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Synergistic enhancement of type I and III collagen production in cultured fibroblasts by transforming growth factor-β and ascorbate

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Transforming growth factor-β (TGF-β) is a prototype of a family of polypeptides that regulates cellular growth and phenotypic differentiation [(1986) Science 233, 532–534; (1987) Cell 49, 437–438]. TGF-β injection induces angiogenesis and fibrosis locally [(1986) Proc. Natl. Acad. Sci. USA 83, 4167–4171; (1987) Science 237, 1333–1336] and stimulates the synthesis of extracellular matrix proteins, fibronectin, collagens, and proteoglycans in vitro in many cell types [(1986) J. Biol. Chem. 261, 4337–4345; (1987) Biochem J. 247, 597–604]. Ascorbate is also known to induce collagen synthesis and to promote wound healing [(1988) J. Invest. Dermatol. 90, 420–424; (1986) Coll. Rel. Res. 6, 455–466]. We report that in cultured human skin fibroblasts, ascorbate and TGF-β synergistically enhance the biosynthesis of type I and III collagens and their steady-state mRNAs. TGF-β alone has no enhancing effect on type III collagen synthesis. The cooperation between ascorbate and TGF-β may be of significance in wound healing and in disorders of fibrosis.

Transforming growth factor-β; Ascorbate; Vitamin C; Collagen; (Dermal fibroblast)

1. INTRODUCTION

Beta type transforming growth factors (TGF-β) are disulfide-linked homo- and heterodimers of two related 12.5 kDa peptide chains, β1 and β2, which bind to cell surface receptors present on many cells [2,9,10]. The β1 and β2 homodimers are the most abundant forms [9]. TGF-β inhibits epithelial and endothelial cells, T and B lymphocytes [5,11], while strongly stimulating the expression of extracellular matrix components [3,5], cell adhesion receptors [12], and protease inhibitors [13]. Clot retraction time is increased in vitro [14], while protease synthesis is decreased [13]. Although TGF-β is known to increase type I collagen synthesis [3,5], the exact mechanism of action is unknown. Steady-state collagen mRNAs are increased, but may result from stabilization rather than a change in the rate of transcription [6,15]. Recent studies have shown that a nuclear factor 1 binding site in the α2(I) collagen gene mediates the transcription of the promoter by TGF-β [16].

The importance of vitamin C (ascorbic acid) in the metabolism of the connective tissue was first studied in 1747 by the British naval surgeon James Lind [17]. Ascorbate is not only a cofactor for the hydroxylation of proline and lysine residues important for crosslinking, but it also increases transcription of type I and III procollagen mRNAs [7].

Although human skin fibroblasts can synthesize both procollagens in vitro [18], adult skin is composed of 80% type I and 15% type III collagen. The latter is more abundant in the fetal stage [19]. Type I collagen is a heterodimer of two α-chains, [α1(I)]$_2$ α2(I), products of genes on chromosomes

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17 [20] and 7 [20]. Type III collagen, $[\alpha 1(III)]_3$, is produced by a gene on chromosome 2 [21]. The tissue-specific regulation of these collagens may depend on nuclear-binding proteins, which could be induced by growth factors such as TGF-β or ascorbate [16].

We investigated whether some of the enhancement of collagen production attributed to TGF-β alone in reported studies [3,6,15] could be partially attributed to the presence of ascorbate in the culture media. We labeled explanted skin fibroblasts from seven donors (ages 11 months to 79 years, $p < 6$) with [^{3}H]proline in the presence of ascorbate, TGF-β, both or media alone at 24 and 72 h. [^{3}H]Proline incorporation into pepsin resistant proteins (collagens) was analyzed following SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and fluorography, using laser densitometry and by trichloroacetic acid (TCA)-precipitable counts.

