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Collagen Synthesis in Cultured Human Skin Fibroblasts: Effect of Ascorbic Acid and Its Analogs

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In confluent human skin fibroblasts maintained in 0.5% serum-supplemented medium, L-ascorbate specifically stimulated the rate of incorporation of labeled proline into total collagenase-sensitive protein, without changing the specific activity of the intracellular free proline. This influence of ascorbate reached a maximum at 30 μM and continued for at least 4 days, resulting in a 4-fold increase. The ascorbate effect occurred in cells at both confluent and subconfluent densities and was evident at all serum concentrations from 0.5–20%. The effect was independent of duration of the radioactive pulse between 2–6 h. D-Ascorbate, D-isoascorbate, and L-dehydroascorbate also stimulated collagen synthesis but at considerably higher concentrations, i.e., 250–300 μM. The stimulation of collagen synthesis by ascorbate and its analogs was accompanied by a decline in prolyl hydroxylase activity and a rise in lysyl hydroxylase activity; again L-ascorbate was found to be most effective. Dimethyltetrahydropterine and L-lactate failed to produce these effects.

It has been known for many years that ascorbic acid plays a vital role in connective tissue growth and repair and, specifically, collagen biosynthesis [1]. Ascorbate is a cofactor for the enzymes catalyzing the synthesis of collagen hydroxyproline and hydroxylysine [2]. Hydroxyproline stabilizes the collagen triple helix under physiologic conditions and consequently promotes secretion [3]. Hydroxylysine participates in the formation of intermolecular cross-links which impart mechanical stability to the collagen fiber [4,5].

The cofactor function of ascorbate provides an apparent explanation for its role in regulation of collagen synthesis, i.e., modulation of secretion through prolyl hydroxylation. We have recently described a specific stimulation of collagen synthesis in human skin fibroblasts by ascorbate [6], one that appears unrelated to hydroxylation. This stimulation is accompanied by an increase in mRNA specific for type I procollagen [7]. Moreover, we have observed a lack of coordination between prolyl hydroxylase activity and collagen synthesis under the influence of ascorbate and a change in lysyl hydroxylase activity in concert with collagen synthesis [6,8]. In the present study, we attempted to further define these influences and examined the specificity of the ascorbate requirement using analogs and other reducing agents.

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MATERIALS AND METHODS

Cell Culture and Labeling

Human skin fibroblasts from a normal 3-day-old male (GM970) were obtained from the Institute for Medical Research, Camden, New Jersey. The cells were seeded into plastic dishes (Falcon) at a density of 75,000 per 35-mm diameter unless otherwise indicated and grown to confluence in Dulbecco's modified Eagle's medium buffered to pH 7.4 with 24 mM sodium bicarbonate and 25 mM Hepes and supplemented with 20% dialyzed calf serum (Grand Island Biological Company) which had been inactivated for 30 min at 56°C. Cultures were then incubated for 72 or 96 h in the medium containing 0.5% serum unless otherwise indicated; the medium was changed daily. A fresh solution of L-ascorbic acid, D-ascorbic acid (kindly provided by Dr. W. E. Scott of the Hoffman-La Roche, Inc., Nutley, New Jersey), D-isoascorbic acid, L-dehydroascorbic acid, 6,7-dimethyl-5,6,7,8-tetrahydropterine, or L-lactic acid was added at the time of medium change. D-Isoascorbic acid and L-dehydroascorbic acid were analyzed by high-pressure liquid chromatography and found to be free of ascorbic acid [9]. Cultures were labeled for the final 6 h of incubation unless otherwise indicated with 20 μCi of L-[2,3-^{3}H]proline (New England Nuclear) per ml of medium. Following labeling, medium was separated from cells and a mixture of protease inhibitors consisting of N-ethylmaleimide, phenylmethylsulfonyl fluoride, and EDTA at 1 mM each was added to the medium. The cell layer was harvested with 0.05% trypsin/0.5 mM EDTA and the cell number in an aliquot of the suspension was determined in a Coulter counter. Cultures were studied in duplicate.

For enzyme studies, cultures were established as described above except cells were seeded at a density of 400,000 per 100-mm plate and harvested without labeling.

Collagen Synthesis

The radioactivity incorporated into the culture medium and the cell layer collagen was determined after digestion with clostridial collagenase (Advance Biofactures) free from detectable nonspecific protease activity, as described [10].

