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ELASTIN BIOSYNTHESIS BY SMOOTH MUSCLE CELLS CULTURED UNDER SCORBUTIC CONDITIONS

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SUMMARY. The proliferation of rat heart smooth muscle cells is unaffected by supplementation of the culture medium with ascorbic acid. The presence or absence of the vitamin has a pronounced effect, however, on the amount of elastin which is produced. Scorbutic cultures incorporate significantly more radioactive valine (an amino acid prevalent in elastin) into proteins present in the extracellular matrix than do supplemented controls, while there was no difference between the systems in the incorporation of labelled methionine (absent from elastin). Deficiency of ascorbic acid appears to result in an enhancement of the biosynthesis and extracellular processing of elastin in this culture system.

INTRODUCTION. The biosynthesis of the native connective tissue proteins, collagen and elastin, involves a number of important post-translational modifications of certain amino acids, in particular, the hydroxylation of proline residues (1-3). The importance of ascorbic acid in this regard has been known for some time and a considerable amount of data has been accumulated using cell culture systems (4-8). The absolute ascorbic acid requirement for the formation of correctly structured individual procollagen molecules and for their subsequent extracellular processing in the process of collagen fibre formation, has accordingly become relatively well understood (3).

In contrast, the role possibly played by ascorbic acid in the biosynthesis of elastin has been poorly documented. It appears that the presence of hydroxyproline in elastin may well be of little or no importance in relation to the biosynthesis of the cross-linked extracellular protein (9,10). Since it is common practice to culture smooth muscle cells in the presence of ascorbic acid (for the promotion of cell division), little work has been done

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on elastin formation by smooth muscle cells in scorbutic culture (11,12).

We have been studying the biosynthesis and degradation of various components of extracellular matrices produced by cultured smooth muscle cells from rat hearts (13,14). Because our cultured cells divide at the same rate in the presence or absence of ascorbic acid, and secrete approximately the same total amount of extracellular matrix proteins under both culture conditions, we decided to use our system to study the biosynthesis of elastin under scorbutic conditions.

MATERIALS AND METHODS

Culture of cells and matrix production: The isolation and culture of rat heart smooth muscle cells and the preparation of radioactively labelled extracellular matrix was carried out as previously described (13,14).

Compositional analysis: Analysis of matrices was carried out, by sequential enzymatic digestion (14), with the following modifications: After incubation of matrix samples with the requisite enzyme, in phosphate-buffered saline (PBS), pH 7.6, containing 1 mM $CaCl_2$, digests were collected and protein determinations carried out according to the method of Lowry et al. (15). The standards used for the protein determinations were bovine serum albumin in the case of digestion with trypsin (Sigma Chemical Co., type III), elastin (Sigma Chemical Co.) in the case of elastase (Worthington ESFF), and rat tail collagen in that of collagenase (Worthington CLSPA).

Cross-linking assay: Assessment of the rate and extent of tropoelastin cross-linking was performed using sequential enzyme digests of matrices laid down by cells which had been pulse-labelled for 4 hrs with L-[2,3,4 ^3H] valine. The portion of the radioactivity that was released by elastase alone, after trypsin treatment was completed, was taken to represent the amount of elastin that had become cross-linked (13) (see fig. 3 for details).

Incorporation of radioisotopes into culture system compartments: The simultaneous incorporation of [^3H] valine and [^{14}C] methionine into different compartments of the rat smooth muscle culture systems was studied in the presence and absence of ascorbic acid. Cultures were incubated in the presence of the labelled compounds (1 μCi/ml culture medium) for 20 hours, after which aliquots of culture media were precipitated by the addition of equal volumes of 20% w/v trichloroacetic acid (TCA) and collected on glass fibre (GFC) filters for the determination of radioactivity. Following the removal of media, culture dishes were washed three times with PBS and the cells lysed with 1% (w/v) sodium dodecyl sulphate (SDS). Lysates were collected and each treated with an equal volume of 2M tris-HCl, pH 8.8, for 1 hr at 37°C to hydrolyse aminoacyl-tRNA. Proteins were then precipitated at 10% (w/v) TCA and collected on GFC filters for counting. Matrix which remained on culture dishes was then washed and dried, prior to dissolution in 2N NaOH (14).

Determinations: Protein determinations were carried out using the method of Lowry et al. (15). Proline/hydroxyproline ratios were determined using a Beckman 119C amino acid analyser.

RESULTS. Rat heart smooth muscle cells grew with an approximate doubling time of 48 hours, when cultured in the presence or absence of ascorbic acid (Fig. 1). This result confirms a finding reported previously by others (6). Light microscopy revealed no differences between the two sets of cultures, but the matrix material produced by scorbutic cultures had a denser, more amorphous appearance, both under phase contrast and scanning electron microscopy (data not shown). After 14 days in culture, the total amount of matrix protein remaining behind on culture dishes after cell lysis and washing was approximately the same in the case of both cultures. However, scorbutic cells required more time before they began to produce detergent-insoluble matrix (Fig. 1). These findings were reproduced in 3 separate experiments.

Compositional analysis revealed that scorbutic cultures produced matrices containing little collagen but approximately double the amount of elastin, when compared with matrices produced by cells cultured in the presence of ascorbic acid (Table 1). The ratios of proline to hydroxyproline were also greatly increased, from 3,5 to 19; this was presumably due to the low level

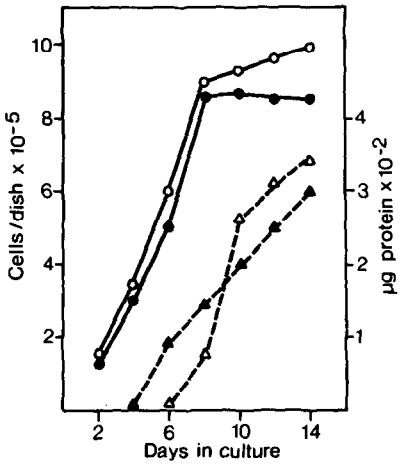


Fig. 1. Kinetics of cell division and matrix protein production in rat smooth muscle cell cultures grown in the presence (closed symbols) and absence (open symbols) of ascorbic acid. Cultures were initiated in 35 mm culture dishes each seeded with 10^5 cells. Ascorbic acid (50 µg/ml) was added daily to the relevant dishes and the medium was changed every second day. Cell numbers (●,○) and protein concentrations (▲, △) were determined as described in Methods.

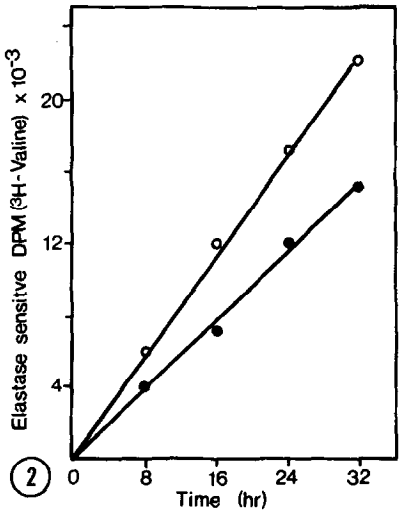
TABLE 1. Compositional analysis of extracellular matrix proteins laid down by rat smooth muscle cells cultured in the presence and absence of ascorbic acid.

Component (%)	+ Ascorbic acid	- Ascorbic acid
Glycoproteins, noncross-linked elastin	22%	22%
Cross-linked elastin	37.5%	70.5%
Collagen	40%	7%
Proline	9.2%	11.6%
Hydroxyproline	2.6%	0.6%

The values for protein composition each represent the means of three separate assays (see Methods). Determinations of proline and hydroxyproline residues were carried out in duplicate (<5% differences) and are the means of the values obtained.

of collagen present in matrices produced in the absence of ascorbic acid, and the fact that the elastin was also under-hydroxylated (16). Analogous cultures treated with α,α'-dipyridyl secrete elastin which contains virtually no hydroxyproline (17,18). Scorbutic matrices were also much less soluble than controls in a number of solvents we have used to dissolve cross-linked material (13) (data not shown).

The rate at which [^{3}H]-valine accumulated in the cross-linked elastin compartment of extracellular matrices was faster in cultures without ascorbic acid than in those supplemented with the vitamin, when measured over short time periods (Fig. 2). This was monitored by culturing cells in the presence of [^{3}H]-valine, harvesting culture dishes and preparing washed dried matrix for enzymatic analysis. Matrices were first digested with trypsin to remove labelled glycoproteins and noncross-linked elastin (13); subsequent elastase treatment released labelled valine from cross-linked elastin. A similar procedure was used to determine the rate at which scorbutic cultures were able to cross-link preformed elastin into the trypsin-insensitive form of the protein (Fig. 3). Both scorbutic and ascorbic acid-treated cultures were pulsed with [^{3}H]-valine, and this was followed by a "cold chase" period (de-



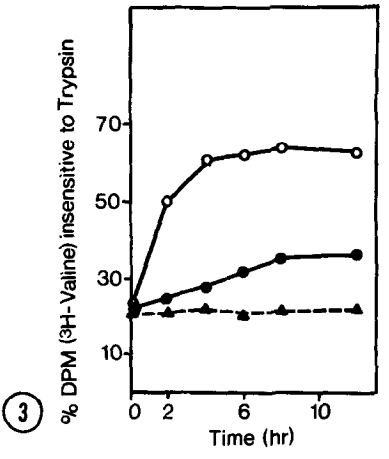


Fig. 2. Incorporation of [^{3}H]-valine into the elastase-releasable component of extracellular matrices produced by scorbutic (O--O) and ascorbutic (●--●) cultures. Cells were seeded in 35 mm dishes at 10^5 cells/dish, and were grown for 9 days as described in Methods. On day 10, the medium was changed and replenished with medium containing [^{3}H]-valine (1 μCi/ml). Dishes were then harvested at the times shown. After removal of medium and preparation of matrices as described in Methods, compositional analysis was performed using sequential enzymatic digestion by trypsin, elastase and collagenase (14).

