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Ascorbic acid preferentially enhances type I and III collagen gene transcription in human skin fibroblasts

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Abstract

Ascorbic acid is a potent stimulator for type I and III collagen expressions in human skin fibroblasts; stimulation of type I and III collagen synthesis and their mRNA levels by ascorbic acid has been reported previously. Nuclear run-on experiments demonstrated that ascorbic acid enhanced the transcription of type I and III collagen genes 4- and 3.4-fold respectively, whereas transcription of type IV collagen was slightly stimulated (1.7-fold). The results suggest that ascorbic acid preferentially enhanced type I and III collagen gene transcription.

Keywords: Collagen; Ascorbic acid; Gene transcription

1. Introduction

Collagen is a major component of the extracellular matrix of human dermal tissue. Several genetically distinct collagen types have been demonstrated in the dermis, and function as a stabilizing scaffold of dermal connective tissues, as well as a regulator of differentiation and migration of dermal cells [1].

Ascorbic acid is an essential cofactor for the enzymes, prolyl and lysyl hydroxylases, catalyzing the synthesis of hydroxyproline and hydroxylysine in collagen. Hydroxyproline acts to stabilize the collagen triple helix; its absence results in structurally unstable collagen which is not secreted from cells at a normal rate [2,3]. It is generally believed that ascorbate modulates collagen production through its effect on prolyl hydroxylation [3]. On the other hand, ascorbate has been shown to take on an additional role in the stimulation of collagen [4,5]. It elevates the steady-state levels of type I and III collagen mRNA [6–8]. Transcriptional activation of type I collagen genes by ascorbic acid 2-phosphate has been reported [9,10].

In this study, we attempted to determine the transcription rate of types I, III and IV collagen genes in the presence or absence of ascorbic acid using nuclear run-on assays.

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2. Materials and methods

2.1. Materials

[^{32}P]-labelled uridine triphosphate (UTP) ($\sim$3000 Ci/mmol) were purchased from Amersham and nitrocellulose filters from Schleicher and Schuell.

2.2. Fibroblast culture and ascorbic acid treatment

Human skin fibroblast culture was established by explant method and grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS). The cells were routinely subcultured every 7 days. Cells in passages 8–10 were used. Cells were treated with 100 μM ascorbic acid for 3 days in DMEM supplemented with 0.5% dialyzed FBS, a condition employed for maximum induction of collagen synthesis [4].

2.3. Nuclear run-on assay

The assay was performed essentially as described previously [11]. Briefly, cells were trypsinized and lysed with a buffer (10 mM Tris-HCl, pH 7.4, 10 mM NaCl, 3 mM $MgCl_2$ and 0.03% NP-40) for 5 min at 4°C, and nuclei were precipitated by centrifugation at 500 $\times$ g for 5 min at 4°C. The nuclei were suspended in the same buffer devoid of NP-40 and re-centrifuged at 500 $\times$ g for 5 min. The nuclei were re-suspended in a storagebuffer (50 mM Tris-HCl, pH 8.0, 40% glycerol, 5 mM $MgCl_2$ and 0.1 mM ethylenediamine tetraacetic acid (EDTA)) and stored at -80°C at a concentration of 10^7 nuclei/100 μl. The nuclei were mixed with an equal volume of a solution containing 10 mM Tris-HCl, pH 7.4, 5 mM $MgCl_2$, 300 mM KCl, 200 units of RNase inhibitor, 2 mM dithiothreitol (DTT), 0.5 mM each of adenosine triphosphate (ATP), cytosine triphosphate (CTP), guanidine triphosphate (GTP) and [α-^{32}P]UTP (120 TBq/mmol, Amersham) and incubated for 20 min at 30°C. The reaction was stopped by DNase I (Epicentre Technology Co.) (10 unit/100 ml) and the sample was deproteinized with proteinase K digestion (Sigma) (100 μg/ml) for 30 min at 42°C. RNA was isolated with phenol/chloroform and precipitated with ethanol, then hybridized with cDNAs in a hybridization solution (6 $\times$ SSC, 0.5% sodium dodecyl sulfate (SDS), 5 $\times$ Denhardt's solution, 100 μg/ml salmon sperm DNA and 50 μg yeast tRNA) for 48 h at 65°C. An equal amount of RNA (approximately 10^6 counts/min) from ascorbate-treated and untreated cells was hybridized in one reaction. The cDNAs used here were human proα_1 (I) (pHf32) [12], proα_2 (I) (pHf677) [13], proα_1 (III) (Hf-934) [14] proα_1 (IV) (pHT21) [15], elastin (pcHEL-2) [16] collagenase [17], β-globin (pRK-1) [18], transferin receptor (pCDTR-1) [19], adenosine deaminase (ADA) (p2Bu) [20] cDNAs rat fibronectin (λrlf-1) [21] and plasmid pBR322 DNA which were isolated and purified as described [22]. One μg of each cDNA was blotted onto nitrocellulose filters using a vacuum manifold (Biorad), denatured with 0.1 M NaOH, neutralized and baked at 80°C for 2 h. The filters were washed with 2 $\times$ SSC four times for 30 min at 65°C and two times for 30 min at room temperature, then air-dried and autoradiographed. Autoradiograms were scanned with a densitometer.

