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Effects of Ascorbate on Insoluble Elastin Accumulation and Cross-Link Formation in Rabbit Pulmonary Artery Smooth Muscle Cultures†

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ABSTRACT: Cultured pulmonary artery smooth muscle cells derived from the medial vessel layer of weanling rabbits were grown in the presence or absence of sodium ascorbate. The connective tissue elements insoluble elastin and collagen were identified and quantified. Formation and accumulation of α-aminoadipic acid γ-semialdehyde (allysine) and the intermolecular cross-links desmosine (Des), isodesmosine (Ides), and aldol condensation product (Aldol) were evaluated from [^{14}C]lysine pulse–chase experiments. [^{14}C]Des, [^{14}C]Ides, peptide-bound [^{14}C]lysine, [^{14}C]allysine, and [^{14}C]Aldol were determined from amino acid analysis. The latter two components were determined after reduction with $NaBH_4$. [^{14}C]Proline conversion to hydroxy[^{14}C]proline and collagenase susceptibility were used to identify and quantify collagen synthesis. Ascorbate dramatically affects insoluble elastin synthesis, accumulation, and cross-link formation. Cells grown in the presence of ascorbate synthesize and accumulate significantly less insoluble elastin than non-ascorbate cultures. Those elastin molecules which do become incorporated into the extracellular matrix in the presence of ascorbate contain a slightly elevated content of hydroxyproline and lysine and, most importantly, are turned over more rapidly.

Several reports on cells in culture have focused on the hydroxylation of proline during the formation of collagen. Such culture studies have included the presence of little or no ascorbate (Peterkofsky, 1972), the use of α,α'-dipyridyl as an inhibitor of prolyl hydroxylase (Hurych & Chvapil, 1965; Rosenbloom et al., 1973; Prockop et al., 1976), and/or the incorporation of proline analogues into the polypeptide backbone (Dehm & Prockop, 1971; Uitto et al., 1976). As expected, significantly less functional collagen is produced under each of these conditions. On the other hand, it appears that excessive amounts of ascorbate in cell cultures is not a problem with regard to collagen hydroxyproline formation (Jeffrey & Martin, 1966). Routinely, cell cultures are treated with sodium ascorbate, 50 μg/mL of media, in order to facilitate hydroxyproline and hydroxylysine formation (Jeffrey & Martin, 1966).

Little attention has been paid to the effect of ascorbate on the formation of hydroxyproline in elastin. One reason relates to the fact that few cell types are capable of synthesizing elastin in culture. Vascular smooth muscle cells (Burke & Ross, 1979; Snider et al., 1981), human umbilical cord vein endothelial cells (Jaffee et al., 1978), and chondroblasts (Quintarelli et al., 1979) have been reported to produce both soluble and insoluble forms of elastin. Bovine vascular smooth muscle cells appear to produce only microfibrillar components (Schwartz et al., 1980), whereas bovine ligamentum nuchae fibroblasts synthesize soluble elastin and the microfibrillar components (Sear et al., 1978, 1981; Mecham et al., 1981). These fibroblasts, however, have been found to incorporate [^{14}C]lysine into desmosines when cultured on a matrix derived from fetal ligament tissue (Mecham, 1981). Recently, DeClerck & Jones (1980) studied the effect of ascorbate on the nature and production of elastin by neonatal rat heart smooth muscle cells. Using sequential enzymatic digestions of the extracellular matrix, they concluded that ascorbate was essential for the production and incorporation of fully hydroxylated collagen into the culture matrix. The amount of insoluble elastin present in the matrix however was inversely proportional to the concentration of ascorbate in the media. Scott-Burden et al. (1979) examined [^{3}H]valine and [^{14}C]methionine incorporation in this system and concluded that a deficiency of ascorbic acid enhanced the biosynthesis and extracellular processing of elastin in these cultures.

Proline hydroxylation appears not to be necessary for secretion of tropoelastin (Rosenbloom & Cywinski, 1976), for oxidative deamination of lysine residue (Narayanan et al., 1977), or for desmosine formation (Narayanan et al., 1978). The presence of α,α'-dipyridyl (Rosenbloom & Cywinski, 1976) or proline analogues (Uitto et al., 1976; Schein et al., 1977a,b) in culture media also does not prevent secretion of tropoelastin from the cell. Two observations have appeared concerning the possible consequences of excess hydroxylation of proline in elastin. One observation suggests that in suspension culture, chick embryonic aortic cells are capable of producing an overhydroxylated tropoelastin when incubated

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with [^{14}C]proline in the presence of ascorbate (Uitto et al., 1976). The other suggests that under appropriate conditions in vitro, purified underhydroxylated tropoelastin can be significantly hydroxylated by treatment with a prolyl hydroxylase system isolated from chick calvaria (Schein et al., 1977a,b). In fact, 44% of the available proline was hydroxylated. In ascorbate-treated and non-ascorbate-treated rat smooth muscle cultures, DeClerck & Jones (1980) found the hydroxyproline content to be the only major difference between the elastins synthesized. These authors suggested that synthesis of elastin molecules was clearly modulated by ascorbate but that insolubility of the molecules was not affected by the vitamin. Scott-Burden et al. (1979) postulate that underhydroxylated elastin may be secreted and cross-linked into insoluble elastin better than hydroxylated monomers. This present study was undertaken to examine the relationship, if any, between cross-link content, hydroxylation, and elastin insolubility in rabbit smooth muscle cell cultures by employing ascorbate as the key probe.