Specific Activity of Intracellular Free Proline

Trypsinized cells were centrifuged and washed 3 times with phosphate-buffered saline. The washed cells were suspended in 0.5 ml of distilled water and disrupted by freeze-thawing 3 times. After precipitating the protein with an equal volume of trichloroacetic acid, the supernatant was desalted on Dowex AG-50 × 5 (100–200 mesh), eluted with 2 M NH_4OH, and evaporated [11]. Proline was separated using the Beckman Model 119 amino acid analyzer and its radioactivity was determined in a liquid scintillation spectrometer. The amount of proline was determined by reaction with ninhydrin [12].

Enzyme Assays

Prolyl and lysyl hydroxylase activities in cell extracts were measured essentially as described [13].

RESULTS

The time course of stimulation of collagen synthesis by L-ascorbate is shown in Fig 1. The medium and cell layer were analyzed separately and the data are presented as the sum. The rate of collagen synthesis increased steadily by about 4-fold over 4 days of the treatment. The rate of noncollagen protein synthesis was not affected by ascorbate.

The effect of ascorbate was also examined in relation to duration of the radioactive pulse from 2–6 h (Table I). Relative

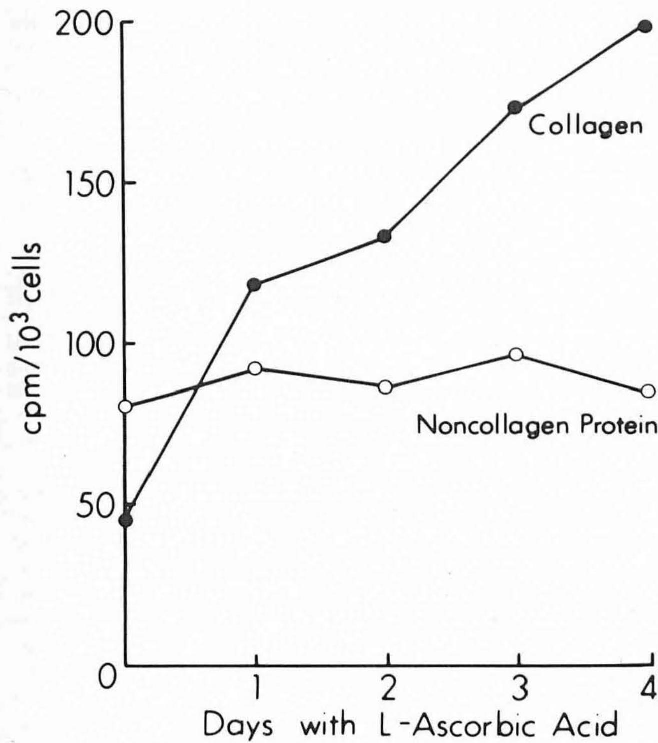

FIG 1. Dependence of collagen synthesis on duration of L-ascorbate administration. Confluent human skin fibroblasts were incubated for 96 h in Dulbecco's modified Eagle's medium supplemented with 0.5% dialyzed calf serum. L-Ascorbic acid at 125 μM was given for various lengths of time before harvest. Cultures were labeled for the last 6 h with L-[2,3-^{3}H]proline (20 μCi/ml/35-mm plate). The radioactivity incorporated into total collagen and noncollagen proteins was determined after digestion with clostridial collagenase.

TABLE I. *Dependence of collagen synthesis on pulse duration*

Ascorbate	Pulse duration (h)	Collagen synthesis (% of total protein synthesis)	Cell pellet collagen (% of total collagen)
−	2	ND[a]	ND[a]
+	2	30.4	32.6
−	3	15.7	92.9
+	3	26.4	19.3
−	4	15.1	83.8
+	4	29.1	13.7
−	5	12.8	83.4
+	5	29.7	11.0
−	6	12.3	74.7
+	6	30.6	9.4

Confluent human skin fibroblasts were incubated for 72 h in Dulbecco's modified Eagle's medium supplemented with 0.5% dialyzed calf serum. Half of the cultures received 100 μM L-ascorbate as indicated. Cultures were labeled with L-[2,3-^{3}H]proline (20 μCi/ml/35-mm plate) for various lengths of time before harvest. The radioactivity incorporated into total collagen and noncollagen proteins was determined after digestion with clostridial collagenase.

[a] Not detectable.

collagen synthesis as well as the magnitude of its induction by ascorbate were similar at all time points. The proportion of collagen-associated radioactivity in the cell pellet, representing mostly intracellular collagen, was strikingly diminished in the presence of ascorbate, in a manner dependent on pulse duration. This observation is consistent with the effect of ascorbate on collagen secretion [14,15].

