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Regulation of procollagen synthesis and processing during ascorbate-induced extracellular matrix accumulation *in vitro*

Danny CHAN, Shireen R. LAMANDE, William G. COLE and John F. BATEMAN*
Department of Paediatrics, University of Melbourne, Royal Children's Hospital, Parkville, Victoria 3052, Australia

Procollagen biosynthesis and matrix deposition were studied in long-term human skin fibroblast cultures exposed to ascorbic acid. Ascorbic acid specifically stimulated types I and III collagen synthesis, reaching a maximum at day 2 and maintaining a specific high rate of production until day 10 of ascorbate exposure, after which collagen production declined. The increased level of collagen synthesis after different exposure times could also be achieved by only brief treatment (10 h) of parallel scorbutic (ascorbic-acid-deficient) cultures with ascorbic acid. This brief exposure did not result in increased collagen mRNA, thus demonstrating that the ascorbate-induced increase in collagen synthesis at all stages of ascorbic acid exposure was due to post-transcriptional mechanisms, most likely a rapid increase in type 1 collagen mRNA translational efficiency. This mechanism, rather than the transcriptional activation, was the primary response and is adequate to explain the ascorbate-induced increase in collagen synthesis. These data also demonstrate that the presence of a collagenous extracellular matrix was not involved in this collagen biosynthetic regulation. During long-term exposure (18 days) to ascorbic acid, a substantial cross-linked collagenous matrix formed, following an approximately sigmoidal time course. The most rapid matrix deposition occurred during the later days of exposure when the rate of collagen synthesis was decreasing, suggesting that the presence of a pre-existing matrix is important for further collagen accumulation. Procollagen was also efficiently processed to collagen during this phase, demonstrating that efficient procollagen processing is an important regulatory event in collagen matrix deposition.

INTRODUCTION

The structure and composition of the extracellular matrix has been shown to have a significant influence on the proliferation, differentiation and phenotypic expression of cells [1]. The major component of the extracellular matrix produced by fibroblasts is type I collagen. It is synthesized as a precursor, procollagen, with *N*- and *C*-terminal propeptide domains. These propeptides are removed during post-translational processing to produce collagen molecules which then undergo fibril formation and cross-linking (for reviews see [2,3]). However, in standard monolayer fibroblast cultures, procollagen secretion and processing and matrix formation are inefficient [4].

Collagen secretion by fibroblast cultures can be increased by the addition of ascorbic acid to the culture medium. Ascorbic acid acts as a cofactor for the enzymic hydroxylation of specific procollagen prolyl and lysyl residues during biosynthesis. This post-translational modification is essential for efficient procollagen helix formation and subsequent secretion from the cell (for reviews see [2,3]). The presence of ascorbic acid has also been shown to increase collagen production in many cell culture systems previously grown under standard culture conditions without ascorbate [5–13]. This stimulatory effect of ascorbate results principally from its action in allowing normal collagen hydroxylation, thus restoring efficient collagen secretion. The secretion of this procollagen accumulated in the scorbutic (ascorbic-acid-deficient) cells has been shown to increase collagen mRNA translation [10,13]. Ascorbate also acts at the level of transcription, specifically increasing collagen mRNA levels [8,10,13,14]. The contributions of transcriptional and post-transcriptional mechanisms in the regulation and maintenance of ascorbate-induced collagen synthesis *in vitro* have not been examined in detail during long-term ascorbate exposure. In this study we examine the mechanism of induction of collagen synthesis by ascorbate in fibroblast cultures during such continuous long-term ascorbate supplementation. Both transcriptional and post-transcriptional stimulation was found, but in contrast with previous studies, at all stages during ascorbate exposure the increase in type I collagen synthesis could be accounted for by post-transcriptional mechanisms alone.

The biochemical characteristics of the matrix accumulated *in vitro* during ascorbate treatment are also of importance in gaining a better understanding of the structure and organization of the extracellular matrix [15]. A recent study by Grinnell *et al.* [16] examined the matrix accumulated after 4–5 weeks of ascorbic acid addition. Although this study reported increased procollagen synthesis, processing and cross-linking in the accumulated collagenous matrix, it did not provide information on how these parameters altered as a collagenous matrix was deposited.

Our studies provide detailed information on the effect of the accumulated endogenous collagenous matrix on collagen gene expression, biosynthesis and processing, and on the dynamics of collagen deposition into the extracellular matrix during continuous long-term exposure to ascorbic acid.