Materials and Methods

Smooth Muscle Cells. Pulmonary artery smooth muscle cells were isolated and grown from the medial layer of the main pulmonary artery obtained from weanling New Zealand rabbits. A slight modification of the method of Faris et al. (1976) was used to separate the intimal and adventitial layers from the medial arterial segments. Dulbecco's modified Eagle's medium containing 2.2 g of sodium bicarbonate/L, 10% fetal bovine serum, 0.1 μM nonessential amino acids, 1 mM sodium pyruvate, 1000 units/mL penicillin G, 10 μg/mL tetracycline hydrochloride, and 1 μg/mL amphotericin B was used throughout the dissection. After attachment to 75-cm^2 tissue culture flasks, the explants were flooded with 10 mL per flask of Dulbecco's medium (D.V. 3.7) containing 3.7 g of sodium bicarbonate/L, 10% fetal bovine serum, 1 μM nonessential amino acids, 1 mM sodium pyruvate solution, 100 units/mL penicillin G, and 100 μg/mL streptomycin and left undisturbed for 1 week in a humidified 5% CO_2/95% air incubator at 37 °C. Refeeding with 20 mL of the same medium occurred on days 7, 10, and 13. On day 16, the cells were subcultivated by detachment with 2 mL of 0.05% trypsin/0.02% EDTA (GIBCO) for 5–8 min at 37 °C. The pooled cells were centrifuged at 600g for 10 min, resuspended, and seeded, 1.5 × 10^6 cells/75-cm^2 flask, into new flasks containing 10 mL of Dulbecco's medium (D.V. 2.2) with 2.2 g of sodium bicarbonate/L but otherwise the same as D.V. 3.7. Twenty-four hours after being seeded and on day 4, the media were replaced with 20 mL of D.V. 3.7. Confluence is reached at approximately day 5, and on day 7, the cells were again detached with trypsin and seeded into new 75-cm^2 flasks as described above (second passage). These second-passage cells were fed with 20 mL of D.V. 3.7 medium 24 h after seeding and then every 3–4 days thereafter. When the cells were grown in the presence of ascorbic acid, sodium ascorbate was added to the maintenance medium at a concentration of 50 μg/mL, prior to each feeding. Each flask was also supplemented once between feedings with 1.0 mg of sodium ascorbate/flask dissolved in 0.5 mL of media.

Pulse Procedure. At the time of pulsing, the spent medium was aspirated off and the cell layer washed 2 times with 5 mL of Ca^{2+}- and Mg^{2+}-free Puck's saline G. Prior to the pulse, the cells were incubated at 37 °C in 5% CO_2/95% air for 1 h with 10 mL of serum-free D.V. 3.7 medium lacking either proline or lysine. The medium in each flask was then replaced with 10 mL of the identical medium containing either 1 μCi/mL [^{14}C]proline (sp act. 250–260 Ci/mol; New England Nuclear) or 1 μCi/mL [^{14}C]lysine (sp act. 312 Ci/mol; New England Nuclear). When the cells were pulsed in the presence of ascorbic acid, both the prepulse medium and pulse medium contained 50 μg/mL sodium ascorbate. A pulse period of 24 h was used in all cases.

Chase Procedures. At the end of the pulse period, the spent radioactive medium was removed, the cell layer was washed twice with 5 mL of Puck's saline G, and 20 mL of D.V. 3.7 medium was added to each flask. The medium was then changed twice weekly for the specified chase period. Cells chased in the presence of ascorbic acid were fed medium containing 50 μg/mL sodium ascorbate. A supplement of 1.0 mg in 0.5 mL of medium was also added to each flask between feedings as described above.

At each harvest time, the medium was removed and each cell layer washed twice with 5 mL of saline. From the [^{14}C]proline-treated cells, the medium and washes from two flasks were combined, dialyzed exhaustively against H_2O, and lyophilized. Cell layers from the same two flasks were harvested with the aid of a rubber policeman, pooled, dialyzed against cold H_2O, and lyophilized. The material was then homogenized in H_2O by a motorized glass homogenizer.

Aliquots representing the equivalent of 0.8 flask of cell layer were analyzed for hot alkali insoluble elastin. The equivalent of 0.2 flask of cell layer was acid hydrolyzed for hydroxy-[^{14}C]proline, [^{14}C]proline, and total nitrogen, 0.3 flask of cell layer was used to determine collagenase-susceptible protein as described by Peterkofsky & Diegelman (1971), and 0.25 flask (×2) of cell layer was used for DNA quantitation. Aliquots representing the equivalent of 0.5 flask of media were analyzed for hydroxy[^{14}C]proline and [^{14}C]proline. The equivalent of 0.5 flask of media was used for determination of collagenase-susceptible protein.

The [^{14}C]lysine-pulsed cells were pooled in groups of four flasks per time point, harvested, dialyzed, lyophilized, and homogenized. These homogenates were aliquoted into samples corresponding to 1.7 flasks (×2) of cell layer for quantitation of ^{14}C-labeled lysine derived cross-links as described below, 0.15 flask of cell layer for total amino acid composition, and 0.2 flask (×2) of cell layer for DNA determinations.

For quantitation of total accumulated lysine-derived cross-links in insoluble elastin, two flasks per time point that had been pulsed and chased with unlabeled lysine were harvested, dialyzed, and lyophilized. Aliquots of homogenate representing 1.0 flask of cell layer were reduced with NaB^3H_4 before hot alkali treatment, 0.2 flask equivalent of cell layer was hydrolyzed for total amino acid composition, and 0.2 flask (×2) of cell layer was used for DNA determinations.

$NaBH_4$ Reduction. α-Aminoadipic acid γ-semialdehyde and the aldol condensation product formed from two residues of the semialdehyde are relatively unstable when exposed to extremes of pH. It had previously been established that reduction of the cell layer with $NaBH_4$ prior to such treatment converts these compounds to the more stable hydroxynorleucine (HNL) and reduced aldol product (Aldol), respectively, allowing quantitation (Lent et al., 1969). NaB^3H_4, which introduces a tritium label into each of the reduced structures, facilitates the identification and measurement of accumulated nonradioactive HNL and Aldol residues (Blumenfeld & Gallop, 1966). For quantitation of peptide-bound [^{14}C]HNL and [^{14}C]Aldol, the reduction is done with nonradioactive $NaBH_4$.

