Effects of Ascorbic Acid on Proliferation and Collagen Synthesis in Relation to the Donor Age of Human Dermal Fibroblasts

Charlotte L. Phillips, Susan B. Combs, and Sheldon R. Pinnell
Department of Medicine, Division of Dermatology, Duke University Medical Center, Durham, North Carolina, U.S.A.

Several events are associated with cellular aging: alterations in the extracellular matrix, loss of the cell's proliferative capacity, and decreased responsiveness to growth factors. In skin, a major component of the extracellular matrix is collagen; an important regulator of collagen synthesis is ascorbic acid, which may also have growth factor–like properties. To investigate the relationship of the extracellular matrix and proliferative capacity to aging, we examined the effects of ascorbic acid on cell proliferation and collagen expression in dermal fibroblasts from donors of two age classes, newborn (3–8 d old) and elderly (78–93 years old). In the absence of ascorbic acid (control) proliferative capacities were inversely related to age; newborn cell lines proliferated faster and reached greater densities than elderly cell lines. However, in the presence of ascorbic acid both newborn and elderly cells proliferated at a faster rate and reached higher densities than controls. To determine whether there are age-related differences in extracellular matrix production and ascorbic acid responsiveness we examined and found that collagen biosynthesis (collagenase-digestible protein) was inversely related to age, but the stimulation by ascorbic acid appeared age independent. The increase in collagen synthesis was reflected by coordinate increases in steady-state proα1(I) and proα1(III) collagen mRNAs, suggesting a pretranslational mechanism. Ascorbic acid appears capable of overcoming the reduced proliferative capacity of elderly dermal fibroblasts, as well as increasing collagen synthesis in elderly cells by similar degrees as in newborn cells even though basal levels of collagen synthesis are age dependent. *Key words: type III collagen/type I collagen/lysyl hydroxylase/lysyl oxidase. J Invest Dermatol 103:228–232, 1994*

Connective tissues, particularly skin, undergo significant alterations during aging, which has led to the hypothesis that cells in the extracellular matrix and the extracellular matrix itself are intimately involved in the aging process (for review, see [1–3]). Independent observations have associated several events with cellular aging, including loss of the cell's proliferative capacity, decreased responsiveness to growth factors, increased responsiveness to growth inhibitors, and alterations in extracellular matrix production [3–5]. The relationship of these different events to the aging process and their interactions with each other are unclear.

A major component of the extracellular matrix is collagen; in skin, type I and type III collagen comprise 85–90% and 8–11% of the total collagen synthesized, respectively [6]. Type I collagen is a heterotrimer of two α1(I) chains and one similar but genetically distinct α2(I) chain, and type III collagen is a homotrimer of three α1(III) chains. Each collagen chain is synthesized as a precursor procollagen chain and these chains assemble into mature collagen molecules *via* a complex series of co- and post-translational processing steps, including association and winding of the correct procollagen α chains, hydroxylation of specific proline and lysine residues (by prolyl and lysyl hydroxylases), glycosylation, cleavage of propeptides, and intra- and intermolecular crosslinking (by lysyl oxidase) to eventually form collagen fibrils of the extracellular matrix [7].

A primary interest of our laboratory has been the regulation of type I and III collagen, particularly by ascorbic acid. Ascorbic acid not only serves as an important cofactor for some of the post-translational processing enzymes but it has also been shown to upregulate type I and III collagen synthesis in human dermal fibroblasts [8]. Previous studies also have suggested that ascorbic acid may act as a growth factor stimulating dermal fibroblasts to proliferate in culture [9–11].

To provide a window for investigating the relationship of the extracellular matrix and the proliferative capacity of a cell during the aging process we examined the effects of ascorbic acid on dermal fibroblasts from different aged donors. *In vitro* studies have shown that the chronologic age of donor tissue is strongly reflected in the behavior of cultured skin-derived cells [12,13]. In this study we demonstrate that even though both the proliferative capacity and basal levels of collagen synthesis are down-regulated in an age-dependent manner, ascorbic acid is capable of both stimulating cell proliferation and upregulating collagen synthesis in dermal fibroblasts regardless of donor age.

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Reprint requests to: Dr. Charlotte L. Phillips, Duke University Medical Center, Department of Medicine, Division of Dermatology, P.O. Box 3135, Durham, NC 27710.