The effect of ascorbate at various cell densities is shown in Table II. Relative collagen synthesis was not affected by the state of confluence of the cells. The response to ascorbate was likewise similar at all cell densities studied.

The effect of ascorbate was also examined at various serum concentrations. As shown in Fig 2, synthesis of collagen as well as noncollagen proteins increased with increasing serum con-

TABLE II. *Effect of ascorbate on collagen synthesis at various cell densities*

Ascorbate	Cell no. × 10^{-6} Initial	Cell no. × 10^{-6} Final	Collagen synthesis (% of total protein synthesis)
−	0.025	0.161	9.4
+	0.025	0.146	32.3
−	0.05	0.316	10.2
+	0.05	0.352	33.4
−	0.10	0.535	11.8
+	0.10	0.497	35.7

Human skin fibroblasts were plated at the indicated densities and grown for 7 days in Dulbecco's modified Eagle's medium supplemented with 20% dialyzed calf serum. Cultures were then incubated for an additional 72 h in 0.5% dialyzed calf serum. Half of the cultures received 100 μM L-ascorbate as indicated. Cultures were labeled for the last 6 h with L-[2,3-^{3}H]proline (20 μCi/ml/35-mm plate). The radioactivity incorporated into total collagen and noncollagen proteins was determined after digestion with clostridial collagenase.

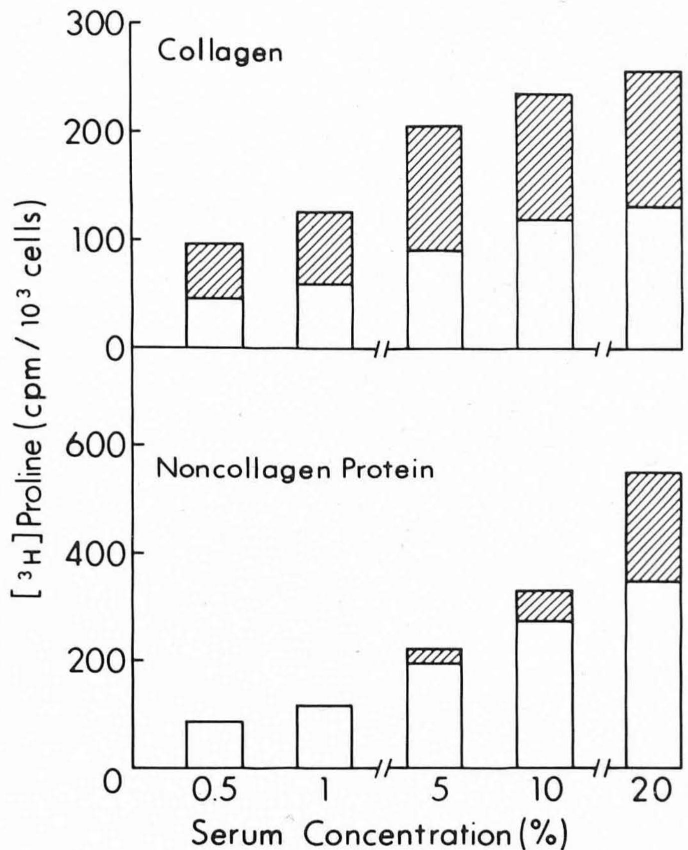

FIG 2. Effect of ascorbate on collagen synthesis at various serum concentrations. Confluent human skin fibroblasts were incubated for 72 h in Dulbecco's modified Eagle's medium supplemented with dialyzed, heat-inactivated calf serum at the indicated concentrations in the absence and in the presence of 100 μM L-ascorbate; the medium was changed daily. Cells were labeled for the last 6 h with L-[2,3-^{3}H]proline (20 μCi/ml/35-mm plate). The radioactivity incorporated into total collagen and noncollagen proteins was measured after digestion with highly purified clostridial collagenase. The *open columns* represent synthesis in the absence of ascorbate and the *hatched columns* the increase in synthesis after adding ascorbate.

centrations from 0.5% to 20%. Ascorbate stimulated the synthesis of collagen at all serum concentrations tested. The synthesis of noncollagen proteins was also stimulated by ascorbate but only at high serum concentrations.