In all cases, a solution of 1.0 mg of $NaBH_4$/0.5 mL of 0.001 N NaOH or 1.0 mg of NaB^3H_4/0.2 mL of 0.001 N NaOH was added to the aliquots of cell layer homogenate. The

reduction was allowed to proceed at 4 °C, pH 9.0, with occassional shaking, for 90 min. The reaction was terminated by the addition of 0.3 mL of 50% acetic acid followed by lyophilization.

Insoluble Elastin Preparation. Insoluble elastin in the cell layer was prepared according to the method of Lansing et al. (1952). Briefly, lyophilized cell layer aliquots were suspended in 5.0 mL of 0.1 N NaOH at 98 °C for 45 min with occasional shaking. Suspensions were centrifuged; the insoluble residue was washed twice with hot H_2O and then lyophilized.

Amino Acid Analysis. Amino acid composition and protein contents were determined by analysis on a Beckman 119CL amino acid analyzer equipped with a Beckman Model 126 data system after hydrolysis in 6 N HCl at 110 °C in vacuo for 22 h.

DNA. The diphenylamine method of Burton (1956) was used to measure DNA in the cell layer. Calf thymus DNA standards and blank solutions were run with the samples.

Quantitation of Radioactivity. Hydroxy[^{14}C]proline and [^{14}C]proline were determined by hydrolyzing the media and cell layer aliquots separately in 6 N HCl at 110 °C in vacuo for 22 h. The hydrolysates were placed on a Beckman 119 amino acid analyzer, and fractions were collected and counted for radioactivity.

Collagenase-susceptible protein in the media and cell layer was determined by digesting the cell layer or media aliquots at 37 °C with bacterial collagenase repurified in this laboratory.

[^{14}C]Desmosines and [^{14}C]lysine incorporated in the insoluble elastin of the cell layer were determined by acid hydrolyzing the insoluble residue remaining after Lansing treatment. The hydrolysates were placed on a Technicon amino acid analyzer equipped with a split stream device. Elution was accomplished by employing a gradient described by Hamilton (1963). The fractions containing desmosine, isodesmosine, and lysine were counted for radioactivity.

Hydroxy[^{14}C]norleucine and ^{14}C-labeled reduced aldol condensation product ([^{14}C]Aldol) in the cell layer were routinely determined by hydrolyzing the $NaBH_4$-reduced insoluble Lansing residues in 2.0 N NaOH at 110 °C for 22 h and analyzing the resulting neutralized hydrolysate on a Technicon amino acid analyzer. A buffer system described by Burns et al. (1965) for separation of lysine-derived reduced cross-links in elastin was employed.

[^{3}H]Hydroxynorleucine and ^{3}H-labeled reduced aldol condensation product ([^{3}H]Aldol) in the Lansing residue from $NaB^{3}H_4$-reduced unlabeled cell layer aliquots were determined by base hydrolysis followed by analysis on a Technicon amino acid analyzer as described above.

Radioactivity was monitored with a Packard Tri-Carb liquid scintillation spectrometer using Beckman formula 963 counting cocktail. This system has a counting efficiency of 40% for tritium and 83% for carbon-14.

Results

Growth of Smooth Muscle Cells. Pulmonary artery smooth muscle cells in culture are not density dependent contact inhibited. They continually divide, forming multilayers in a typical "hills and valleys" pattern. DNA levels and total nitrogen continue to accumulate with time in culture. When cultures of rabbit pulmonary artery smooth muscle cells are grown in the presence of ascorbate from the onset of second passage, the DNA levels are approximately 1.2 times higher than for those cells grown in the absence of ascorbate. This observation is consistent with data from other laboratories (Schwartz et al., 1981).

Table I: Effect of Ascorbate on Collagen Synthesis for the Day 14 Pulse

	dpm × 10^{-5}/flask					
	cell layer[a]			media[a]		
	group A	group B	group C	group A	group B	group C
proline	18	16	–	1.7	3.0	–
	13	–	29	2.3	–	4.8
OH-proline	0.8	2.3		0.2	1.6	
	0.5	–	3.4	0.3	–	2.5
	Percent Collagen Synthesis					
hydroxylation[b]	1.8	5.7	–	5	30	–
	1.5	–	4.7	5	–	29
collagenase[c]	3.3	3.3	5.0	9	18	19

[a] Group A, cells grown in the absence of ascorbate, pulsed in the absence of ascorbate; group B, cells grown in the absence of ascorbate, pulsed in the presence of ascorbate; group C, cells grown in the presence of ascorbate, pulsed in the presence of ascorbate. [b] Percent collagen synthesized (hydroxylation) = {2[OH-proline (dpm)] × 100}/{5.4[proline (dpm) − OH-proline (dpm)] + 2[OH-proline (dpm)]}. [c] Percent collagen synthesized (collagenase) = {[collagenous (dpm)] × 100}/{5.4[noncollagenous (dpm)] + [collagenous (dpm)]}.

Effect of Ascorbate on Collagen Synthesis and Hydroxylation. Smooth muscle cells derived from one set of explants were subcultured into second passage and evaluated for proline hydroxylation and collagen synthesis in the presence and absence of ascorbate. Flasks of cells were divided into three groups. Group A was grown in the absence of ascorbate from the time of seeding into second passage and pulsed in the absence of ascorbate as described under Materials and Methods. Group B was grown in the absence but pulsed in the presence of ascorbate while group C was grown and pulsed in the presence of the vitamin. Pulse labeling studies with [^{14}C]proline were done on day 14 after seeding for a duration of 24 h. Percent hydroxylation, collagenase-susceptible protein, and relative collagen synthesis occurring in both the cell layer and media are shown in Table I.

