Vitamin A Antagonizes Decreased Cell Growth and Elevated Collagen-Degrading Matrix Metalloproteinases and Stimulates Collagen Accumulation in Naturally Aged Human Skin[1]

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Damage to human skin due to ultraviolet light from the sun (photoaging) and damage occurring as a consequence of the passage of time (chronologic or natural aging) are considered to be distinct entities. Photoaging is caused in part by damage to skin connective tissue by increased elaboration of collagen-degrading matrix metalloproteinases, and by reduced collagen synthesis. As matrix metalloproteinase levels are known to rise in fibroblasts as a function of age, and as oxidant stress is believed to underlie changes associated with both photoaging and natural aging, we determined whether natural skin aging, like photoaging, gives rise to increased matrix metalloproteinases and reduced collagen synthesis. In addition, we determined whether topical vitamin A (retinol) could stimulate new collagen deposition in sun-protected aged skin, as it does in photoaged skin. Sun-protected skin samples were obtained from 72 individuals in four age groups: 18–29 y, 30–59 y, 60–79 y, and 80+ y. Histologic and cellular markers of connective tissue abnormalities were significantly elevated in the 60–79 y and 80+ y groups, compared with the two younger age groups. Increased matrix metalloproteinase levels and decreased collagen synthesis/expression were associated with this connective tissue damage. In a separate group of 53 individuals (80+ y of age), topical application of 1% vitamin A for 7 d increased fibroblast growth and collagen synthesis, and concomitantly reduced the levels of matrix-degrading matrix metalloproteinases. Our findings indicate that naturally aged, sun-protected skin and photoaged skin share important molecular features including connective tissue damage, elevated matrix metalloproteinase levels, and reduced collagen production. In addition, vitamin A treatment reduces matrix metalloproteinase expression and stimulates collagen synthesis in naturally aged, sun-protected skin, as it does in photoaged skin. ***Key words: fibroblast/gelatinase/interstitial collagenase/type I procollagen/type II procollagen. J Invest Dermatol 114:480–486, 2000***

Skin becomes thin, dry, pale, and finely wrinkled with the passage of time (Smith *et al*, 1962; Lavker, 1979, 1995; West, 1994). In aging skin, the normal stages of epidermal differentiation are preserved, but epidermal thinning, associated with decreased numbers of keratinocytes, is observed histologically. The dermis also thins in aging skin, the result of reduction in the amount and organization of connective tissue (Smith *et al*, 1962; Lavker, 1979, 1995). Skin connective tissue is comprised primarily of fibrillar collagen bundles and elastic fibers, along with a complex array of proteoglycans and other extracellular matrix molecules. Dermal fibroblasts are imbedded within the matrix (Wenstrup *et al*, 1991). Collagen and elastin impart strength and resiliency to skin, and their degeneration with aging causes skin to become fragile, with easy bruising and loss of youthful appearance.

Ultraviolet (UV) irradiation from the sun damages human skin and causes premature skin aging (photoaging) (Kligman, 1969). Clinically, photoaged skin differs from sun-protected, naturally aged skin by having a thickened and rough appearance, with course wrinkles and mottled pigmentation. A hallmark of photoaged skin (not seen in sun-protected aged skin) is the presence of amorphous elastotic material (Lavker, 1995). Damage to the collagen bundles that constitute the bulk (90% wet weight) of skin connective tissue is another prominent feature of photoaged skin. We have shown that UV irradiation induces synthesis of matrix metalloproteinases (MMP) in human skin *in vivo* (Fisher *et al*, 1996, 1997). We have proposed that MMP-mediated collagen destruction accounts, in large part, for the connective tissue damage that occurs in photoaging. In addition, we have reported that collagen synthesis is reduced in photoaged human skin (Griffiths *et al*, 1993; Talwar *et al*, 1995).

Traditionally, differences rather than similarities between naturally aged and photoaged skin have been emphasized (Smith *et al*, 1962; Lavker, 1979, 1995). We envisioned, however, that collagen damage in natural aging may arise, as it does in photoaging, from elevated MMP expression with a concomitant

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Abbreviations: AP-1, activation protein-1; MMP, matrix metalloproteinases; MMP-1, matrix metalloproteinase-1 (interstitial collagenase); MMP-2, matrix metalloproteinase-2 (72 kDa gelatinase, gelatinase A); MMP-9, matrix metalloproteinase-9 (92 kDa gelatinase, gelatinase B).

[1]The authors declared in writing to have no conflict of interest.