MATERIALS AND METHODS

Cell Lines Normal human dermal fibroblast cell lines were obtained from the following: the Human Genetic Mutant Cell Repository (Institute for Medical Research, Camden, NJ), cell lines 671 (3 d old, male: AG1519) and 672 (3 d old, male: AG1523); the cell lines 722 (8 d old, female), 882 (78

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years old, male), 663 (88 years old, female), and 655 (93 years old, female) were from explants.

Cell Culture Fibroblast cells were seeded in tissue culture dishes (Falcon, Lincoln Park, NJ) at densities of 150,000 cells/35-mm diameter dish (for collagen synthesis assay), 100,000 cells/60-mm diameter dish (proliferation study), and 430,000 cells/100-mm diameter dish (Northern blot hybridization analysis), and grown to confluence in Dulbecco's modified Eagle's medium (DMEM) buffered to pH 7.4 with 24 mM sodium bicarbonate and 25 mM Hepes and supplemented with 20% heat-inactivated bovine serum (GIBCO BRL, Gaithersburg, MD). The atmosphere was humidified and maintained at 37°C in 5% carbon dioxide and 95% air.

Measurement of Collagen Synthesis Dermal fibroblasts were incubated under nonproliferating conditions in DMEM supplemented with 0.5% dialyzed bovine serum in the presence or absence of 100 μM ascorbic acid for 72 h, and collagen and non-collagen synthesis was determined as described [8].

Northern Blot Hybridization Analysis Total RNA was isolated, using the procedure of Chomczynski and Sacchi [14], from the cells in 100-mm diameter dishes that had been washed twice with phosphate-buffered saline (PBS), and the medium changed to DMEM supplemented with 0.5% dialyzed bovine serum in the presence or absence of 100 μM ascorbic acid for 72 h. The steady-state RNA levels were determined by Northern blot hybridization analyses [8]. The following cDNAs were used for hybridization: proα1(I) collagen (Hf-677) [15], proα1(III) collagen (Hf-934) [16], lysyl hydroxylase [17], lysyl oxidase [18], and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) [19]. The hybridization signals were visualized by autoradiography. The relative levels of the specific mRNAs were quantitated by densitometry using the LKB 2202 ultrascan laser densitometer. Total RNA concentrations were determined by absorbance at 260 nm.

RESULTS

Ascorbic Acid Stimulated Proliferation of Newborn and Elderly Cells We examined six human dermal fibroblast cell lines for their proliferative capacity in relation to *in vivo* age. Because of the inherent variation between individuals we decided in this pilot study to work with only two age classes, one at each end of the age spectrum, newborn (3–8 d old) and elderly (78–93 years old), and in each age class to use three cell lines derived from three different donors. As depicted in Fig 1 the newborn cell lines *(A)* grew at a faster rate and reached higher densities than the elderly cell lines *(B)*. The elderly cell lines (*in vivo* aged) appeared to exhibit decreased proliferative capacity, reaching a plateau. By day 13 the newborn and elderly cell densities had increased from 1.0×10^5 cells/100 mm culture dish to $1.0–2.4 \times 10^6$ and $0.2–0.6 \times 10^6$ cells/100-mm culture dish, respectively.

The proliferative capacity of the newborn and elderly cell lines was also examined in the presence of ascorbic acid to determine whether ascorbic acid exhibits growth factor–like activity in cultured dermal fibroblasts (Fig 1*A,B*). In the presence of 100 μM ascorbic acid both newborn and elderly cells were induced to proliferate at a greater rate and reach higher densities than in the absence of ascorbate. By day 13 the densities of the newborn and elderly cell lines had increased by 167–303% and 208–315% of control (cells grown in the absence of ascorbate), respectively. The presence of continuous ascorbic acid treatment appears capable of stimulating elderly cells to proliferate, suggesting that it may have growth factor activity and that this activity may be age independent.

Ascorbic Acid Upregulation of Collagen Synthesis To determine whether the upregulation of collagen production by ascorbic acid is age dependent we quantitated collagen synthesis in the presence and absence of ascorbic acid under nonproliferating conditions. Our results (Table I) demonstrate that the relative levels of collagen synthesis by dermal fibroblasts from different aged donors (three newborn donors [3–8 d old] and three elderly donors [78, 88, and 93 years old]) appear inversely related to age; the relative level of collagenase-digestible protein synthesized by cultured dermal fibroblasts from newborn and elderly cell lines was $10.5 \pm 0.9\%$ and $6.2 \pm 1.2\%$ (mean $\pm$ SD), respectively (Student t test, $p = 0.008$). Even though the elderly cell lines synthesized reduced basal levels of collagen, their collagen production was upregulated by ascorbate

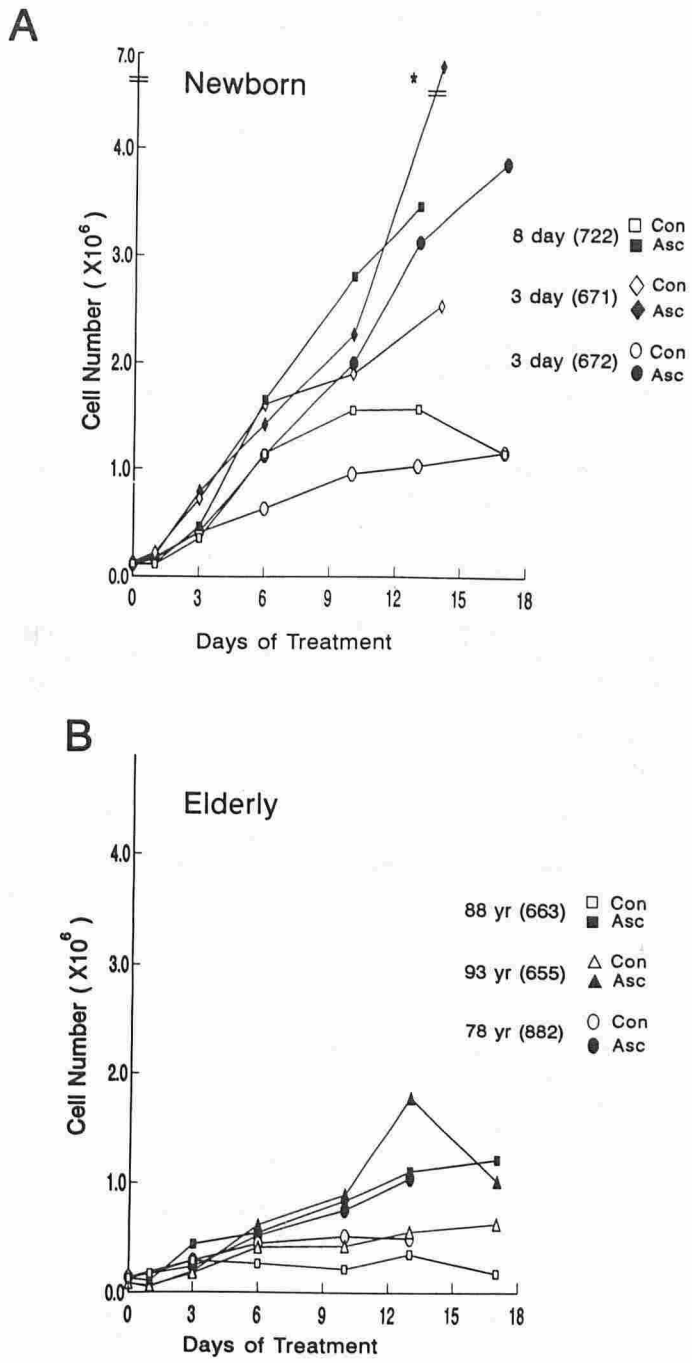

Figure 1. Cultured dermal fibroblasts from newborn (3–8-day-old) and elderly (78–93 years old) donors proliferate at a faster rate and reach higher densities in the presence of 100 μM ascorbic acid. In parallel experiments dermal fibroblasts from three newborn cell lines *(A)* and three elderly cell lines *(B)* were seeded at a density of 100,000 cells/60-mm dish and cultured in the presence *(closed symbols)* and the absence *(open symbols)* of 100 μM ascorbic acid for up to 17 d. At the indicated times the cells were harvested and counted (* 6.7×10^6 cells).

to the same degree as the newborn cell lines, by twofold; the relative level of collagenase-digestible protein synthesized by cultured newborn and elderly fibroblasts increased to $19.9 \pm 1.0\%$ and $12.4 \pm 2.0\%$, respectively. These results suggest that even though the basal levels of collagen synthesis in newborn and elderly fibroblasts appear age dependent the upregulation of collagen synthesis by ascorbic acid appears age independent.