In groups A and B, the total radioactivity incorporated into the cell layers was similar. The degree of proline hydroxylation in these two groups, however, differed significantly. On the basis of hydroxy[^{14}C]proline formation, the percent collagen synthesized by the cells in group A appeared to be 3 times less in their extracellular matrix on day 14 than that in group B. When the cells were grown and pulsed in the presence of ascorbate (group C), 2 times more total radioactivity was incorporated into the cell layer than in either group A or group B whereas the percent hydroxyproline formed is approximately equal to, or slightly lower than, that in group B. The data obtained from the media of each of these groups were consistent with the cell layer data.

Effects of Ascorbate on Elastin Synthesis and Accumulation. Second-passage smooth muscle cells subcultivated from the same set of explants were divided into two groups. One group was grown with 50 μg/mL sodium ascorbate in the media from the onset of second passage as described under Materials and Methods, and the other group was grown without ascorbate. On day 14 a portion of the cultures from both groups was pulsed with [^{14}C]lysine and chased as described under Materials and Methods. The remaining cultures were sham pulsed with nonradioactive lysine and then chased. Those cells grown in the presence of ascorbate were pulsed and chased in the presence of ascorbate while those not grown in ascorbate were pulsed and chased without the vitamin. Cells

Table II: Amino Acid Composition of Lansing Residue

	residues per 1000								
	day 7		day 14		day 21		day 28		day 36
	−ASC	+ASC	−ASC	+ASC	−ASC	+ASC	−ASC	+ASC	−ASC
Hyp	3	11	3	13	6	14	9	15	7
Asp	28	64	24	65	8	17	10	29	8
Thr	17	25	13	25	11	12	11	15	10
Ser	19	30	17	27	13	14	13	17	11
Glu	45	94	40	82	21	30	23	45	21
Pro	119	93	126	95	149	120	158	119	150
Gly	275	189	270	214	287	305	260	278	279
Ala	201	140	220	165	238	215	231	194	235
Val	99	99	107	94	116	104	119	99	118
Cys	0	0	0	4	0	0	0	0	0
Met	5	9	6	5	5	6	7	8	5
Ile	26	35	21	28	18	25	21	26	23
Leu	65	80	62	71	57	62	62	64	62
Tyr	28	28	29	12	30	24	32	28	30
Phe	24	32	23	28	21	21	22	24	21
Lys	17	37	13	29	4	10	4	13	3
His	7	15	5	12	2	5	3	7	2
Arg	17	32	15	26	9	10	10	16	9
Des + Ides	0.98	0.76	0.98	0.75	1.25	1.25	1.25	1.25	1.25

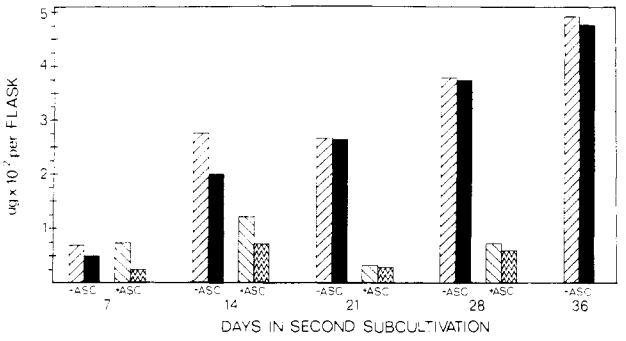

FIGURE 1: Effect of ascorbate on the accumulation of insoluble elastin with time: (right-hatched bar) absence of ascorbate, Lansing insoluble residue; (solid bar) absence of ascorbate, theoretical value based on desmosine content; (left-hatched bar) presence of ascorbate, Lansing insoluble residue; (saw-toothed bar) presence of ascorbate, theoretical value based on desmosine content (see Materials and Methods).

exposed to [^{14}C]lysine were harvested 24 h, 1 week, 2 weeks, and 3 weeks after the pulse and evaluated for elastin accumulation and cross-link formation. The total elastin content in the cultures was defined as the total weight of the amino acids obtained from the Lansing residue of the cell layer homogenate. The theoretical elastin content was calculated from the observation that there are 1.25 residues of desmosine plus isodesmosine per 1000 residues in purified elastin from cell cultures (see 21-day cultures in Table II). This value is, of course, lower than that for elastin obtained from animal sources.

Figure 1 shows the total accumulation of elastin in the ascorbate- and non-ascorbate-treated cultures. Experimental values calculated from the amino acid composition of the Lansing residues are compared to theoretical values calculated from the corresponding desmosine content. For both conditions of ascorbate, the actual amount of Lansing residue on days 7 and 14 is significantly higher than the theoretical amount of elastin. It should be noted that while equal amounts of Lansing residue are accumulated at day 7, in both non-ascorbate and ascorbate cultures, the ascorbate-treated cultures have significantly less (2.5 times) accumulated residue by day 14. By day 21, the experimental and theoretical values of elastin within the respective culture groups are not significantly different. However, there is a dramatic difference in the quantity and accumulation pattern of elastin with the presence of ascorbate in the cultures. In the absence of ascorbate, the amount of Lansing residue increases with time while in the ascorbate-treated cultures the amount of accumulated elastin, albeit low, reaches a maximum at day 14. The amino acid composition and desmosine content of these Lansing residues indicate that what is accumulating in these cultures is mature elastin. However, an increase in the hydroxyproline and lysine contents is present. At day 28, insoluble elastin still is present in these cultures, but the quantity is 6-fold less than that in the flasks of cells grown in the absence of ascorbate. The amino acid compositions of the Lansing residues at the various times of culture are shown in Table II. No hydroxylysine was found in any of the samples regardless of ascorbate treatment. Compositions on days 7 and 14 contain higher than expected values for the acidic residues. This could be explained by the presence of immature elastin which is only partially resistant to hot alkali treatment, or the presence of unusual quantities of the microfibrillar component. By day 21, insoluble elastin is clearly evident in both conditions of ascorbate. Although there are differences in the amino acid compositions of the treated and nontreated cell layer Lansing residues, desmosine and isodesmosine cross-links are found in both. It is of importance to note that during the time course of this study, both proline and hydroxyproline are continuously accumulating in the cell layers regardless of ascorbate treatment (Figure 2). The amount of hydroxyproline accumulated in the cell layers grown in the presence of ascorbate is 2.5 times that present in the absence of ascorbate. However, the percent of the total hydroxyproline that remains insoluble after the Lansing treatment is significantly greater in the cell layers grown in the absence of ascorbate. Table III gives the calculated accumulations of collagen and elastin in the two culture systems. The data are derived from the percent of hydroxyproline present in the various insoluble residues. Clearly, the accumulation of collagen is greater in the ascorbate-treated cultures. The insoluble elastin in these same cultures, however, is relatively small while in the nonascorbate cultures elastin accumulation is quite significant.