Fig. 3. Assay of the rate at which elastin becomes cross-linked into trypsin-non-releasable matrix elastin. Cultures were grown as described in Figure 2. On day ten, after a medium change, cultures were labelled for 4 hours with [^{3}H]-valine. The medium was removed, the cells washed three times with PBS, and cold medium added. Dishes were harvested at the times shown (zero time being the start of the chase period) and matrices prepared and analysed by sequential enzyme digestion as described in Figure 2. Trypsin-non-releasable material (% of total radioactivity released, after trypsin treatment, by elastase) produced by scorbutic culture O--O; trypsin-non-releasable material produced by ascorbutic culture ●--●; trypsin non-releasable material produced by scorbutic cultures in the presence of β-aminopropionitrile ▲--▲ .

tails in Fig. 3). Matrices were then harvested at the times shown in the figure and subjected to sequential enzymatic digestion by trypsin and then elastase. Since trypsin digested the glycoprotein components of the matrix, as well as the noncross-linked tropoelastin molecules, at the end of the pulse period the initial amount of radioactivity not released by trypsin (but released by elastase) was low (Fig. 3). As elastin became progressively more cross-linked, there was an increase in the percentage of radioactive

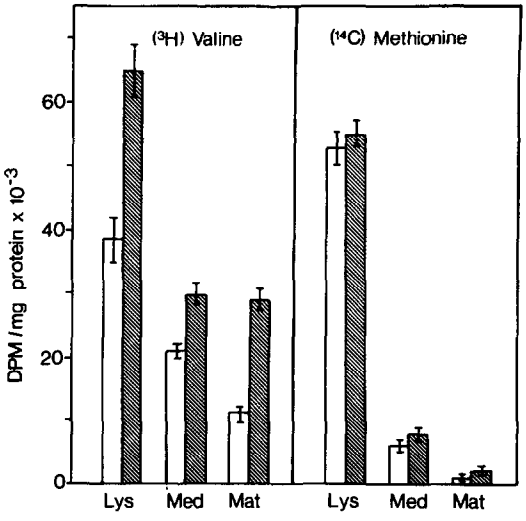


Fig. 4. Simultaneous incorporation of [^{3}H]-valine and [^{14}C]-methionine into various compartments of rat smooth muscle cultures. Cells were grown as described in Fig. 2. On day 10, the medium was changed and replenished with radioactive medium for 20 hours. The various compartments of the culture system were then collected as described in Methods, and assayed for radioactivity. Error bars represent the standard errors of the means of six determinations, and results are expressed as dpm/mg protein in each specific compartment. Abbreviations:- Lys - lysates; MED - culture medium; MAT - matrix proteins.

material insensitive to trypsin but released by subsequent elastase digestion (Fig. 3). In the presence of β-aminopropionitrile, the trypsin-insensitive material remained insignificant due to the action of this inhibitor on lysyl oxidase. The cross-linking of elastin proceeded at a faster rate in cultures deprived of ascorbic acid, and equilibrium was reached after four hours, compared with eight hours in the case of ascorbic acid-supplemented cultures (Fig. 3).

Since it has been established by a number of workers that collagen biosynthesis in the absence of ascorbic acid results in an accumulation of procollagen peptides in the culture medium without an overall decrease in collagen production by scorbutic cells, we tried to establish whether our cultures showed an actual increase in the intracellular rate of elastin biosynthesis by use of radiolabelled valine (19). Scorbutic cells incorporated much more valine (+70%) into intracellular protein than did vitamin-supplemented cultures,

which parallelled the increased valine incorporation which occurred into total matrix proteins, and into the proteins of the medium. However, the incorporation of methionine, which is not present in elastin (20), into all these compartments was the same in both cases. The accumulation of labelled peptides in the culture medium represents steps in the paths of secretion and cross-linking of the connective tissue proteins (3). These results show that the increased valine incorporation which occurred in the absence of ascorbate was not a result of an overall increase in protein synthesis by cells under scorbutic conditions. In other words, the rat smooth muscle cells under scorbutic conditions synthesized valine-containing connective tissue proteins (i.e. elastin) specifically at a faster rate than did ascorbic acid-supplemented controls.

DISCUSSION. We have confirmed that ascorbic acid is required in culture systems for the correct formation of stable collagen molecules and for their subsequent cross-linking into collagen fibres (3, inter alia). However, this requirement for ascorbic acid does not apply in the case of elastin secretion and deposition. The fact that only 5-8% of the proline residues in normal elastin are hydroxylated suggests that these residues do not play a prominent part in the structural organisation of elastin. The biosynthesis and secretion of elastin in the presence of the prolyl hydroxylase inhibitor, 3,3'dipyridyl, has already been shown to proceed normally (9,10). These authors have even suggested that proline hydroxylation in elastin is just a coincidental "accident" due to the presence of susceptible peptide sequences at the site of biosynthesis of collagen chains in connective tissue secretory cells. Our data show that elastin, which is also underhydroxylated in the absence of ascorbic acid, may be secreted and cross-linked into insoluble elastin at least as well as, if not better than, hydroxylated monomers.

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