The concentration vs effect relationship for stimulation of collagen synthesis by ascorbate and its analogs is shown in Fig 3. Relative collagen synthesis increased sharply and reached a maximum at 30 μM L-ascorbate. D-Ascorbate and D-isoascorbate also stimulated collagen synthesis; however, in contrast to L-ascorbate the response occurred at higher concentrations and reached comparable values at 250–300 μM. L-Dehydroascorbate was least effective in stimulating collagen synthesis. Dimethyltetrahydropterine and L-lactate were ineffective (not shown).

The specific activity of the intracellular free proline was measured to see whether the increase in label incorporation under the influence of ascorbate was related to a possible change in the uptake of proline or turnover of the proline pool. No differences were found between ascorbate-treated and untreated cultures (Table III), in line with the observation that the incorporation of radioactivity into noncollagen protein was not affected by ascorbate (Fig 1).

The influence of ascorbate, its analogs, and other reducing agents on prolyl and lysyl hydroxylase activities was examined (Table IV). Prolonged exposure of cells to L-ascorbate, D-ascorbate, D-isoascorbate, or L-dehydroascorbate resulted in a 2- to 4-fold increase in lysyl hydroxylase activity, with L-ascorbate being most effective. Treatment with dimethyltetrahydropterine or L-lactate had no effect on the activity. By contrast, prolyl hydroxylase activity was reduced by L-ascorbate, D-isoascorbate, L-dehydroascorbate, and D-ascorbate by 75, 37, 31, and 9%, respectively. It should be noted that in these experiments prolyl hydroxylase activity was assayed after incubation of the extracts with cofactors [13] in order to completely hydroxylate the endogenous substrate, which otherwise would compete with radioactive substrate in the assay. A similar problem would not occur in lysyl hydroxylase assay with radioactive substrate, for the collagen that is synthesized in the absence of added ascorbate is not deficient in hydroxylysine [6,16,17].

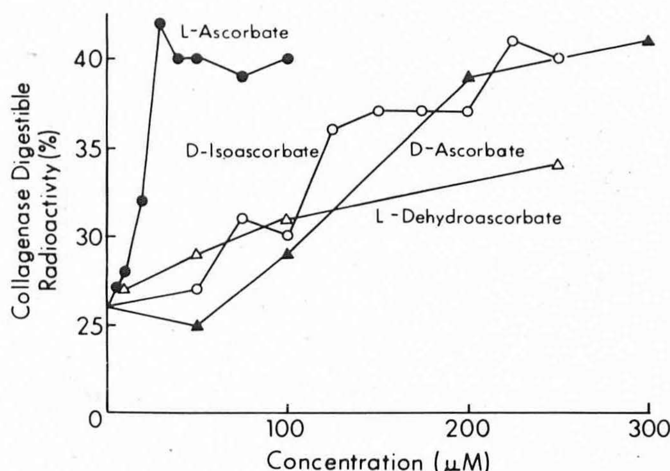

FIG 3. Dependence of collagen synthesis on concentration of ascorbate and its analogs. Confluent human skin fibroblasts were incubated for 72 h in Dulbecco's modified Eagle's medium supplemented with 0.5% dialyzed calf serum. L-Ascorbic acid, D-ascorbic acid, D-isoascorbic acid, or L-dehydroascorbic acid at various concentrations was given for the last 24 h. Cultures were labeled for the final 6 h with L-[2,3-^{3}H] proline 20 μCi/ml/35-mm plate). The radioactivity incorporated into total collagen and noncollagen proteins was determined after digestion with clostridial collagenase. The figure is a composite of 2 separate experiments and the control values have been normalized for ease of comparison.

TABLE III. *Effect of ascorbate and isoascorbate on specific activity of intracellular free proline*

Treatment	Proline specific activity (cpm/nmol)
None	544
L-Ascorbate, 24 h	495
L-Ascorbate, 96 h	582
D-Isoascorbate, 96 h	594

Confluent human skin fibroblasts were incubated for 96 h in Dulbecco's modified Eagle's medium supplemented with 0.5% dialyzed calf serum. L-Ascorbic acid or D-isoascorbic acid at 125 μM was present for the entire duration or the last 24 h. Cultures were labeled for the final 6 h with L-[2,3-^{3}H]proline (20 μCi/ml/35-mm plate). Free proline was isolated from the deproteinized lysates of washed cells as described in *Materials and Methods.*

TABLE IV. *Effect of ascorbate, its analogs, and other reducing agents on prolyl and lysyl hydroxylase activities*