MATERIALS AND METHODS

Materials

L-[5-^{3}H]Proline (15.7 Ci/mmol), [5,6-^{3}H]UTP (40.9 Ci/mmol) and [α-^{32}P]dCTP (3000 Ci/mmol) were purchased from Amersham Australia Pty. Ltd., Sydney, N.S.W., Australia. Dulbecco's modified Eagle's medium (DMEM) and foetal calf serum were purchased from Flow Laboratories Australia, Stanmore, N.S.W., Australia. Pepsin, sodium ascorbate and bacterial collagenase (type I) were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A. Bisbenzimid H 332558 was obtained from Riedelde–de Haenag Seelze, Hannover, Germany. Riboprobe vectors pSP64, pSP65 and SP6 polymerase were purchased from Promega Biotec, Madison, WI, U.S.A. Restric-

Abbreviations used: SSC, 15 mM-trisodium citrate/0.15 M-NaCl; DMEM, Dulbecco's modified Eagle's medium.

* To whom correspondence should be addressed.

tion endonucleases and nick-translation kits were obtained from Boehringer, Mannheim, Germany. Nitrocellulose membranes were obtained from Schleicher and Schuell, Keene, NH, U.S.A. All other chemicals were commercially available and of analytical grade.

Cell culture

Human skin fibroblasts from a normal 2-year-old child were cultured using previously described methods [17]. To minimize variability, a single batch of medium was used throughout the experiments. Approx. 1×10^6 cells (passage 7) were seeded into 175 cm^2 dishes and grown to confluency in DMEM containing 10% (v/v) foetal calf serum. At confluency (day 0) the culture were divided into three experimental groups and grown for a further 17 days: 1, cells were grown in DMEM containing 10% (v/v) foetal calf serum, and supplemented daily with 0.25 mM-ascorbic acid (+); 2, cells were grown without ascorbic acid (−); and 3, cells were grown in the absence of ascorbic acid, but ascorbic acid was added during radioactive labelling (−/+).

Collagen biosynthetic labelling

At the relevant times, the medium was removed and replaced with 19.8 ml of DMEM containing 10% (v/v) dialysed foetal calf serum and either with (+) or without (−) 0.25 mM-ascorbic acid. After 4 h, 0.2 ml of DMEM containing 100 μCi of L-[5-^{3}H]-proline was added to the medium and the incubation was continued for a further 6 h. The final concentration of proline in the medium was 0.1 mM. Following incubation, the cell layer and medium fractions were treated separately as previously described [17,18]. Briefly, procollagens from the medium fractions were precipitated with ammonium sulphate at 25% saturation. The precipitate was redissolved in 2 ml of 50 mM-Tris/HCl, pH 7.5, containing 0.15 M-NaCl and the protease inhibitors 5 mM-EDTA, 10 mM-*N*-ethylmaleimide and 1 mM-phenylmethanesulphonyl fluoride. In some experiments, samples of procollagens were precipitated with 75% (v/v) ethanol and subjected to limited pepsin digestion (100 μg/ml, 4 °C, 16 h) to remove non-helical sequences. Collagen synthesis was quantified by the incorporation of radioactivity into electrophoretically resolved collagen bands [19]. Non-collagen protein synthesis was calculated by subtracting the collagen radioactivity from the total protein radioactivity precipitated by 75% (v/v) ethanol from the cell layer and medium fractions. Collagen and non-collagen radioactivities were corrected for their different imino acid content [20].

Extraction of collagen from the extracellular matrix

The cell layers were scraped into 2×5 ml of 50 mM-Tris/HCl, pH 7.5, containing 0.15 M-NaCl and proteinase inhibitors. The cell fractions were disrupted by sonication and extracted overnight at 4 °C [17,18]. Insoluble material was removed by centrifugation and the procollagen in the supernatant (soluble fraction) was precipitated with ammonium sulphate (25% saturation) and redissolved in 2 ml of 50 mM-Tris/HCl (pH 7.5)/0.15 mM-NaCl containing protease inhibitors. Insoluble collagen in the pellet was further extracted with 0.5 M-acetic acid containing 0.1 mg of pepsin/ml for 16 h at 4 °C. Digestion was terminated by lyophilization. In some experiments, the pellet was extracted with 0.5 M-acetic acid alone to study collagen processing.

SDS/polyacrylamide-gel electrophoresis

Collagen chains were analysed by 5% (w/v) PAGE with a 3.5% (w/v) polyacrylamide stacking gel. Sample preparation, electrophoresis conditions, Coomassie Brilliant Blue staining and fluorography of radioactive gels are described elsewhere [17,18].