2. MATERIALS AND METHODS

2.1. *Experimental conditions*

Triplicate cultures (5×10^5 cells, $p < 6$) were plated and switched to media alone (DMEM plus 0.5% calf serum) or ascorbate (50 μg/ml, Sigma Chemical Company, St. Louis, MO, USA), TGF-β (200 pM, Collaborative Research Incorporated, Bedford, MA, USA) or both and cultured for 72 h with fresh media daily and then 2,3,4-[^{3}H]proline (1 mCi/ml, Amersham, Arlington Heights, IL, USA) was added for a 24 h labeling period. Cell numbers did not vary significantly.

2.2. *Assays for pepsin-resistant proteins (collagens)*

Procollagens from media and cell pellets were extracted and digested with pepsin as previously described [22]. The samples were divided for analysis by 10% TCA precipitation and scintillagraphy (LKB 1211 Rackbeta liquid scintillation counter) and by 5.0% SDS-polyacrylamide gel electrophoresis followed by fluorography and scanning densitometry (LKB 2202 Ultroscan Laser Densitometer and LKB 2190 GelScan software).

2.3. *Northern blot analysis*

Confluent human skin fibroblast cultures from each of 4 cell lines were stimulated for 24 h as described in fig.1. Total RNAs were extracted using guanidine isothiocyanate as described [23] and 15 μg of total RNA was loaded per lane on a 1.0% agarose formaldehyde gel. The RNA was transferred to nitrocellulose paper [24] and hybridized to [^{32}P]dCTP (3000 mCi, Amersham, Arlington Heights, IL, USA) oligo-labeled cDNA probes [25]. The cDNA probe for $\alpha 1$(I), Hf-404, was kindly provided by F. Ramirez, the cDNA probe for $\alpha 1$(III), PDT 1010, by B. de Crombrugghe, and the cDNA probe for β-tubulin (control) by F. Cabral.

3. RESULTS

3.1. *Effect of TGF-β and ascorbate on type I and III collagen production*

As shown in fig.1, pepsin-resistant proteins labeled with [^{3}H]proline are increased by ascorbate, TGF-β, and both over control media in two representative cell lines. There was no increase in the cell numbers in triplicate cultures. Both the $\alpha 1$ and $\alpha 2$ chains of type I collagen were increased by TGF-β and ascorbate. The $\alpha 1$ chain of type III