Table I. Effect of Ascorbic Acid on Relative Collagen Synthesis in Dermal Fibroblasts from Newborn and Elderly Donors

Cell Strain	% Collagen[a] −ASC[b]		% Collagen[a] +ASC (100 μM)[c]		Fold Increase Over Control	
Newborn	Mean ± SD		Mean ± SD		Mean ± SD	
3 d (671)	10.2		19.0		1.9	
3 d (672)	11.5	10.5 ± 0.9	19.7	19.9 ± 1.0	1.7	1.9 ± 0.2
8 d (722)	9.8		20.9		2.1	
Elderly						
78 years (882)	6.3		10.4		1.7	
88 years (663)	5.2	6.2 ± 1.2	12.2	12.4 ± 2.0	2.4	2.0 ± 0.3
93 years (655)	7.5		14.4		1.9	

[a] Expressed as proportion of total protein synthesis devoted to collagen. Data have been corrected to take into account the increased proline content.
[b] Cells cultured under nonproliferating conditions, in DMEM supplemented with 0.5% dialyzed fetal bovine serum for 72 h.
[c] Cells cultured under nonproliferating conditions, in DMEM supplemented with 0.5% dialyzed fetal bovine serum in the presence of 100 μM ascorbic acid for 72 h.

Northern Blot Hybridization Analysis of Type I and Type III Collagen, Lysyl Hydroxylase, and Lysyl Oxidase To investigate the mechanism of the ascorbic acid regulation of collagen synthesis more closely, we examined the steady-state RNA levels of proα1(I) collagen and proα1(III) collagen, as well as the collagen post-translational modifying enzymes lysyl hydroxylase and lysyl oxidase by Northern blot analyses (Fig 2). The proα1(I) collagen gene transcribes two distinct mRNA species of approximately 5.8 and 4.8 kilobases (Kb) [20]; the proα1(III) collagen gene also transcribes two distinct mRNA species of 5.4 Kb and 4.8 Kb [16]. For each case the transcripts are colinear, with the longer transcripts using alternative polyadenylation signals that are further downstream, 3′, from the polyadenylation signals of the shorter transcripts. The differences in expression of type I collagen RNAs between the newborn and elderly fibroblasts do not appear greater than the differences between individuals within either of the specific age classes; the relative densiometric units for the 4.8 Kb mRNA of the newborn and elderly cell lines were $11.35 \pm 3.35 \times 10^3$ and $15.84 \pm 2.43 \times 10^3$ (mean ± SEM; Student t test, $p = 0.34$), and for the 5.8 Kb mRNA were $6.24 \pm 1.37 \times 10^3$ and $5.72 \pm 1.23 \times 10^3$ ($p = 0.79$), respectively. This appeared to be true also for proα1(III) collagen RNAs. The relative densiometric units for the 4.8 Kb mRNA of the newborn and elderly cell lines were $24.03 \pm 4.46 \times 10^3$ and $27.87 \pm 1.46 \times 10^3$ ($p = 0.47$), and for the 5.4 Kb mRNA were $7.79 \pm 1.79 \times 10^3$ and $9.63 \pm 1.77 \times 10^3$ ($p = 0.51$), respectively. There do not appear to be age-dependent changes in steady-state RNA levels of proα1(I) or proα1(III) collagen, suggesting that the decrease in collagen synthesis in the elderly cells cannot be accounted for by pretranslational events.

Treatment of the newborn and elderly cell lines for 72 h with 100 μM ascorbic acid resulted in coordinately increasing the steady-state levels of RNA for both proα1(I) and proα1(III) collagen in both the newborn and elderly cell lines. The amount of this induction in the steady-state levels of proα1(I) and proα1(III) collagen mRNAs (Table II) paralleled the twofold increase seen in collagen synthesis. The steady-state levels of both the 5.8- and 4.8-Kb mRNAs of type I collagen were increased but not to the same degree. In newborn and elderly fibroblasts the 5.8-Kb mRNA was stimulated by 2.04 ± 0.24 and 2.22 ± 0.32 fold (mean ± SEM), respectively, and the 4.8-Kb mRNA was stimulated by only 1.59 ± 0.18 and 1.26 ± 0.16 fold, respectively. This effect was observed also with the steady-state levels of both the 5.4- and 4.8-Kb mRNAs of type III collagen. These results suggest that the increase in collagen production by cultured dermal fibroblasts in the presence of ascorate can be accounted for by increased type I and type III collagen mRNAs, *via* a coordinate pretranslational mechanism, and that the induction by ascorbate is age independent.