Extraction of RNA and DNA from fibroblasts

At appropriate times, cells were released by digestion with bacterial collagenase (3 mg/ml) for 10 min at 37 °C followed by trypsin (2.5 mg/ml) in 0.54 mM-EDTA for 10 min at 37 °C. Foetal calf serum was then added to 10% (v/v) to inactivate trypsin and cells were then washed three times with Dulbecco's phosphate-buffered saline. Cytoplasmic RNA was extracted from the cell pellets as previously described [21]. Cell nuclei were resuspended in 1.0 ml of $1 \times$SSC buffer (15 mM-sodium citrate/0.15 M-NaCl) and sonicated, and the cell DNA content was quantified using the Hoescht 332558 specific fluorescence procedures [22].

mRNA quantification

To produce RNA hybridization standards for quantification of mRNA levels, a 1460 bp *Eco*RI fragment of α1(I) cDNA (Hf677, [23]) and a 1420 bp *Pvu*II–*Eco*RI fragment of α2(I) cDNA (Hf32, [24]) were cloned into the SP6 expression vectors pSP65 and pSP64 respectively [25], so that sense-strand RNAs would be generated in the transcription reaction. The plasmids were linearized and 3 μg of each was transcribed by the SP6 polymerase reaction as described previously [26] in the presence of 5 μCi of [^{3}H]UTP. The concentration of RNA produced in the transcription reaction was calculated from the radioactivity incorporated into the RNA, the specific radioactivity of the [^{3}H]UTP and the sequence and size of the cDNA templates.

Portions of the cytoplasmic RNA samples were applied in duplicate to six separate nitrocellulose membranes as previously described [21]. α1(I) and α2(I) RNA hybridization standards were applied in the range $(5–80) \times 10^7$ copies per dot, along with 10 μg of yeast tRNA. Approx. 100 ng of purified insert cDNAs for collagen α1(I) [23], α2(I) [24], α1(III) [27], fibronectin [28] and β-actin [29] were labelled with [^{32}P]dCTP by nick-translation [30] and hybridized to the filter-bound RNA for 16 h at 42 °C in 50% (v/v) formamide under standard conditions [21]. After hybridization the β-actin-cDNA-probed filter was washed in $0.5 \times$SSC/1% (w/v) SDS, first at 42 °C and then at 65 °C for 30 min. The other cDNA-probed filters were washed with $0.1 \times$SSC/1% (w/v) SDS at 42 °C and then at 65 °C for 30 min. Other filters were probed with ^{32}P-labelled oligo(dT) to measure the total poly(A)$^+$ mRNA content. Synthetic $(dT)_{30}$ was hybridized and the filter was washed as described elsewhere [31]. Specific hybridization of the probes to the RNA was quantified by excision of the dots followed by scintillation counting.

RESULTS

Effect of ascorbic acid on cell proliferation

In the presence of ascorbic acid, the fibroblasts proliferated more rapidly than cells cultured without ascorbate addition (Fig. 1). The ascorbate-treated cells did not show any tendency to reach a growth plateau within the 18 day culture period.

Collagen accumulation in the extracellular matrix

Immunofluorescence staining of the extracellular matrix with a specific type I collagen antibody showed that, in the absence of ascorbic acid, type I collagen remained largely intracellular. In the presence of ascorbic acid, a dense collagenous extracellular matrix gradually formed which increased with prolonged culture (results not shown). The extensive collagen accumulation in ascorbic acid-treated cultures was confirmed by detailed biochemical analyses (see Figs. 2 and 3). Two matrix fractions were

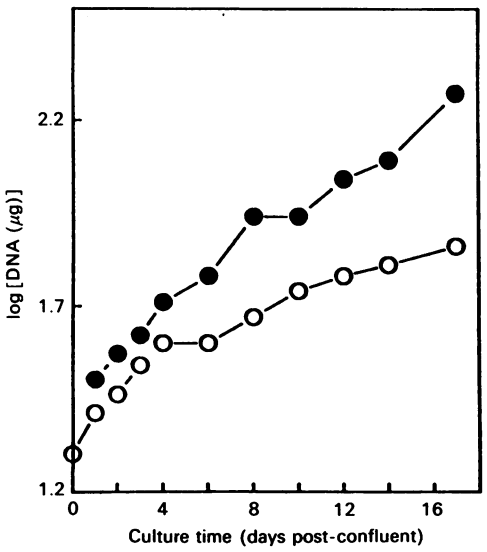

Fig. 1. Growth characteristics of human skin fibroblasts in culture

Cells were grown in DMEM containing 10% (v/v) foetal calf serum to confluent density and were then grown in the absence (○) or presence (●) of 0.25 mM-ascorbic acid (see the Materials and methods section for details). DNA content was used as a measure of cell growth and was quantified using specific fluorescence procedures.

examined, a soluble fraction which was readily extracted with 50 mM-Tris/HCl, pH 7.5, containing 0.15 M-NaCl, and an insoluble collagen fraction which was solubilized only after limited pepsin digestion. These fractions were analysed on 5% polyacrylamide gels. When ascorbic acid was added to the culture, collagen with covalently cross-linked β-components was laid down in an insoluble matrix (Fig. 2). The proportion of cross-linked collagen increased with prolonged culture so that by 14–17 days the collagen pattern showed a remarkable similarity to pepsin-extracted collagen from skin. Quantification of the collagen deposition in the extracellular matrix revealed an approximately sigmoidal time course (Fig. 3) with the most rapid accumulation occurring after 8 days of culture in ascorbic acid.