Effect of Ascorbate on Cross-Link Formation. As described under Materials and Methods, total accumulation of reducible elastin cross-links in those cell cultures not undergoing [^{14}C]lysine labeling was determined by reduction with NaB^3H_4

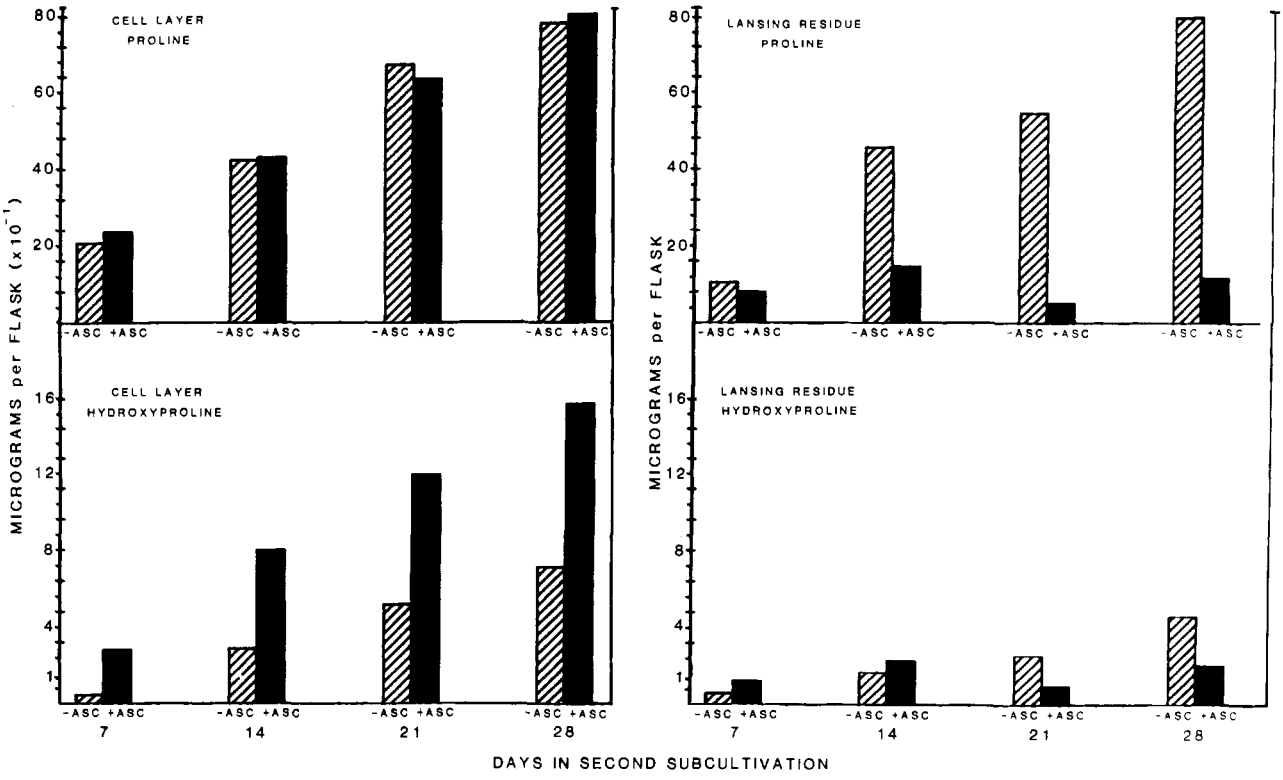

FIGURE 2: Effect of ascorbate on the accumulation of proline and hydroxyproline in the whole cell layer and elastin residue (Lansing) of the cell layer: (right-hatched bar) absence of ascorbate; (solid bar) presence of ascorbate. Note the 10-fold difference in the value of the *y* axis in the cell layer vs. that in the Lansing residue.

Table III: Accumulation of Elastin and Collagen with Time of Culture

days in culture	ascorbate		no ascorbate	
	collagen[a]	elastin[b]	collagen[a]	elastin[c]
7	230.9	57.2	35.3	56.6
14	659.8	128.9	230.0	224.4
21	1008.0	36.3	421.0	352.3
28	1321.9	97.1	567.0	660.8
35	–	–	795.0	668.8

[a] All collagen determinations assumed that hydroxyproline accounted for 8.23% (by weight). This was calculated from an estimate of 100 hydroxyprolyl residues/1000 amino acid residues. No corrections were made for the possible occurrence of underhydroxylated collagen in the non-ascorbate cultures. Values are micrograms of collagen per flask. [b] Accumulated elastin was calculated on the assumption that 1.7% of the weight of elastin is hydroxyproline. This is based on the finding that approximately 13 hydroxyprolyl residues per 1000 amino acid residues are present (see Table II). Values given are micrograms of elastin per flask. [c] Accumulated elastin was calculated on the assumption that 0.7% of the weight of elastin is hydroxyproline. This is based on the finding that approximately 5 hydroxyproline residues per 1000 amino acid residues are present (see Table II). Values given are micrograms of elastin per flask.

before Lansing treatment and base hydrolysis. Figure 3 shows the total amounts of [^{3}H]hydroxynorleucine ([^{3}H]HNL) and ^{3}H-labeled reduced aldol condensation product ([^{3}H]Aldol) as a function of days in culture. Cultures grown in the absence of ascorbate show a continual accumulation of both [^{3}H]HNL and [^{3}H]Aldol in their Lansing residue. When the cells are grown with ascorbate, there is a dramatic decrease in the amount of the two lysine-derived aldehyde components compared to that in the non-ascorbate cultures. A slight increase in [^{3}H]HNL and [^{3}H]Aldol accumulation is observed between days 7 and 14. However, maximum HNL and Aldol levels are reached at day 14, similar to the observations for total insoluble elastin accumulation.