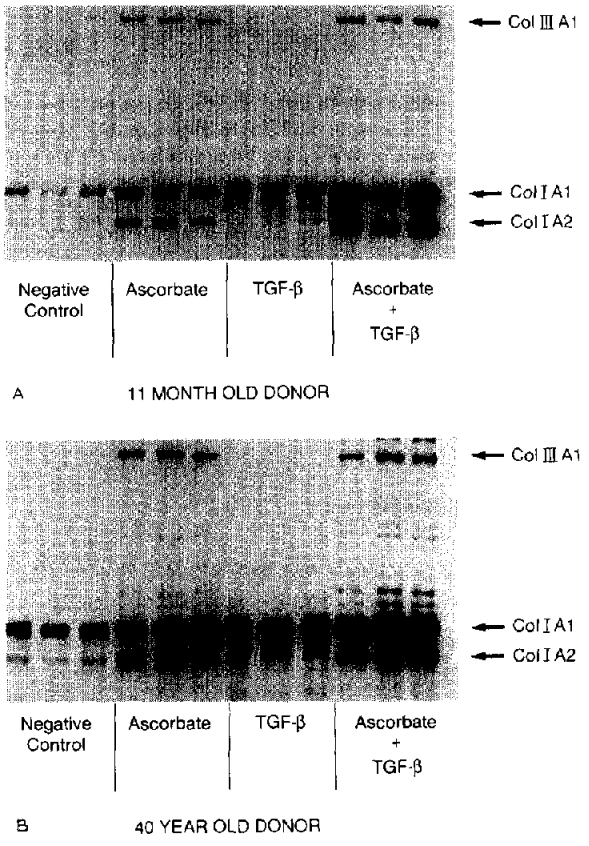

Fig.1. Effects of ascorbate and TGF-β on types I and III collagen synthesis by cultured human dermal fibroblasts. Compared with the negative controls, both ascorbate alone and TGF-β alone resulted in enhanced production (4- to 8-fold) of $\alpha 1$(I) and $\alpha 2$(I) collagen chains, versus a 22-fold increase with both together. No $\alpha 1$(III) was seen with TGF-β alone compared to a faint amount (negative control) and a prominent band with ascorbate alone. Other non-collagen proteins were not seen following pepsin treatment although faint, incompletely processed procollagen chains are present in some lanes. There was synergism with both ascorbate plus TGF-β. The autoradiographs (11 m o and 40 y o) are representative of the seven cell lines studied.

showed enhanced synthesis with both TGF-β and ascorbate but was significantly induced by ascorbate alone over controls. Both laser densitometry and scintillation counts of pepsin-resistant, TCA-precipitable proteins (table 1 and fig.2), showed synergism of collagen production with TGF-β and ascorbate combined. Using the three way analysis of variance, ANOVA, the synergism was significant within the triplicate samples of each of the seven different cell lines and additionally, using the mean values for the lines collectively ($p < 0.05$). There was an age-related decrease in response to TGF-β and ascorbate, but this was not statistically significant ($p = 0.08$).

3.2. *Effect of TGF-β and ascorbate on steady-state mRNAs for type I and III collagens*

To determine whether there was synergistic enhancement of the steady-state mRNAs for α1(I),

Table 1

[^{3}H]Proline incorporation into pepsin resistant proteins (collagens)

	Control	Ascorbate	TGF-β	TGF-β and ascorbate combined
Scintillation counts – total collagen in media samples radioactivity in cpm				
11 m o	11320	18268	29962	67223
1 y o	18246	54919	42400	191549
40 y o	27474	48355	47827	69265
45 y o	5198	10321	4072	12657
64 y o	13563	20072	49380	90447
74 y o	16218	18772	19902	86980
79 y o	12546	21434	28779	31239
Densitometer measurements – type I and type III combined				
11 m o	67	697	175	2654
1 y o	11	2115	95	4846
40 y o	470	2628	781	3759
45 y o	0	131	43	504
64 y o	226	1017	942	5088
74 y o	24	18	22	3236
79 y o	183	844	1491	2307

Human skin fibroblasts explanted from seven donors were plated, stimulated and the [^{3}H]proline-labeled pepsin-resistant proteins were extracted as described in fig.1 [22]. The cpm represent the mean of triplicate samples for each condition. The synergistic effects of TGF-β and ascorbate on pepsin resistant, TCA precipitable proteins were confirmed by laser densitometry measurements of fluorographs of SDS-polyacrylamide gels. Two cell lines (11 m o and 40 y o) were repeated with similar results (not shown)

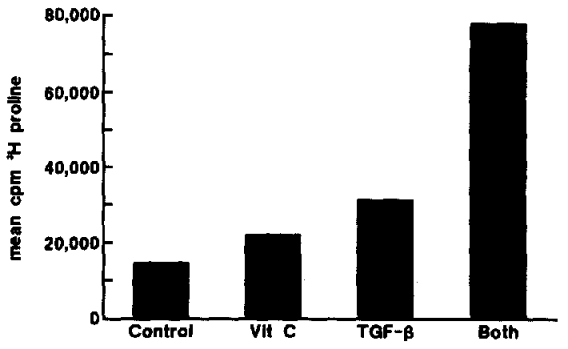

Fig.2. Synergistic effect of ascorbate and TGF-β on total collagen production in cultured human skin fibroblasts. The data was analyzed using 3-factor analysis of variance [Vitamin C, TGF-β, and age group (<10 years, 10–50 years, and >50 years)]. Vitamin C and TGF-β showed significant increases in counts ($p \ll 0.001$), while age group showed a nonsignificant decrease in counts ($p = 0.08$). The only significant interaction was vitamin C by TGF-β ($p = 0.006$). Upon rerun using $\log_{10}$ (counts) instead of counts, vitamin C and TGF-β continued significant ($p \ll 0.001$) while the interaction of vitamin C and TGF-β became not significant ($p = 0.392$) indicating a 'multiplicative effect' and thus synergism [28].

