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REGULATION OF COLLAGEN SYNTHESIS BY ASCORBIC ACID.

ASCORBIC ACID INCREASES TYPE I PROCOLLAGEN mRNA.

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SUMMARY: Ascorbic acid specifically stimulates collagen production in cultured human skin fibroblasts, an effect that appears to be independent of its cofactor role in prolyl and lysyl hydroxylation. In order to investigate the level of regulation of ascorbic acid on collagen synthesis, we have translated mRNA in a cell-free system derived from rabbit reticulocytes. Total RNA was prepared from normal human skin fibroblasts and similar fibroblasts which had been exposed to 100 uM ascorbic acid for four days. Ascorbic acid treatment resulted in a twofold stimulation of procollagen mRNA whereas non-collagenous mRNA was unchanged. These results reveal that ascorbic acid has a preferential stimulating effect on type I procollagen mRNA.

Introduction

The fact that ascorbic acid is essential for collagen synthesis has been known for years. Its importance in the hydroxylation of proline to hydroxyproline and of lysine to hydroxylysine has been thought to account for its primary influence (1). Hydroxyproline is required for collagen helix formation (2), and subsequent secretion (3). Hydroxylysine is essential for collagen intermolecular crosslinking (4).

In recent years however, studies from our laboratory (5) and others (6) have shown that ascorbic acid has a specific stimulatory effect on collagen synthesis, which is apparently unrelated to the degree of hydroxylation of proline and lysine. Murad et al. showed that ascorbic acid stimulated collagen synthesis without influencing non-collagen proteins and that this effect was independent of hydroxylation (5), or the level of prolyl hydroxylase (6).

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In this work, we have examined the possibility that ascorbic acid specifically stimulates procollagen mRNA by measuring the translation of human skin fibroblast RNA in rabbit reticulocyte lysate.

Experimental Procedures

Cell Culture: Human skin fibroblasts from a normal newborn (GM 970, Institute for Medical Research) were grown in 150 mm plastic dishes (Falcon) at an initial density of 0.8 $x10^6$ cells/dish in Dulbecco's modified Eagle's medium buffered with 24 mM sodium bicarbonate and 25 mM Hepes and supplemented with 20% dialyzed calf serum (Grand Island Biological Company). After the cells reached confluence, they were further incubated with and without 100 uM L-ascorbic acid in the presence of 0.5% dialyzed calf serum for 4 days. Ascorbic acid was prepared fresh and media was changed daily. Cells were detached with 0.05% trypsin/0.5 mM EDTA and cell number was determined in a Coulter counter.

Extraction of RNA from Fibroblasts: Total RNA was extracted from cultured fibroblasts in 6 M guanidine hydrochloride (7,8). Extracted RNA was dissolved in distilled water at a concentration of 1 ug/ul and stored at -70°C.

Cell Free Translation and Assay of Collagenous Proteins: Proline-free rabbit reticulocyte lysate was obtained from Bethesda Research Laboratories. The concentration of Mg acetate and K acetate in the translation reaction was adjusted to give a final concentration of 1.75 mM and 120 mM, respectively (9). 35 uCi of L-[2,3,4,5-^{3}H] proline (specific activity 110 Ci/mmol) (Amersham) which had been concentrated to 7 mCi/ml was added to each reaction mixture. The translation reaction was carried out in a total volume of 30 ul at 30°C for 90 min. and was stopped by incubating with RNase (30 ug/tube) (Type II-A Sigma) at 37°C for 10 min. Collagenous proteins produced by cell free translation were analyzed by two different methods. Half of the sample (15 ul) was heat denatured (100°C, 2 min.) in an equal volume of sample buffer (20 mM Tris-HCl pH 6.8, 2% SDS) and loaded on a 7.5% polyacrylamide gel slab (10). After electrophoresis, translation products were autoradiographed (11) for 48 hrs. at -70°C and Pro α_1(I) and Pro α_2(I) bands were scanned with a Gelman ACD-5 gel scanner within a linear range (0-$3x10^4$ cpm/48 hr. exposure). The rest of the sample (15 ul) was diluted with 20 mM proline and dialyzed with collagenase digestion buffer (25 mM Tris-HCl, 0.33 M Ca acetate pH 7.4) containing 1 mM N-ethylmaleimide at 4°C for 18 hrs. After dialysis, an aliquot was digested with 10 units of clostridial collagenase (Form III, Advance Biofactures) at 37°C for 4 hrs (12), and collagenase resistant proteins were precipitated with 25% trichloroacetic acid/1.25% tannic acid at 4°C. The precipitate was washed with 5% trichloroacetic acid/0.25% tannic acid twice and dissolved with 0.1 M Tris-HCl pH 7.4 containing 0.5% SDS 10 mM dithiothreitol at room temperature 2 hrs. Both fractions were counted after adding Aquasol-2.

Results and Discussion

No remarkable difference in cell number and recovery of total RNA was observed by ascorbic acid treatment as shown in Table 1.

Preliminary experiments to optimize the translation of procollagen mRNA showed that the ratio Pro α_1(I)/Pro α_2(I) decreased below 2.0 when

TABLE I

Recovery of RNA from Cultured Fibroblasts

	total RNA (ug/dish)	cell number ($x10^{-6}$/dish)	total RNA (ug/10^6 cell)
- ascorbate	89.9	5.8	15.5
+ ascorbate	85.2	5.6	15.2

Values are average of two individual experiments.

more than 2 ug/reaction of total RNA was added (data not shown) (13). For this reason, we employed amounts of RNA ranging from 0.1-1.0 ug.