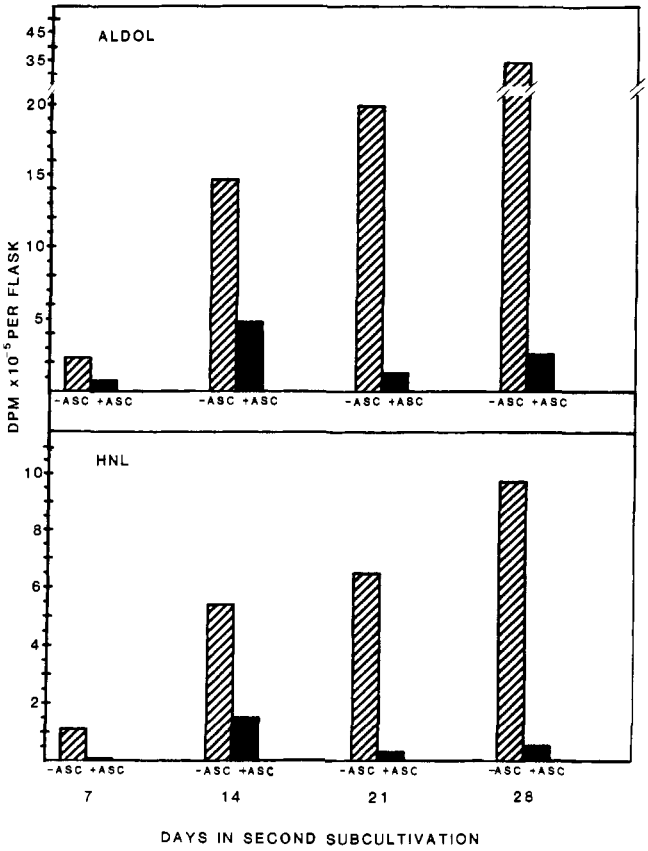

FIGURE 3: Effect of ascorbate on accumulation of ^{3}H-labeled reduced aldol condensation product and hydroxynorleucine in insoluble elastin (Lansing residue): (right-hatched bar) cells grown in the absence of ascorbate; (solid bar) cells grown in the presence of ascorbate.

Analyses of the [^{14}C]lysine pulse–chases were carried out as described under Materials and Methods. Formation of

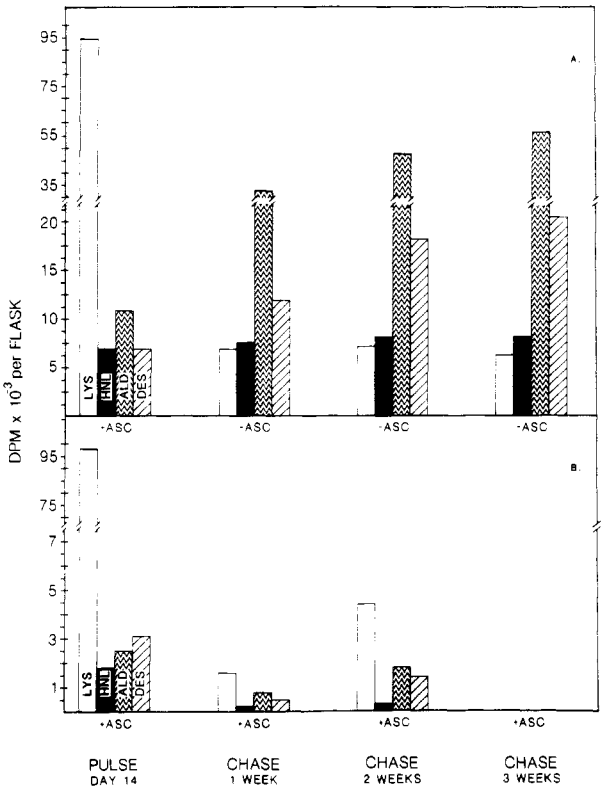

FIGURE 4: Synthesis and accumulation of lysine-derived cross-links during pulse–chase with [^{14}C]lysine: (A) cells grown in the absence of ascorbate; (B) cells grown in the presence of ascorbate. Note differences in the y-axis scales. (Open bar) [^{14}C]lysine; (solid bar) hydroxy[^{14}C]norleucine; (saw-toothed bar) ^{14}C-labeled aldol condensation product; (right-hatched bar) [^{14}C]desmosine + [^{14}C]isodesmosine. The pulse was carried out on 14-day-old second subcultivaton cells, and the chase was monitored weekly as described in the text.

[^{14}C]desmosine and [^{14}C]isodesmosine was quantitated from nonreduced acid hydrolysates of the Lansing residues, while α-amino[^{14}C]adipic acid δ-semialdehyde and the [^{14}C]Aldol condensation product were evaluated from base hydrolyzates of $NaBH_4$-reduced Lansing residue. Figure 4 contains the data for ^{14}C-labeled HNL, Aldol, desmosine plus isodesmosine (DES), and peptide-bound lysine formed during pulse–chase periods in the presence and absence of ascorbic acid.

A 24-h pulse on day 14 shows the presence of all four lysine-derived cross-links in the Lansing residue of the cell layers during both ascorbate conditions, albeit with lower concentrations in the ascorbate-treated cultures.