A striking feature was the demonstration of extensive processing of procollagen to collagen in the soluble fraction after 10 days of culture in the presence of ascorbic acid. Fully processed collagen was also demonstrated in the insoluble matrix of these cultures by extraction with 0.5 M-acetic acid/4.0 M-urea (results not shown). The processing of procollagen to collagen in ascorbic acid-treated cultures coincided with the rapid deposition phase of collagen into the insoluble matrix (Fig. 3), suggesting that collagen accumulation may be at least in part controlled at the level of procollagen processing.

Effect of ascorbic acid on collagen synthesis

Daily supplementation with ascorbic acid stimulated types I and III collagen synthesis when compared with cells grown in the absence of ascorbic acid. There was an approx. 6-fold increase in the production of type I collagen after 2 days of ascorbic acid supplementation (Fig. 4*a*). This stimulation was maintained until about day 10, after which collagen synthesis gradually declined. This decline coincided with the rapid accumulation phase of collagen in the extracellular matrix (Fig. 3). Type III collagen synthesis followed a similar pattern to that of type I collagen, but the stimulation by ascorbate was greater than that of type I collagen (Fig. 4*b*).

When scorbutic cells were exposed for only a short time (10 h) to ascorbic acid (−/+ ascorbate), the cells produced a type I collagen synthetic pattern very similar to that of the cells which were exposed continuously to ascorbic acid (Fig. 4*a*). Type III collagen synthesis was also stimulated by short-term exposure to ascorbic acid, but to a level significantly lower than in cells grown continuously in ascorbic acid (Fig. 4*b*).

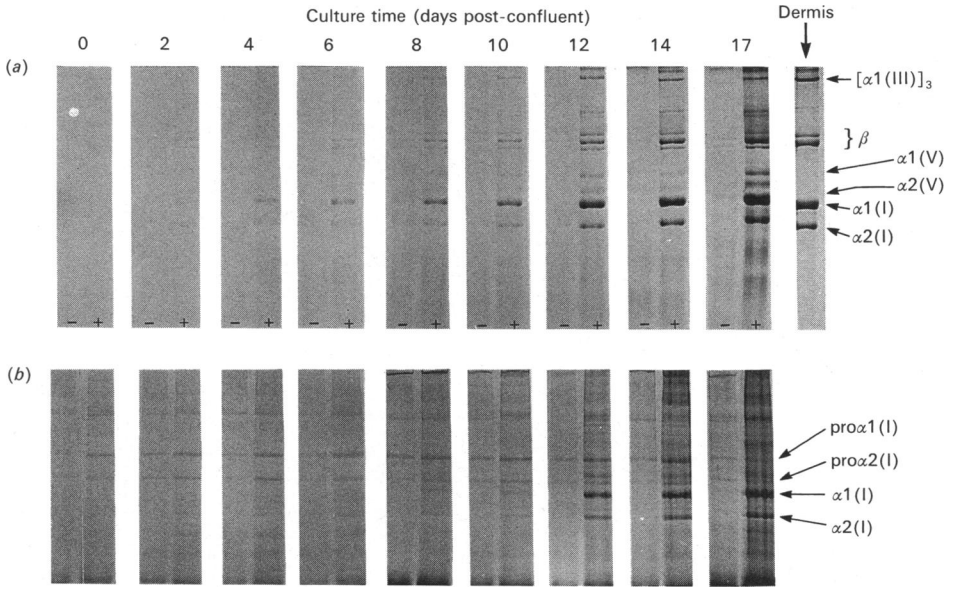

Fig. 2. Effect of ascorbic acid on deposition, cross-linking and processing of fibroblast type I collagen

Cells were grown from confluent density in the absence (−) or presence (+) of 0.25 mM-ascorbic acid. Collagen in the cell fraction was extracted with 50 mM-Tris/HCl (pH 7.5)/0.15 M-NaCl/5 mM-EDTA/10 mM-*N*-ethylmaleimide/1 mM-phenylmethanesulphonyl fluoride (cell soluble fraction; *b*). The residual material was digested with pepsin (0.1 mg/ml) in 0.5 M-acetic acid (cell insoluble fraction; *a*). The collagens extracted in these fractions were analysed on SDS/polyacrylamide gels and stained with Coomassie Brilliant Blue. The migrations of type I procollagen proα1, proα2, α1, α2 chains and cross-linked α-chain dimers (β-components) and type V collagen α1(V) and α2(V) chains are shown. A standard pepsin-soluble collagen extract from human dermis was used for comparison.