Treatment	Prolyl hydroxylase activity (%)	Lysyl hydroxylase activity (%)
None	100	100
L-Ascorbate	25	378
D-Ascorbate	91	217
D-Isoascorbate	63	268
L-Dehydroascorbate	69	212
L-Lactate	104	110
Dimethyltetrahydropterine	111	90

Confluent human skin fibroblasts were incubated for 72 h in Dulbecco's modified Eagle's medium supplemented with 0.5% dialyzed calf serum. Treatments at 100 μM each were given for the entire duration. Dimethyltetrahydropterine was given in 0.1% dimethylsulfoxide; the controls received 0.1% dimethylsulfoxide only. Prolyl and lysyl hydroxylase activities in cell extracts were measured by tritium-release assays. The values refer to specific activities. The data are derived from 2 separate experiments.

DISCUSSION

Studies with whole animals as well as tissue and cell cultures have established a regulatory role for ascorbate in collagen biosynthesis [1,18]. Recently, there has been some concern with regard to the level at which ascorbate acts to stimulate collagen synthesis. Earlier studies [19–22] relied on measurement of hydroxyproline in collagen, reflecting the sum of collagen polypeptide synthesis and its hydroxylation. Subsequently, the role of ascorbate in collagen synthesis was reexamined by measuring the incorporation of proline into collagenase-sensitive protein, a procedure independent of hydroxylation. These studies [23–27] generally showed no effect of ascorbate on collagen polypeptide synthesis and attributed the beneficial effect of ascorbate to hydroxylation of the polypeptide chain and consequently secretion. In contrast, the studies presented here show a marked stimulation of collagen polypeptide synthesis in human skin fibroblasts by L-ascorbate. This effect of ascorbate ocurred over a period of several days and thus is slow compared to its effect on hydroxylation, which is completed within a matter of hours [14]. It is worth emphasizing that the present studies were carried out on confluent cells whose growth had been arrested by 0.5% dialyzed serum. Such cells remain in stable nonmitotic condition for several weeks and are metabolically functional [28]. A positive regulation of collagen polypeptide synthesis by ascorbate has been reported for primary cultures of avian tendon maintained in low serum concentration [29]. These observations are consistent with the known reduction of collagen polypeptide synthesis in scorbutic animals [1,30].

The stimulation of collagen synthesis by ascorbate was

equally evident at high serum concentrations. The ascorbate effect therefore appears to be unrelated to possible cellular starvation for some serum components. Serum had its major effect on noncollagen protein synthesis, in agreement with previous studies [31].

The stimulation of collagen synthesis by ascorbate was independent of cell density. These results differ from those with primary avian tendon cells in which stimulation of relative collagen synthesis by ascorbate was greater at confluent densities [32]. This difference might be due to the cell type or culture conditions. We have previously reported diminished relative collagen synthesis in subconfluent human skin fibroblasts due to an effect on noncollagen protein synthesis [10]. In the present study, relative collagen synthesis was similar at all cell densities studied. The difference in results may relate to higher serum concentration used in the earlier study (5% vs 0.5%) and the accompanying stimulation of noncollagen protein synthesis.

The relative rate of collagen production remained unchanged over the entire duration of the radioactive pulse, both in the absence and in the presence of ascorbate. This observation suggests that the ascorbate effect is not simply due to a change in degradation or accumulation. As mentioned earlier, collagen secretion is stimulated by ascorbate [14,15]. It is not yet clear whether secretion is linked to the increase in collagen-specific mRNA induced by ascorbate [7], although such an association has been suggested [32].

D-Ascorbate and D-isoascorbate also stimulated collagen synthesis similar to L-ascorbate but at 8 to 10 times higher concentrations, in accord with their antiscorbutic potencies [33,34]. Ascorbate is concentrated by cultured fibroblasts [14,35] and the observed difference may be related to stereospecificity in cellular transport. Thus, both D-ascorbate and D-isoascorbate are as active as L-ascorbate in the in vitro prolyl hydroxylase reaction [36,37]. The small but significant stimulation of collagen synthesis by L-dehydroascorbate, the oxidized form of the vitamin, is consistent with its antiscorbutic properties [38,39] and provide reason to suggest that the stimulation of collagen synthesis by L-ascorbate is independent of its reducing property, in marked contrast to the hydroxylation reaction.