The chase period after the day 14 pulse shows a continual increase in the formation of ^{14}C-labeled cross-links in the absence of ascorbate with a concomitant decrease in the [^{14}C]lysine content during the initial part of the chase.

By far, the [^{14}C]Aldol represents the highest quantity of radioactivity during the chase period in the non-ascorbate-treated cultures. [^{14}C]HNL remains somewhat constant. The [^{14}C]DES display a continual increase in radioactivity over the entire 3-week chase period. [^{14}C]Lysine, on the other hand, decreases rapidly after the pulse and then remains relatively constant. The specific activity of the [^{14}C]lysine continuously decreases, suggesting a dilution with nonradiolabeled, newly synthesized elastin. The ^{14}C-labeled lysine must represent the characteristic amount of lysine incorporated into the tropoelastin molecule during the time of pulse that will remain as peptide-bound lysine in mature elastin. The increase in radioactivity of the various cross-links appears to be reaching a maximum by the third week of the chase.

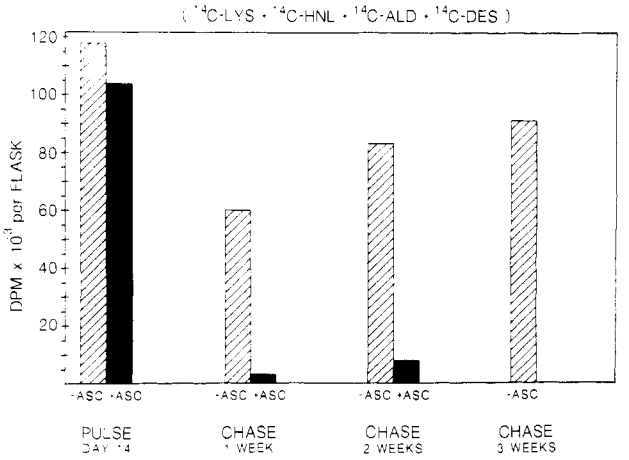

FIGURE 5: Total [^{14}C]lysine content (lysine + cross-links) of insoluble elastin during pulse–chase experiment: (right-hatched bar) absence of ascorbate; (solid bar) presence of ascorbate.

The data from the chase period in the ascorbate-treated cultures are most interesting. As shown in Figure 4B, there is no increase above the pulse levels in the amount of accumulated [^{14}C]lysine-derived cross-links. In fact, the amount of the cross-links tends to decrease. This occurs in spite of the presence of very significant quantities of [^{14}C]lysine in the Lansing residue after the pulse period. This [^{14}C]lysine does disappear during the chase period but obviously is not converted to cross-links. Such data would suggest that the tropoelastin that is synthesized during the day 14 pulse in the presence of ascorbate, and is identified with the Lansing residue, seems to be less able to become incorporated into the extracellular matrix in the form of cross-linked elastin.

Figure 5 serves to illustrate this point. Examination of the total [^{14}C]lysine plus ^{14}C-labeled cross-links incorporated into elastin at various times during the chase suggests that the elastin synthesized in the presence of ascorbate is not incorporated into the cell layer to any significant extent.

Discussion

In cell culture studies of connective tissue synthesis, it is common practice to add sodium ascorbate to the culture medium to ensure complete hydroxylation of the precursor molecules (Jeffrey & Martin, 1966; Peterkofsky, 1972). Since elastin has the potential to be overhydroxylated in vitro (Uitto et al., 1976; Schein et al., 1977a,b), it was of interest to employ a well-defined cell culture system to investigate the consequence of ascorbate treatment on elastin biosynthesis. Cultured rabbit pulmonary artery smooth muscle cells are an appropriate choice since they display an ability to synthesize and accumulate both collagen and elastin (Faris et al., 1976; Snider et al., 1981). In addition, these cells are not dependent upon added ascorbate for overall growth and therefore offer a system in which to study the effects of the vitamin on collagen and elastin production simultaneously.

Coacervation of tropoelastin, the soluble precursor of elastin, at 37 °C results in the condensation of these non-cross-linked molecules into a fibrillar structure (Urry et al., 1979; Cox et al., 1974). It has been suggested that this coacervation or some other aggregate form is a prerequisite for enzymatic cross-linking (Narayanan et al., 1977, 1978). Urry and co-workers (Urry et al., 1979) have shown that a polymer of a synthetic pentapeptide $(Val\text{-}Pro\text{-}Gly\text{-}Val\text{-}Gly)_n$ is capable of forming a coacervate at 37 °C similar in mechanism to tropoelastin. By incorporation of varying amounts of hydroxyproline in place of proline into this polymer, the authors demonstrated that

the hydroxyproline-containing polymers have significantly higher temperature requirements for coacervation.

Several of the observations presented in this paper support and expand upon the studies previously described by others. As expected, the continual presence of ascorbate in the cell cultures results in an increase in collagen synthesis and accumulation and proline hydroxylation well above that which can be accounted for by an increase in DNA.

In the absence of ascorbate, this amount of insoluble collagen accumulated in the matrix is still quite measurable (Table III) even though the ability of the cultures to synthesize hydroxyproline is greatly impaired due to the lack of the vitamin (Table I). The results for elastin synthesis and accumulation are even more striking. From the data presented, it is clear that much less elastin is accumulated in the extracellular matrix of the ascorbate-treated cultures than in the matrix of those cultures not treated with ascorbate. The insoluble elastin matrix produced in the presence of ascorbate contains a somewhat higher content of hydroxyproline and lysine and slightly less lysine-derived cross-links than the non-ascorbate cultures.