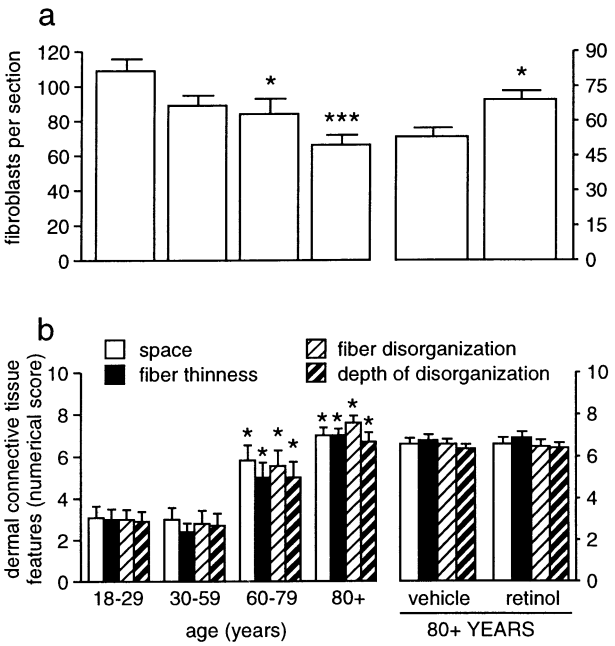


Figure 1. Skin connective tissue alterations increase with increasing age and are partially improved by retinol treatment. (*a*) Histologic sections of skin from persons of varying age were analyzed for the number of fibroblasts in the dermis. Skin samples from n = 11, 10, 11, and 10 persons were analyzed in the four age groups, respectively. *$p < 0.05$ *versus* 18–29 y old group. ***$p < 0.001$ *versus* 18–29 y age group. Fibroblasts were also quantitated in 17 persons 80+ y of age who were treated with 1% retinol and its vehicle for 7 d. *$p < 0.05$ *versus* vehicle-treated skin. (*b*) Degree of skin connective tissue alteration was assessed on a 1–9 scale (1 = least, 9 = most) in histologic sections from persons of varying age as in *A*. *$p < 0.05$ *versus* 18–29 y old group. Connective tissue features were also assessed in 17 persons 80+ y of age who were treated with 1% retinol and its vehicle for 7 d. All values are mean ± SEM.

reduction in collagen synthesis; this was because: (i) both types of skin aging display prominent connective tissue damage; (ii) dermal fibroblasts from aged skin display increased levels of collagenase (Millis *et al*, 1989, 1992; West *et al*, 1989; Burke *et al*, 1994; Bizot-Foulon *et al*, 1995; Ricciarelli *et al*, 1999); and (iii) oxidant stress is thought to play a key part in both photoaging (Brenneisen *et al*, 1998) and natural aging (Sohal and Weindruch, 1996). In this study we report that with increasing age, MMP levels are increased and collagen synthesis is decreased in sun-protected human skin *in vivo*. Furthermore, we find that vitamin A (retinol), which inhibits UV induction of MMP (Fisher *et al*, 1996, 1997) and stimulates collagen synthesis in photoaged skin (Griffiths *et al*, 1993), similarly reduces MMP expression and enhances collagen synthesis in sun-protected, naturally aged skin. These data demonstrate that the pathophysiology of natural skin aging and photoaging share some common underlying mechanisms. These data also demonstrate the potential of topical retinoid therapy for reversing connective tissue-destructive events in natural skin aging just as it does in photoaging.

MATERIALS AND METHODS

Study population The study population consisted of 72 individuals grouped according to age as follows: 18–29 y, 30–59 y, 60–79 y, and 80 y and older. An additional (completely separate) group of 53 individuals, all of whom were 80 y or older, were treated topically for 7 d with 1% retinol and its vehicle (95% ethanol and propylene glycol; 7:3 vol/vol) on different sun-protected buttock skin sites. The retinol and vehicle were applied under occlusion to prevent drug loss and to prevent exposure to light. Replicate 4 mm full-thickness punch biopsies of untreated, or retinol-treated and vehicle-treated sun-protected buttock skin were obtained from each individual. All procedures involving human subjects were approved by the University of Michigan Institutional Review Board, and all subjects provided written informed consent.

Histology and morphometry Sections (5 μm) from formalin-fixed skin samples were stained with hematoxylin and eosin and blinded. The number of cells present in the dermis of each section was determined, taking care not to include cells associated with epithelial structures or capillaries. In selected sections, it was determined that cells which were counted did not stain with antibodies to keratin (epithelial cells) or smooth muscle α-actin (myofibroblasts and smooth muscle cells). The cells thus characterized were operationally defined as fibroblasts, and this term will be used throughout the manuscript to describe the cells. The same sections were scored for four markers of connective tissue alteration: (i) fiber spacing; (ii) fiber thinness; (iii) fiber fragmentation; and (iv) depth of fiber fragmentation, using a scale of 1–9 for each parameter.

Vehicle-treated and retinol-treated skin was also stained with a monoclonal antibody (MIB-1; Immunotech, Westbrook, ME) to the proliferation-associated antigen Ki-67 (Key *et al*, 1993).

Proliferation assays Skin biopsies were cut into small fragments (15–20 fragments per biopsy) and each fragment placed in a well of a 48-well dish. The fragments were incubated for up to 1 mo in Dulbecco's modified minimal essential medium containing nonessential amino acids and 10% fetal bovine serum at 37°C in a humidified atmosphere containing 5% CO_2. The number of tissue fragments from which fibroblasts were isolated (defined as spindle-shaped cells which were reactive with vimentin, but which did not stain with antibodies to keratin or with antibodies to smooth muscle α-actin) was determined, and expressed as a percentage of the total number of tissue fragments incubated. We have previously shown that isolation of fibroblasts from tissue fragments can be used as a reliable means for quantitating growth potential of fibroblasts within the tissue (Varani *et al*, 1994a, b).

MMP assays Skin samples were frozen in liquid nitrogen immediately after collection, and kept frozen at –80°C until used for analyses. Skin samples were crushed under liquid nitrogen in mortar and pestle and homogenized in 20 mM Tris (pH 7.6), 5 mM $CaCl_2$. