
Gly	329	348	332
Ala	203	220	228
Val	90	96	138
Pro	115	142	117
HPp	26	0	16
Ile	25	23	25
Leu	62	59	60
Tyr	22	20	6
Phe	15	13	29
Thr	33	29	10
Ser	25	22	10
Asp	26	13	8
Glu	27	16	16

Table 5. *Composition of matrices produced by human fibroblasts and bovine endothelial cells in the presence and absence of ascorbic acid*

Cells were grown for 21 days with or without the daily addition of ascorbic acid. Matrices labelled with [^3H]proline were prepared by aq. NH_3 lysis and analysed by sequential enzymic digestion with trypsin, elastase and collagenase.

Cell type	Ascorbic acid addition ($\mu\text{g}/\text{ml}$ per day)	Total radioactivity (c.p.m./35 mm dish)	Percentage of total radioactivity released by:			Residual radioactivity (%)
			Trypsin	Elastase	Collagenase	
Human fibroblasts	50	110000	27.5	5.3	63.7	3.5
(HF ₂)	0	5500	74.0	9.0	7.0	10.0
Bovine endothelial	50	45000	70.1	1.7	26.1	2.1
cells (A ₁ Cl ₁)	0	14000	87.0	5.5	3.0	4.5

types was also examined. Human skin fibroblasts and bovine endothelial cells produced matrices that were essentially composed of glycoprotein(s) and collagen in the presence of 50 μg of ascorbic acid/ml per day (Table 5). No insoluble collagen was present in the matrices produced under ascorbic acid-free conditions. No appreciable quantities of elastin were produced by these two cell types in either the absence or the presence of ascorbic acid.

Discussion

Since the description of scurvy and the discovery of its treatment with lemon juice by Lind in 1753, the role of vitamin C in the synthesis and integrity of connective tissue proteins has been extensively investigated. Most mammals, with the notable exceptions of guinea pig and man, are able to synthesize ascorbic acid. Scorbutic guinea pigs have therefore been used in many studies *in vivo* to investigate the effects of this vitamin on connective-tissue biosynthesis. Barnes (1975) showed that ascorbic acid deprivation in guinea pigs was associated with a 5–10% reduction in hydroxylation of proline and impairment of collagen synthesis.

Collagen has also been shown to be produced *in vitro* by many types of cultured fibroblasts, including 3T3 mouse fibroblasts (Goldberg *et al.*, 1963), L929 mouse skin fibroblasts (Gribble *et al.*, 1969), chick-embryo tendon fibroblasts (Kao *et al.*, 1977) and human fibroblasts (Green & Goldberg, 1964; Gay *et al.*, 1976). The production of collagen by cultured endothelial cells (Howard *et al.*, 1976), smooth-muscle cells (Burke & Ross, 1977) and chondrocytes (Benya *et al.*, 1979) has also been reported. However, different types of collagen are produced by these cells. Whereas human skin fibroblasts secrete type-I and type-III collagen (Gay *et al.*, 1976), bovine endothelial cells produce type-IV collagen similar to that of basement-membrane collagen (Howard *et al.*, 1976). Preliminary data indicate that the R9 and R22 cells produce a mixture of types-I and -III, collagen as has been reported for other smooth-muscle cells (Mayne *et al.*, 1978).

It has been shown that ascorbic acid modulates collagen production by cells in culture (Kao *et al.*, 1977; Schwartz & Bissel, 1977; Burke & Ross, 1977; Faris *et al.*, 1978) and that the presence of hydroxyproline is essential for the folding of the precursor chains into the correct triple-helical structure of mature collagen (Grant & Prockop, 1972a,b,c; Berg & Prockop, 1973). Several studies have demonstrated the role of ascorbic acid as a cofactor of proline hydroxylase and lysine hydroxylase and have shown that an underhydroxylated form of collagen is produced in the absence of ascorbic acid (Bates *et al.*, 1972a,b,c; Levene & Bates, 1975; Barnes, 1975; Cardinale *et al.*, 1975). This collagen has different physical properties from mature normal collagen and cannot be converted into insoluble collagen. Whether or not the synthesis of underhydroxylated collagen is blocked in the absence of ascorbic acid is more controversial (Bates *et al.*, 1972b; Barnes, 1975).