It is our hypothesis that the elastin data from the ascorbate-treated cultures may be explained by the alteration of the two phenomena briefly alluded to above, namely, the hydroxylation of proline and the cross-linking of lysine. We speculate that the majority of tropoelastin molecules synthesized in the presence of ascorbate become overhydroxylated and therefore require higher temperatures for maximum coacervation (or fiber formation) and stabilization. Those highly hydroxylated elastin molecules which become bound to the matrix in spite of the presence of ascorbate probably cannot assume an appropriate fiber orientation for cross-link formation and thus can be more easily solubilized by either nonspecific proteolysis (Sandberg et al., 1975) and/or hot alkali treatment. Note that only 8% of the ^{14}C label incorporated into the hot alkali derived insoluble elastin from ascorbate-treated cultures during the 24-h pulse period remains after the 2-week chase, while 70% of the ^{14}C label incorporated during the pulse period is present in the elastin from cells grown in the absence of ascorbate (Figure 5). Such data clearly demonstrate that in the ascorbate-treated cultures the majority of the elastin molecules incorporated into the cell layer during the pulse period are not processed into the insoluble matrix via the normal cross-linking events. Interestingly enough, the portion of elastin in the ascorbate-treated cultures which remains insoluble does have reasonable levels of cross-links. This suggests that some tropoelastin units may not be as extensively hydroxylated as others and thus are capable of being cross-linked. These data are consistent with those others (DeClerck & Jones, 1980; Scott-Burden et al., 1979) in which trypsin was employed as the probe to evaluate cross-linked vs. non-cross-linked elastin in the extracellular matrix of ascorbte- and non-ascorbate-treated rat heart cell cultures.

An important point to be made from our studies and those of others is that, as originally suggested by DeClerck & Jones (1980), one can manipulate a smooth muscle cell culture with ascorbate and alter its extracellular matrix. Yet it cannot be expected that all smooth muscle cells will behave in the same manner. In contrast to rabbit cells, cultured calf smooth muscle cells appear to require ascorbate in their growth medium in order to maintain their morphology (Schwartz et al., 1981; Mazurkowitz et al., 1980). This ascorbate treatment decreases population doubling time and increases general protein synthesis (Schwartz et al., 1981). Microfibrillar protein is accumulated in these cultures during either ascorbate or non-ascorbate conditions but becomes the major matrix component in the absence of ascorbate. However, insoluble elastin is not deposited in either case (Schwartz et al., 1981).

Other studies, not necessarily related to ascorbate, also suggest a relationship between the extracellular milieu and connective tissue synthesis. Cells derived from bovine ligamentum nuchae do not synthesize insoluble elastin unless they are cultured on a preexisting elastin matrix (Mecham et al., 1981; Mecham, 1981). The observations presented here offer insights into the specific effects of ascorbate on extracellular matrix synthesis and the role of hydroxyproline in cross-link formation and accumulation of insoluble elastin. These studies, as well as those from other laboratories, suggest an increasingly important role for the extracellular matrix of cultured cells in defining cell functions.

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Protein Synthesis Initiation Factors from Human HeLa Cells and Rabbit Reticulocytes Are Similar: Comparison of Protein Structure, Activities, and Immunochemical Properties†

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ABSTRACT: Five initiation factors, eIF-2, eIF-3, eIF-4A, eIF-4B, and eIF-5, were purified from human HeLa cells. Methods of protein fractionation and assays for initiation factors which had been developed for rabbit reticulocytes were found to be suitable for HeLa factors. The initiation factors from HeLa cells are similar to or indistinguishable from the corresponding rabbit reticulocyte factors with respect to specific activities, molecular weights as determined by sodium dodecyl sulfate–polyacrylamide gel electrophoresis, and subunit structure. The molecular weight of eIF-3 particles from both species is about 410 000 as determined by equilibrium sedimentation analytical centrifugation. The partial protease fragmentation patterns of corresponding proteins also are similar and indicate that the primary sequences of the factors are related in the two species. Antisera raised in goats against rabbit eIF-3 and human eIF-2, eIF-4A, and eIF-4B cross-react with the cognate factors from both species. On the basis of immunoblotting techniques, eIF-4A is highly conserved, eIF-2α, eIF-3, and eIF-4B are somewhat less conserved, and eIF-2β is the least conserved of the proteins examined. The functional, structural, and immunological results are all consistent with the view that initiation factors from different mammalian cells are very similar.

Initiation of protein synthesis in mammalian cells is promoted by a large number of proteins called initiation factors. Elucidation of the roles played by these factors has been most extensive with those purified from rabbit reticulocytes. At least 10 different initiation factors have been purified and characterized (Schreier et al., 1977; Merrick, 1979; Benne & Hershey, 1978; Sonenberg et al., 1979; Dasgupta et al., 1979; Amesz et al., 1979). Rabbit reticulocytes are a good source of initiation factors because the cell lysate is an extremely active system for protein synthesis and large amounts of the cells can be obtained. However, the reticulocyte is a terminally differentiated cell which has lost its nucleus and is incapable of cell division. Thus, these cells are unsuitable for studying protein synthesis in different physiological states. We have chosen to study human HeLa cells grown in suspension culture. The cells are easily grown, may be synchronized, and can be infected by a number of animal viruses. Furthermore, the cost of growing the cells is comparable to that for obtaining rabbit reticulocytes. We report here the purification of five eukaryotic initiation factors (eIF)[1] from HeLa cells, eIF-2, eIF-3, eIF-4A, eIF-4B, and eIF-5, and show that they resemble closely the corresponding rabbit reticulocyte factors in all respects tested.

Materials and Methods

Growth of HeLa Cells. HeLa strain S3 cells were grown in 3-L spinner culture bottles (Bellco) in up to 2.5 L of Joklik's modified minimal essential medium supplemented with 5% fetal calf serum or calf serum (Flow Laboratories). The cells were harvested in exponential growth phase at a density of (7–8) $\times 10^5$ cells/mL by centrifugation, washed twice in PBS

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[1] Abbreviations: eIF, eukaryotic initiation factor; PMSF, phenylmethanesulfonyl fluoride; $NaDodSO_4$, sodium dodecyl sulfate; BME, 2-mercaptoethanol; Hepes, *N*-(2-hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid; DEAE, diethylaminoethyl.

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