The requirement of ascorbate for stimulation of collagen synthesis appears to be specific as it could not be satisfied by other reducing agents, namely dimethyltetrahydropterine and L-lactate. Dimethyltetrahydropterine has been found to replace ascorbate in the prolyl hydroxylase reaction in vitro [36,40,41] as well as in vivo [30]. Short incubation of cells with L-lactate at high concentrations has been reported to stimulate prolyl hydroxylase activity [42], in a manner similar to ascorbate [8,13,42].

An interesting finding in this study was the decline in prolyl hydroxylase activity and the rise in lysyl hydroylase activity following treatment of human skin fibroblasts with ascorbate or its analogs. It is not known whether the reduced prolyl hydroxylase activity in ascorbate-treated cells is due to a diminished amount of the enzyme or conversion of the active enzyme to the inactive form. It is noteworthy in this regard that prolyl hydroxylase activity in extracts of L-ascorbate-treated cells appears to be thermally less stable and in extracts of D-ascorbate-treated cells appears to be thermally as stable as in extracts of untreated cells (unpublished results), an observation consistent with the relative effectiveness of the two isomers in reducing the enzyme activity. Recent studies have provided evidence for a translational control of collagen synthesis by aminoterminal peptides of procollagen [43,44]. It is conceivable that a similar negative feedback mechanism might exist for regulation of prolyl hydroxylase activity. The reciprocal modulation of collagen synthesis and prolyl hydroxylase activity by ascorbate as described here would be consistent with such a mechanism. The mechanism by which ascorbate stimulates lysyl hydroxylase activity in intact cells and the physiologic significance of this phenomenon is less clear. An activation of the enzyme appears unlikely considering the long time needed for maximal response to ascorbate [8] and the lack of ascorbate effect in cell extracts [13]. Thus, ascorbate may increase the level of lysyl hydroxylase activity by altering the enzyme synthesis and/or degradation, possibilities that remain to be explored.

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Retinoic Acid Inhibition of Collagenase and Gelatinase Expression in Human Skin Fibroblast Cultures. Evidence for a Dual Mechanism

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Human skin fibroblast cultures have been employed to study the effects of a variety of vitamin A analogues (retinoids) on the expression of two enzymes involved in collagen degradation in the skin, collagenase and a gelatinolytic protease. In normal and recessive dystrophic epidermolysis bullosa fibroblast cultures, retinoic acid compounds were effective inhibitors of the accumulation of both enzymes in the culture medium with half-maximal inhibitions occurring at 0.25–1 μM for collagenase and at 3–6 μM for the gelatinolytic protease. Various retinoids exhibited differing degrees of inhibitory actions, so that at a 1 μM concentration, relative inhibitions were: 13-*cis*-retinoic acid > all-*trans*-retinoic acid > aromatic retinoid (Ro 10-9359) >> retinol. The retinoic acid-mediated decrease in collagenase activity was accompanied by a parallel decrease in immunoreactive collagenase protein, suggesting that the retinoic acids were acting to inhibit synthesis of the enzyme. However, an additional effect of these agents was encountered. Although the retinoids themselves had no direct collagenase inhibitory action, medium derived from cultures maintained in these retinoids showed direct inhibitory capacity which was dependent both on the concentration of retinoic acid and on the length of time in culture. The results suggest that the retinoic acids modulate collagenase in vitro by two mechanisms: by decreasing the synthesis of enzyme protein and by modulating the expression of an inhibitory molecule.

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Abbreviations:
CM-cellulose: carboxymethylcellulose
DMSO: dimethylsulfoxide
Hepes: N-2-hydroxyethyl-piperazine-N′-2-ethanesulfonic acid
RDEB: recessive dystrophic epidermolysis bullosa

Collagenase is the rate-limiting enzyme in collagen degradation and is crucial for the initial characteristic cleavage of the collagen molecule [1]. Following this cleavage, at physiologic temperature the individual collagen chains become denatured [2] and are further degraded by the gelatin-specific neutral protease, gelatinase [3–7]. For complete collagen degradation to occur in the skin, it is likely that the concerted, but sequential, actions of at least two enzymes—collagenase and gelatinase—are required [7–9].

Although vitamin A (retinol) and its derivatives (retinoids) constitute a class of compounds with many diverse actions, particularly on epithelial cells [10], their effects are not limited to this cell type. Thus, it is of interest that Brinckerhoff and coworkers [11,12] have shown that both naturally occurring all-*trans*-retinoic acid and the synthetic retinoid, 13-*cis*-retinoic acid, inhibit collagenase expression in cultures of mesenchymal cells derived from human rheumatoid synovium. In other sys-