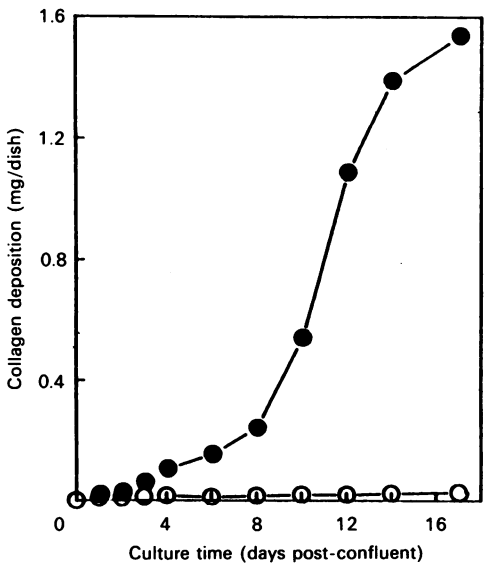

Fig. 3. Time course of collagen deposition in the extracellular matrix

Skin fibroblasts were grown to confluent density and then cultured in the absence (○) or presence (●) of 0.25 mM-ascorbic acid (see the Materials and methods section for details). Collagen deposited in the cell insoluble fractions (solubilized by limited pepsin digestion) was analysed by SDS/PAGE and quantified by densitometry scanning. Collagen is expressed as mg of collagen extracted per 175 cm^2 tissue culture dish.

Non-collagen protein synthesis was largely unaffected by the presence of ascorbic acid (Fig. 5*a*) as the synthetic patterns were similar for the three experimental conditions (+, − and −/+). The specific induction of collagen synthesis by ascorbic acid was further demonstrated when collagen synthesis was expressed as a percentage of the total cell protein synthesis (Fig. 5*b*).

Effect of ascorbic acid on collagen mRNA levels

To determine whether the stimulation of collagen synthesis was related to increased transcription or translation, collagen mRNA levels were measured using cytoplasmic RNA dot-blots. When exposed to ascorbic acid, cells contained more poly(A)$^+$ RNA (results not shown). In addition to this general increase, mRNA levels for the *COL1A1*, *COL3A1* (Fig. 6) and *COL1A2* (results not shown) genes were specifically increased. mRNA levels for actin and fibronectin were also measured, but were not significantly altered in the presence of ascorbic acid (results not shown). This specific increase in collagen mRNA corresponded to the pattern of the collagen protein response. However, the increase in collagen mRNA (2–3-fold) was not proportional to the increase in collagen protein synthesis (6–7-fold). The extent of transcriptional stimulation of the three collagen genes was $\alpha1(III) \geqslant \alpha1(I) > \alpha2(I)$. The transcriptional activation of *COL3A1* was most apparent during the first 4 days of exposure, after which there was little difference between the scorbutic and ascorbate-treated cultures. The greater stimulation of $\alpha1(I)$ mRNA over $\alpha2(I)$ mRNA was reflected in the $\alpha1/\alpha2$ ratio within the cell. In the absence of ascorbic acid, the $\alpha1(I)/\alpha2(I)$ mRNA ratio was 1.06 ± 0.19 (mean ± S.D., $n = 5$) compared with 2.16 ± 0.25 ($n = 4$) in cells grown in the presence of ascorbic acid for up to 12 days. Thus, in the presence of ascorbic acid, the expression of these two genes is co-ordinately regulated at a pre-translational level at all time points to maintain an approximate 2:1 ratio. However, the ratio of 1:1 observed in the absence of ascorbic acid suggested that the co-ordinate regulation of these two genes is not obligatory.

An important finding was that short-term exposure (−/+ ascorbate) of cells to ascorbic acid (10 h) did not significantly alter the $\alpha1(I)$ mRNA levels (Fig. 7). The levels of the $\alpha2(I)$ and $\alpha1(III)$ mRNAs were studied and also did not show significant changes after 10 h of ascorbic acid treatment (results not shown). This finding suggests that the increased rate of collagen synthesis during short-term ascorbic acid exposure must be due to altered post-transcriptional processes.