The differences observed in the steady-state levels of lysyl hydroxylase and lysyl oxidase mRNAs between the two age classes also do not appear to be any greater than the differences between any individuals within the specific age class. In the presence of ascorbic acid the steady-state levels of lysyl hydroxylase mRNAs were slightly elevated, by 1.37 ± 0.18 and 1.40 ± 0.25 fold over control for the newborn and elderly cell lines, respectively, whereas the steady-state levels of lysyl oxidase mRNAs appeared unchanged in both newborn and elderly cell lines. Interestingly, the expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH), a house-

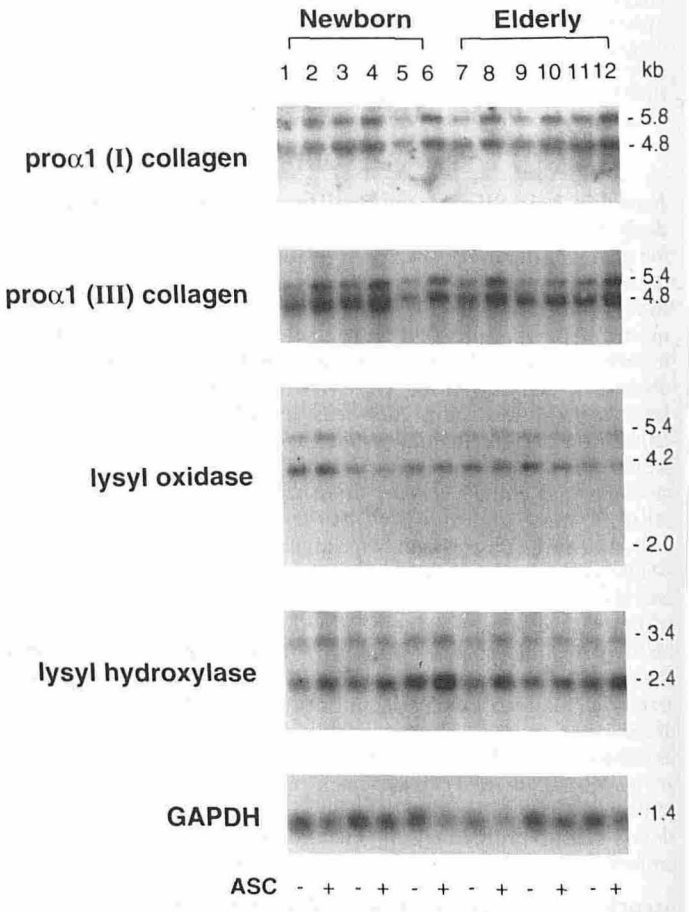

Figure 2. Ascorbic acid increases the steady-state levels of proα1(I) and proα1(III) collagen mRNA in an age-independent manner. Northern blot hybridization analysis of proα1(I) and proα1(III) collagen mRNA expression in dermal fibroblast from three newborn donors (*lanes 1,2,* 3 days, 671; *lanes 3,4,* 3 days, 672; *lanes 5,6,* 8 days, 722) and three elderly donors (*lanes 7,8,* 93 years, 655; *lanes 9,10,* 88 years, 663; *lanes 11,12,* 78 years, 882). Confluent cells were incubated 72 h in DMEM, 0.5% fetal bovine serum alone *(lanes 1,3,5,7,9,11),* or with the addition of 100 μM ascorbic acid *(lanes 2,4,6,8,10,12).* Total RNA, 2 μg, was size fractionated by electrophoresis in a formaldehyde 1% agarose gel, transferred to a Gene-screen plus membrane, and hybridized to the following ^{32}P-radiolabeled cDNA: proα(I) collagen, proα1(III) collagen, lysyl hydroxylase, lysyl oxidase, and GAPDH. The sizes of the specific transcripts are indicated.

Table II. Effect of Ascorbic Acid on Steady-State mRNA Levels in Dermal Fibroblasts from Newborn and Elderly Donors

mRNAs	Ratio = (Ascorbate-Treated Steady-State mRNA Levels[a,b])/(Control Steady-State mRNA Levels) Newborn (3–8 d) (mean ± SEM)	Elderly (78–93 years) (mean ± SEM)
proα1(I) collagen		
5.8 kb	2.04 ± 0.24	2.22 ± 0.32
4.8 kb	1.59 ± 0.18	1.26 ± 0.16
proα1(III) collagen		
5.4 kb	2.46 ± 0.32	1.97 ± 0.22
4.8 kb	1.52 ± 0.14	1.24 ± 0.09
Lysyl oxidase[c]	0.97 ± 0.08	0.87 ± 0.15
Lysyl hydroxylase[d]	1.37 ± 0.18	1.40 ± 0.25
GAPDH[e]	0.68 ± 0.11	0.72 ± 0.07

[a] Cells cultured under nonproliferating conditions, in DMEM supplemented with 0.5% dialyzed fetal bovine serum for 72 h in the presence and absence of 100 μM ascorbic acid.
[b] The relative mRNA levels were determined by densitometry of the autoradiographs in Fig 2.
[c] Combined densities from 5.4 kb and 4.2 kb mRNAs of lysyl oxidase.
[d] Combined densities from 3.4 kb and 2.4 kb mRNAs of lysyl hydroxylase.
[e] Glyceraldehyde-3-phosphate dehydrogenase.

keeping protein whose expression was predicted to be unaffected by ascorbic acid, demonstrated a definite reduction in steady-state GAPDH mRNA levels in both the newborn and elderly cell lines.

DISCUSSION

Previous studies have suggested that ascorbic acid may have growth factor activity [9–11]. We examined the ability of dermal fibroblasts from three newborn donors and three elderly donors to proliferate in the presence and absence of ascorbic acid. We chose to include cells from elderly donors because one of the cellular manifestations of aging is the loss of a cell's proliferative capacity, which is hypothesized to result from cells becoming less responsive to certain growth factors [4,5]. In the absence of ascorbic acid, newborn cell lines grew faster and reached higher densities than the elderly cell lines [9,21]. The elderly cell lines exhibited a decreased proliferative capacity. However, the addition of ascorbic acid to the cultures encouraged both the newborn and elderly cell lines to proliferate. Chan *et al* had previously observed that newborn fibroblasts proliferated more rapidly in the presence of continuous ascorbic acid treatment [10]; in this report we demonstrate that ascorbic acid is also capable of stimulating elderly cells to proliferate. The elderly cells still had the ability to respond to ascorbic acid even though in the absence of ascorbate they had reached a plateau in their growth curve and were no longer dividing. This is in contrast to observations in other *in vivo* aged and/or *in vitro* aged cells where there was the loss of responsiveness to other growth factors [4,5,22]. Phillips *et al* demonstrated that as the normal human diploid cell line went into senescence it became progressively less responsive to platelet-derived growth factor, epidermal growth factor, dexamethasone, insulin, and transferrin [5]. This suggests that either the elderly cells used in our study were not sufficiently close to senescing to have lost their ability to respond to ascorbate or that ascorbate acid stimulates proliferation *via* a different mechanism than the other growth factors, and that this mechanism is age independent.

The inverse relationship between collagen synthesis and age of cells, whether *in vivo* or *in vitro* aged, has been well documented [23–26]. This relationship has also been observed with another connective tissue component, elastin [27,28]. It has been suggested that the decrease in collagen synthesis is a reflection of an overall decrease seen in protein synthesis with *in vitro* and *in vivo* aging [23]. However, Martin *et al* had also demonstrated that fibronectin, another connective tissue component, increases with age, contradicting this hypothesis [24].

The differences in the basal levels of expression of proα1(I) and proα1(III) collagen steady-state RNA between the newborn and elderly age classes were not greater than those seen between individuals in the same age class, suggesting that the decreased collagen synthesis in elderly cells cannot primarily be accounted for by decreased steady-state collagen RNA levels. These results at first appear to contrast the results from some previous studies [21,29], but may actually reflect differences between *in vivo* and *in vitro* aging, senescence, and/or the inherent variation seen between individuals. Furth demonstrated, using *in vitro* aged WI-38 cells (human fibroblasts derived from embryonic lung tissue), a decrease in steady-state proα1(I) and proα2(I) collagen mRNAs in senescent cells [29]. Takedo *et al,* using *in vitro* aged fetal fibroblasts as well as *in vivo* aged fibroblasts (51 and 80 years old), observed reduced levels of steady-state type I and type III collagen mRNAs with *in vitro* aging [21], as well as significantly lower levels of collagen RNA in *in vivo* aged 51 and 80 year old fibroblasts relative to the fetal fibroblasts. In examining other matrix components including proteoglycans and fibronectin, Takeda *et al* concluded that although *in vitro* and *in vivo* aging were very similar, they were not equivalent. The results reported here using *in vivo* aged dermal fibroblasts from two different age classes (newborn and elderly) suggest that the reduced basal levels of collagen synthesis seen in elderly cells are not due to reduced steady-state levels of type I and type III collagen RNA but are due to post-translational regulatory events. The role of post-translational regulatory events was previously postulated from an *in vitro* aging study using porcine skin fibroblasts, in which the decreased production of extracellular collagen by *in vitro* aged pig fibroblasts appeared to be the result of both a slight decrease in type I collagen synthesis and a significant increase in intracellular collagen degradation [24].