Two different quantitative assays for measuring procollagen mRNA were employed. In one case the translation mixture was electrophoresed and the gels were scanned following fluorography (Fig. 1). In the other case the translation mixture was digested by highly purified bacterial collagenase under conditions of undetectable nonspecific proteolytic activity. Both of them gave essentially identical results, which revealed ascorbic acid stimulated type I procollagen mRNA approximately 2 times (1.7-2.5). No effect of ascorbate was detected on noncollagen mRNA (Fig. 2).

Pro α_1(I) and Pro α_2(I) bands in the autoradiogram of translation products were identified by bacterial collagenase digestion and comparison with authentic type I procollagen chains from human skin fibroblasts (Fig 1). The position of migration of authentic type I procollagen chains was slightly slower than that of the two major bands seen in the translation products, probably due to the lack of post- translational modification.

This study reveals that procollagen mRNA is increased in the presence of ascorbate. Although our experiments do not resolve the level of regulation of collagen synthesis by ascorbate, it would appear that the ascorbate-directed changes in collagen production and procollagen mRNA levels are linked. In many previous studies procollagen mRNA levels have been correlated with collagen production (14-18).

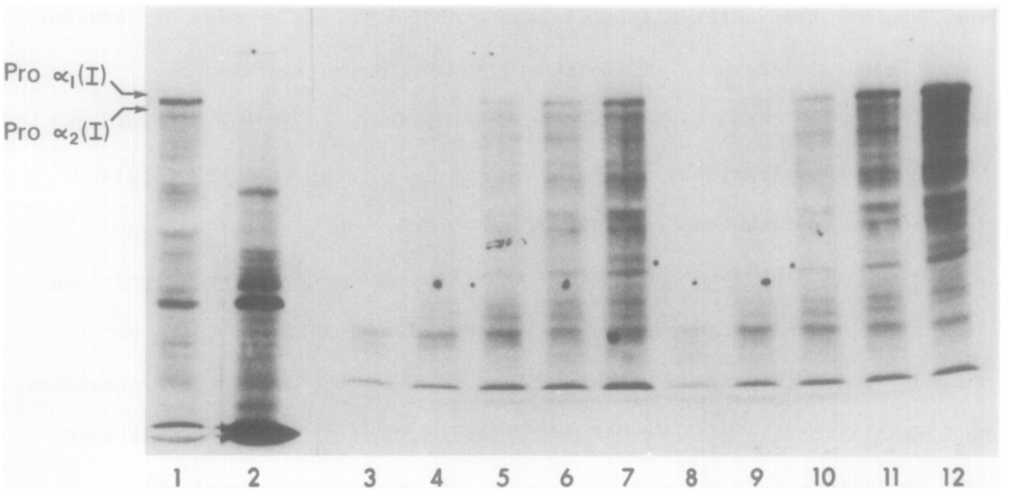

Fig 1. SDS polyacrylamide gel electrophoretic patterns of cell-free translation products with total RNA extracted from cultured fibroblasts treated with and without ascorbic acid. Lane 1 and 2, without and with bacterial collagenase digestion prior to electrophoresis; lane 3-7, 0, 0.1, 0.25, 0.5 and 1.0 ug of RNA was added to the translation reaction. RNA was isolated from fibroblasts untreated with ascorbate; lane 8-12, same as lane 3-7 except RNA was isolated from ascorbate-treated fibroblasts. Arrows show migration position of authentic Pro α_1(I) and Pro α_2(I) collagen purified from normal skin fibroblasts by DEAE cellulose chromatography.

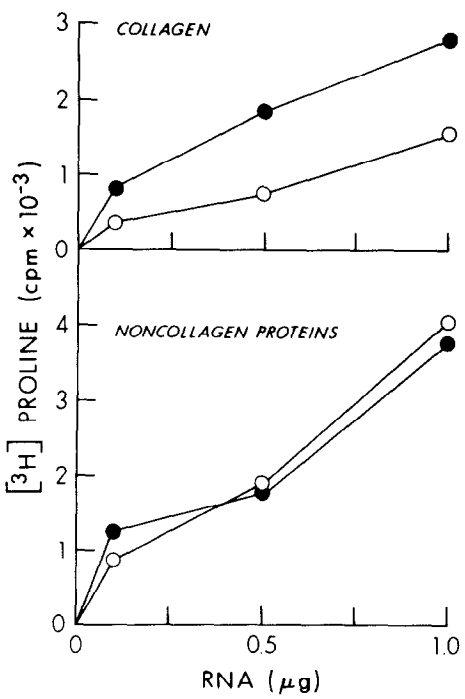

Fig 2. Cell-free translation in rabbit reticulocyte lysate of total RNA extracted from ascorbate-treated (•) and control (o) fibroblasts. Collagen and noncollagen proteins were determined by digestion with bacterial collagenase.

Whether the increase in procollagen mRNA level is primary or secondary is not clear. Ascorbate is important in many post-translational modifications. The ascorbate effect observed here could not be explained by changes in hydroxylation, secretion, intracellular or extracellular degradation, unless they were linked to altered synthesis of collagen.

Although ascorbate could exert translational control, we observed no direct influence of ascorbic acid on translational activity (data not shown). This indicates that ascorbic acid does not function by increasing the translational efficiency of procollagen mRNA. Increased procollagen mRNA could result from a change in gene copy number, increased gene transcription, altered processing of procollagen mRNA precursors or altered degradation of mRNA. Experiments designed to test these possibilities are now possible using recently isolated human collagen gene probes.

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