DISCUSSION

These studies provide a detailed examination of the mechanism of induction by ascorbate of skin fibroblast collagen production and deposition into the extracellular matrix in both short-term

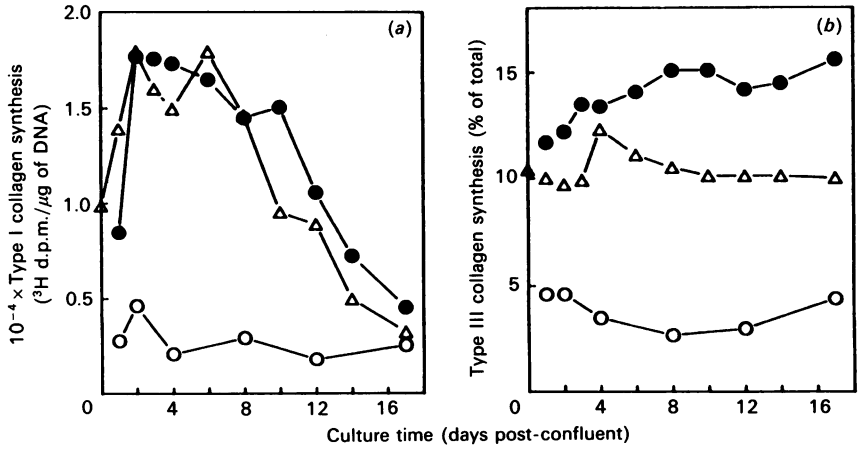

Fig. 4. Effect of ascorbic acid on type I and type III collagen synthesis

Cells were grown from confluent density and labelled with [5-^{3}H]proline for 6 h. The incorporation of [^{3}H]proline into type I and type III collagen was measured by scintillation counting following SDS/PAGE, as described in the Materials and methods section. (*a*) Type I collagen synthesis expressed as d.p.m. of [^{3}H]proline incorporated into type I collagen/μg of DNA; (*b*) type III collagen synthesis as a percentage of total collagen synthesis. The symbols represent the following cell cultures: ●, cells were grown and labelled in the presence of ascorbic acid; ○, cells were grown and labelled in the absence of ascorbic acid; △, cells were grown in the absence of ascorbic acid, but ascorbic acid was added during radioactive labelling.

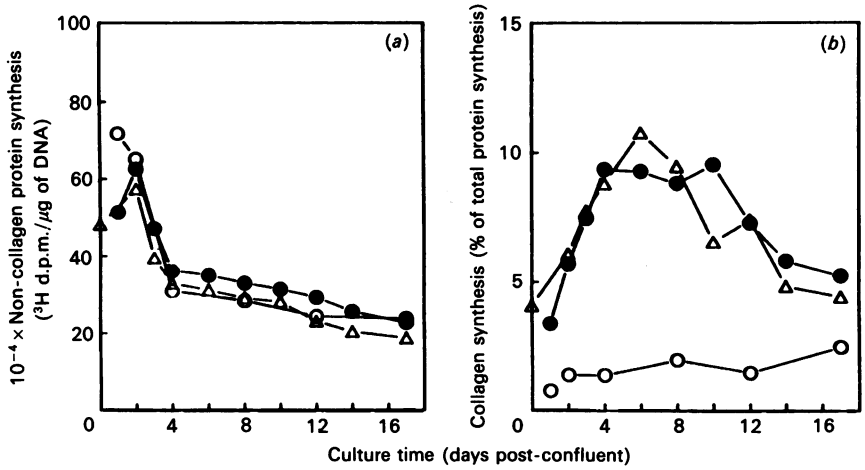

Fig. 5. Specific stimulation of collagen protein synthesis by ascorbic acid

Non-collagen protein synthesis was measured by the difference between [5-^{3}H]proline incorporation into total protein and total collagen synthesis (see the Experimental section for details). (*a*) Non-collagen protein synthesis/μg of DNA; (*b*) total collagen synthesis expressed as a percentage of total protein synthesis. The symbols represent the following cultures: ●, cells grown and labelled in the presence of ascorbic acid; ○, cells grown and labelled in the absence of ascorbic acid; △, cells grown in the absence of ascorbic acid, but ascorbic acid was added during radioactive labelling.