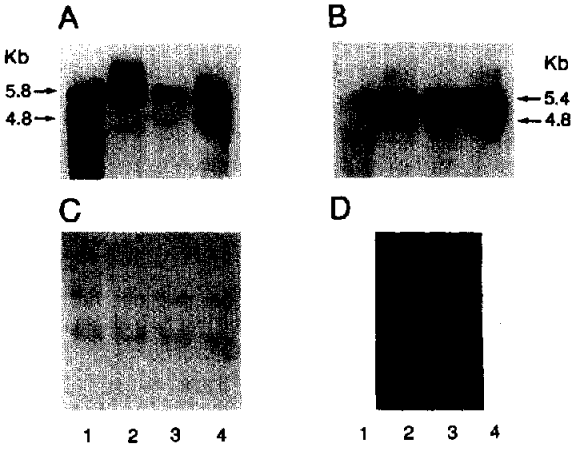

Fig.3. Northern blot analysis of the effect of ascorbate and TGF-β on type I (α1) and type III collagen mRNA in cultured human skin fibroblasts. (A) Hybridization with a Col 1 A1 cDNA probe; (B) hybridization with a Col 3 A1 cDNA probe; (C) equal hybridization to β-tubulin cDNA control probe; (D) total RNA. Laser densitometry showed a 1.5-fold increase in Col 1 A1 (5.8 plus 4.8 kb) with ascorbate and 3.5-fold increase with ascorbate and TGF-β, while Col 3 A1 (5.4 plus 4.8 kb) was increased 1.5-fold by TGF-β and 2.5-fold by ascorbate and TGF-β. That the increase in collagen production was greater than the rise in mRNA levels suggests an effect of ascorbate and TGF-β on mRNA stabilization and/or on a post-translational event.

α2(I), and α1(III), confluent skin fibroblasts were cultured as above, and the mRNAs were extracted with guanidine isothiocyanate as described [23]. Fig.3 shows the steady-state mRNAs, analyzed by Northern blotting. Steady-state type I and III collagen mRNAs were increased 3.5-fold and 2.5-fold, respectively, with TGF-β and ascorbic acid combined. Unexpectedly, type III mRNA was also increased with TGF-β alone although there was no apparent enhancement of the biosynthesis.

4. DISCUSSION

In a study examining the effects of 1,25-dihydroxyvitamin D_3 on types I and III collagen synthesis, a similar pattern of collagen regulation was found [26]. Type I but not type III collagen synthesis was stimulated by 1,25-dihydroxyvitamin D_3; however, analysis by Northern blot hybridization indicated that both type I and III procollagen mRNAs were increased 4-fold after a 24 h exposure. Of note, agents that stimulate bone resorption, such as 1,25-dihydroxyvitamin D_3, parathyroid hormone, and interleukin-1, increase TGF-β activity in organ cultures of fetal rat or neonatal mouse calvaria [27]. Whether TGF-β is a direct mediator of action of these agents is unknown.

The regulation of collagen synthesis by TGF-β is complex, and probably involves changes in translational efficiency as well as enhanced transcription. Previous studies have shown that type I collagen and fibronectin steady-state mRNAs are increased in the presence of TGF-β. The mechanism may not involve increased translation but rather stabilization of mRNAs [6]. Vitamin C increases both types I and III collagen mRNA translation by 2- to 3-fold, and may also decrease its degradation [7]. The synergistic enhancement of types I and III collagen biosynthesis by ascorbate and TGF-β may result from an increase in translation and/or a decrease in mRNA degradation. Finally, vitamin C could hypothetically interact with TGF-β induced nuclear binding proteins at the level of the collagen gene enhancer.

Our results suggest that attention to the presence of ascorbate in experimental systems used to test the effects of TGF-β would be critical, and may contribute to the development of clinical protocols for improved wound healing.

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