Our studies have been concerned with all of the insoluble matrix components produced by cultured cells, whereas most of the above reports have concentrated only on collagen production. Also, the proteins were produced in sufficient quantities by the smooth-muscle cells to allow their actual quantification. We have therefore been able to investigate the overall effects of ascorbic acid and to confirm and extend the above results. We clearly demonstrated that ascorbic acid is essential for the production of insoluble fully hydroxylated collagen and its incorporation into the extracellular matrix produced by smooth-muscle cells, endothelial cells or fibroblasts. The absence of immature trypsin-sensitive insoluble collagen under ascorbate-free conditions was also confirmed by the use of a proteinase-free collagenase preparation on a native matrix. We also demonstrated a strong connection between the ascorbic acid concentration and the amount of insoluble collagen present in the matrices. The production of collagen by rat smooth-muscle cells was not stopped in the absence of ascorbic acid, as shown by the presence of collagenase-sensitive peptides in the culture medium. These soluble peptides secreted in the absence of ascorbic acid were markedly under-

hydroxylated, as shown by their high proline/hydroxyproline ratio. In contrast, the insoluble collagen secreted in the presence of various ascorbic acid concentrations was fully hydroxylated or only slightly underhydroxylated (4–7%). These results confirm the suggestion of Barnes (1975) that a more than 5–10% decrease in hydroxyproline content prevents the formation of the triple-helical structure of collagen.

Although elastin production *in vitro* has been demonstrated in several cell systems (Ross, 1971; Gimbrone & Ramzi, 1975), much less is known on the effects of ascorbic acid on the biosynthesis of this protein. The formation of hydroxyproline is, however, not essential for the biosynthesis and secretion of this protein, since tropoelastin synthesis continues in chick aortic cells incubated in the presence of the prolyl hydroxylase inhibitor $\alpha\alpha'$ -bipyridine (Uitto *et al.*, 1976). Our results demonstrate that ascorbic acid has an important modulating effect on elastin production by rat smooth-muscle cells. An inverse correlation between the amount of elastin present in the matrix and the concentration of ascorbic acid in the medium was observed. Amino acid analysis of elastase-susceptible proteins present in matrices synthesized in the presence and in the absence of ascorbic acid were similar to published values for insoluble elastin (Rucker & Tinker, 1977), and the only major difference between the two types of elastin was their hydroxyproline content. The production of an underhydroxylated form of elastin has also been demonstrated in ascorbic acid-deficient guinea pigs (Barnes *et al.*, 1970). Mature elastin is formed from soluble elastin subunits, which are cross-linked into a fibrous structure. Allysine, allysine aldol, lysinonorleucine, dehydrolysinonorleucine and desmosines, all derived from lysine, are the major cross-linking amino acids (Rucker & Tinker, 1977). Hydroxylysine, a cross-linking amino acid of collagen, is not found in elastin (Tanzer, 1973). Hydroxyproline is present at a lower concentration in elastin than in collagen and has not been shown to be essential for the stability of the elastin molecule as it has been demonstrated for collagen (Berg & Prockop, 1973). It is thus not surprising that ascorbic acid does not affect elastin insolubility.

The use of cultured rat smooth-muscle cells is a simple and rapid technique to obtain relatively large amounts of extracellular matrix, which should therefore allow further studies on the biosynthesis of all the connective-tissue proteins. The cells also produce milligram quantities of underhydroxylated elastin when grown in the absence of ascorbic acid which could be used for structural and degradation studies. Preliminary results in our laboratory have demonstrated that the underhydroxylated elastin is more resistant to digestion by cultured human tumour cells.

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