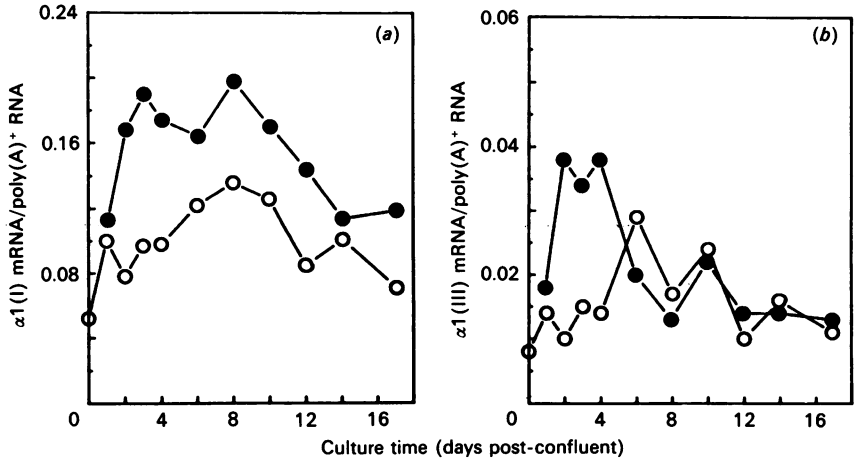

Fig. 6. Effect of ascorbic acid on α1(I) (*a*) and α1(III) (*b*) mRNA levels

Cells were grown to confluent density and then were grown in the absence (○) or presence (●) of 0.25 mM-ascorbic acid. Total mRNA was extracted and transferred on to nitrocellulose paper (see the Experimental section for details). Specific hybridization to cDNA probes was quantified by scintillation counting. The filters were also hybridized with [^{32}P]oligo(dT) and the levels of α1(I) and α1(III) mRNA were expressed as a ratio relative to total poly(A)$^+$ mRNA content.

and long-term culture experiments. As reported in other cell culture systems, the effects of ascorbate are pleiotrophic [7,8,10,12,13]. The immediate effect (within 10 h) of ascorbate addition is to rapidly increase the rate of type I collagen protein production in scorbutic cultures to the same levels as in those treated continuously with ascorbate. This increase occurs without the alteration in the steady-state levels of type I collagen mRNA, thus indicating that the stimulation is a post-transcriptional process. Such post-transcriptional mechanisms are altered mRNA translational efficiency or availability and collagen degradation. Whereas some studies have reported that the high rate of rapid intracellular collagen degradation in scorbutic cultures was decreased by ascorbate addition [32,33], other studies have shown no difference in collagen degradation between fibroblasts cultured with and without ascorbate [12]. Detailed studies *in vivo* [34,35] have also demonstrated that ascorbate deficiency does not cause increased collagen degradation in guinea pigs.

Since rapid intracellular collagen degradation was not established in these cultures, the relative contributions of degradation and altered mRNA translatability is not known, but it is likely that both mechanisms may play a role in the rapid stimulation of collagen production. Increased translational efficiency of type I collagen mRNA in response to ascorbate has been noted previously in avian tendon cell cultures [10], but the translational response was much slower, taking 36 h to reach a maximum. In our studies with human skin fibroblasts, ascorbate induced maximal type I and III collagen production within 10 h. It is proposed [10,36] that in the absence of ascorbic acid, the procollagen synthesized by the cells is poorly hydroxylated and not secreted, and this accumulation of unhydroxylated procollagen within the rough endoplasmic reticulum represses

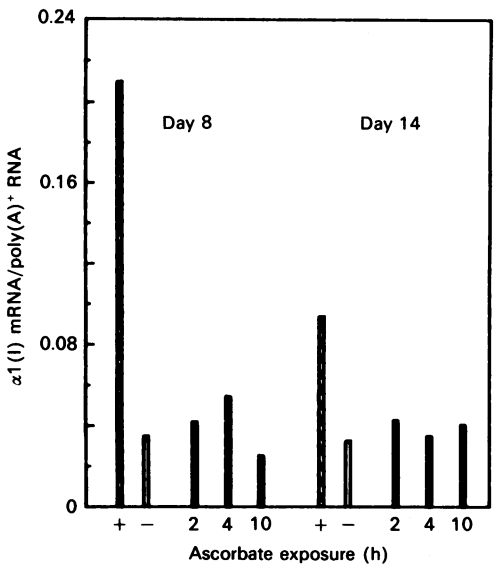

Fig. 7. Effect of short-term ascorbic acid treatment on steady-state level of α1(I) mRNA

Cells were grown from confluent density in the absence of ascorbic acid (−) for an additional 8 and 14 days. The cells were then subjected to 2, 4 or 10 h of exposure to 0.25 mM-ascorbic acid. RNA was isolated and dot-blot hybridization with an α1-(I)-specific cDNA probe was used to quantify α1(I) mRNA levels as described in the Experimental section. α1(I) mRNA was expressed relative to total poly(A^+) mRNA. (+) represents α1(I) mRNA levels of cells grown continuously in the presence of 0.25 mM-ascorbic acid.