It has been postulated that collagen synthesis and cell proliferation in fibroblasts and osteoblasts may be linked [30].* Harada *et al* demonstrated, using the osteoblast cell line MC3T3-E1, that ascorbic acid stimulated proliferation as well as collagen synthesis, and that the stimulatory effect of ascorbic acid on proliferation is blocked by inhibitors of collagen synthesis [30]. Investigations using benzoic acid hydrazide to inhibit collagen synthesis in dermal fibroblasts demonstrated similar results, suggesting that endogenously synthesized collagen may play a role in fibroblast proliferation.* Even though our data are not sufficient for a conclusion, our results are consistent with the hypothesis that collagen synthesis and cell proliferation are associated; with each condition in our study (*in vivo* age or the presence or absence of ascorbic acid) the proliferative response paralleled the collagen synthetic response.

Ascorbic acid, an important regulator of collagen expression, appears to increase collagen biosynthesis in an age-independent manner. Even though there were reduced basal levels of collagen synthesis in elderly cells they were still capable of being induced by the same degree as newborn cells. In both the elderly and newborn cell lines this induction could be accounted for by increased steady-state RNA levels of proα1(I) and proα1(III) collagen, suggesting a pretranslational mechanism, which may be different and independent of the mechanisms controlling basal levels of collagen expression. TGF-β1 is a known potent stimulator of collagen synthesis and has been shown to increase collagen synthesis *via* pre- and post-translational mechanisms [8,31]. In previous studies using newborn dermal fibroblasts and examining the role of ascorbic acid and TGF-β1 we had also noted the preferential increase of the larger transcripts of both proα1(I) and proα1(III) collagen by ascorbate and TGF-β1 [8]. Using Swiss Mouse 3T3 cells, Penttinen *et al* observed that the TGF-β1 stimulation of proα1(I) collagen mRNAs also exhibited a preferential increase of the larger transcript [31]. We have now shown that this differential yet coordinate pattern of induction of type I and type III collagen mRNAs can also occur in the elderly (*in vivo* aged) cells, and thus appears independent of donor age.

In both the newborn and elderly cell lines ascorbic acid treatment resulted in small equivalent increases in the steady-state RNA levels of lysyl hydroxylase. Even though ascorbic acid is an important cofactor of lysyl hydroxylase it still remains to be determined

* Murad S, Walker LC, Tajima S, Phillips CL, Pinnell SR: Benzoic acid hydrazide: a novel inhibitor of fibroblast proliferation and collagen synthesis in cell culture (abstr 461). *J Invest Dermatol* 96:608A, 1991.

whether or not the increase in lysyl hydroxylase mRNA is due to ascorbic acid directly or secondarily to the increased collagen production. In contrast, steady-state levels of lysyl oxidase mRNA were not affected by ascorbic acid in either newborn or elderly cell lines.

The fibroblasts in this study were incubated with physiologically significant concentrations of ascorbic acid (100 μM). Finglas *et al* reported the following concentrations of ascorbic acid in plasma from healthy adults, aged 20–64 years (n = 46), to range from 3 to 93 μM (mean 50 μM), and for elderly adults, aged 64–74 years (n = 74), to range from 4 to 89 μM (mean 40 μM) [32]. With this in mind, the results reported here may have important implications for wound healing in the elderly, in whom there are often seen increased difficulties and prolonged healing times of wounds [1]. If, *in vivo*, ascorbic acid is capable of overcoming the decreased proliferative capacity of dermal fibroblasts in aged skin and concurrently inducing both type I and type III collagen synthesis, it may prove very beneficial to the wound-healing process.

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