3. Results

There was no significant difference in the yield of nuclei isolated from the ascorbate-treated and control cells. To optimize the incorporation of UTP, pilot assays were performed with a range of nuclei (10^5–5 $\times$ 10^7) isolated from ascorbate-treated and control cells. The results demonstrated that nuclei less than 5 $\times$ 10^7 were well correlated with the amount of incorporated radioactivity (not shown). There was no significant difference in the incorporation of UTP between the nuclei isolated from ascorbate-treated and control cells (0.7 $\pm$ 0.6 vs. 0.5 $\pm$ 0.3 counts/min/nuclei, respectively).

Nuclear run-on assays demonstrated that the transcription of proα_1 (I), proα_2 (I) and proα_1 (III) genes was enhanced 3–4-fold by ascorbic acid treatment, whereas the transcription of proα_1 (IV) was stimulated only 1.7-fold. Non-collagenous protein genes, the fibronectin gene, as well as housekeeping genes, transferin receptor and adenosine deaminase, were mostly constant (Fig. 1 and Table 1). No significant signals for elastin

and collagenase cDNAs were detected in this experimental system.

4. Discussion

The data in the present study demonstrates that ascorbate enhanced the transcription of types I and III collagen genes 3–4-fold, and slightly enhanced type IV collagen gene transcription (1.7-fold). This is in agreement with previous reports that ascorbic acid increases types I and III collagen mRNA levels [6,7] and type IV collagen synthesis [23]. The reason why the degree of the stimulation of type IV collagen gene transcription is lower than that of types I and III collagens is not clear. Type IV collagen has a function distinct from interstitial types I and III collagens and forms the framework of basement membrane [1]. In addition, one paper described that type IV collagen underhydroxylated with α, α′ dipyridyl, a potent inhibitor for prolyl hydroxylase, was secreted at a normal rate, whereas the secretion of underhydroxylated types I and III collagens was reduced [24]. These facts suggest that gene expression of type IV collagen and interstitial types I and III collagens may be differently controlled by ascorbic acid.

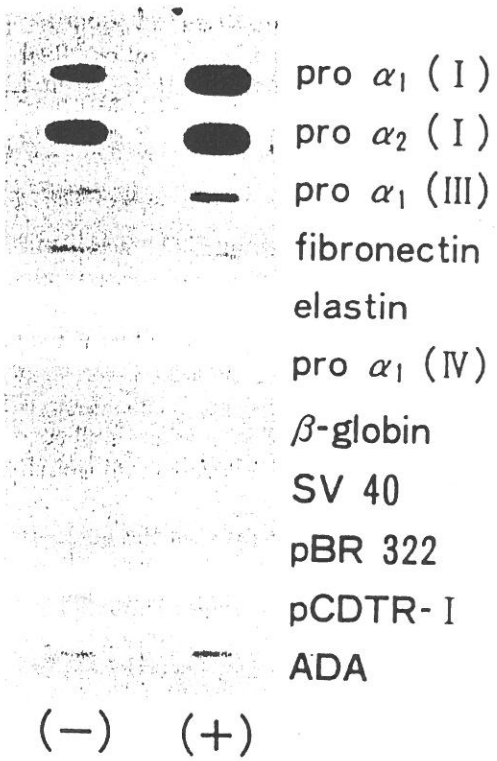

Fig. 1. Cells were treated without or with ascorbate (100 μM) for 72 h. Nuclei were isolated from the cells and mRNA precursors synthesized in vitro were hybridized with proα$_1$ (I), proα$_2$ (I) proα$_1$ (III), proα$_1$ (IV) and other noncollagenous protein DNAs. The filters were washed and autoradiographed.

Table 1
Transcriptional activity of collagen after ascorbic acid treatment

DNA	Ratio (ascorbate treated/untreated)
Proα$_1$ (I)	4.7 ± 0.8
Proα$_2$ (I)	4.0 ± 0.8
Proα$_1$ (III)	3.47 ± 1.0
Proα$_1$ (IV)	1.7 ± 0.6
Fibronectin	0.85 ± 0.14
Transferrin receptor	1.3 ± 0.16
Adenosine deaminase	1.0 ± 0.05

Indicated are the transcriptional activities in ascorbate-treated cells relative to untreated cells. Values are mean ± standard error ($n = 5$) except proα$_1$ (IV) ($n = 3$). Data for elastin and collagenase cDNAs are omitted from this table because their signals are below detectable levels.

The transcriptional ratio of α$_1$ (I) to α$_2$ (I) gene was less than 1. This is in contrast to the previous reports in which the ratio was always 2 [9,10]. A possible explanation may be that the primary transcript of α$_1$ (I) is not extended enough to be detected by the cDNA probe, which is near the 3′ end.

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