collagen synthesis. The addition of ascorbic acid brings about normal hydroxylation of the procollagen and subsequent secretion, so removing the translational block and rapidly increasing collagen protein synthesis. Thus it is apparent that the stimulation of the collagen mRNA levels during continuous exposure of fibroblasts to ascorbic acid was not necessary to achieve the higher levels of collagen protein synthesis. Cells exposed to ascorbate for only 10 h synthesized collagen at a rate comparable with that in cells continuously exposed at all stages of growth. This clearly demonstrated that post-transcriptional regulation can quantitatively account for the increased rate of collagen protein production at all stages of fibroblast culture in the presence of ascorbate.

In cultures continuously exposed to ascorbate, the steady-state mRNA levels for type I and III collagen increased from day 2 to 4 and remained at a high level until about day 10, after which they declined. The mechanism by which ascorbic acid modulates collagen mRNA levels is uncertain. It is possible that it may have a direct effect on the transcription of collagen genes, or on mRNA stability as demonstrated in primary avian tendon cells [8,10]. The formation of a collagenous matrix during ascorbate treatment may also play a role in increasing mRNA transcription, as suggested by Zern *et al.* [14].It is unclear what the relationship of this increased transcription to increased protein synthesis in ascorbate-treated cultures is, since in our experiments a post-transcriptional mechanism was shown to be sufficient to account for the increase in type I collagen production. However, in previous studies, increased transcription has been suggested as the primary mechanism by which collagen synthesis is up-regulated during long-term ascorbate exposure [10,12], but these studies did not examine mRNA transcriptional efficiency changes at different times of exposure. Thus it is possible that in these studies also, increased translational efficiency, rather than the increased levels of mRNA, may be able to quantitatively account for the increased synthesis.

The finding that the rate of collagen synthesis was the same in cultures grown continuously in the presence of ascorbate or only briefly exposed to ascorbate on the same day of culture suggested that the rate of synthesis of cells at different times in culture is tightly controlled and apparently dependent in some way on the duration of the culture period. Although the mechanism is unknown, it is intriguing to speculate on the role of the accumulated extracellular matrix. Since the cells exposed briefly to ascorbate did not have a significant collagenous matrix, these data demonstrate that the presence of a collagenous matrix does not, in itself, play a role in this collagen biosynthetic regulation. The accumulation of other matrix components such as hyaluronic acid and glycosaminoglycans may play some regulatory role. The biochemical nature of the other components of the fibroblast ascorbate-induced matrix has not yet been studied in detail, and will be of importance in defining extracellular matrix effects on cell phenotypic expression.

During long-term culture with ascorbate, a substantial cross-linked collagen matrix formed around the cells. Other recent studies on osteoblast [37] and fibroblast [16] cultures also demonstrated this accumulation of a collagen matrix. The collagenous matrix accumulated most rapidly with cultures maintained in ascorbate beyond 8 days, although during this period collagen synthesis and mRNA declined. Thus, with extended culture, although less collagen is being produced, its incorporation into the insoluble extracellular matrix is much more efficient. This has also been observed in osteoblasts [37] and may reflect a co-operative phenomenon whereby the presence of a pre-existing collagenous matrix makes further collagen deposition more efficient [15].

Although the short-term addition of ascorbic acid to cultured fibroblasts allows the synthesis of normally hydroxylated procollagen, processing to the functional collagen molecule was poor, as commonly observed in standard monolayer cell culture studies. Procollagen processing is thought to occur close to the cell surface [38], and in standard culture of fibroblasts procollagen processing is minimal, as procollagen is secreted and diluted into the excessive volume of the culture medium. However, after 10 days of culture in the presence of ascorbic acid, the procollagen synthesized was efficiently processed to collagen and collagen deposition into the matrix was maximal, demonstrating that the onset of efficient processing is an important regulatory event in collagen matrix deposition. The inability of unprocessed procollagen to form collagen fibrils *in vitro* [39] further emphasizes this critical need for correct processing.

This study has demonstrated the effect of ascorbic acid in stimulating collagen deposition in human skin fibroblast cultures. The specific increase in collagen production is due to post-transcriptional regulatory mechanisms. The primary mechanism probably involves the restoration of normal collagen secretion, thus removing translational inhibition. The extent of collagen accumulated in the extracellular matrix did not affect the rate of collagen production, and the increased deposition in long-term culture apparently resulted from regulation at the level of procollagen processing and possibly fibril assembly. The long-term culture of fibroblasts and other cell types with ascorbate should provide a powerful methodology for examining the regulation and organization of the extracellular matrix.

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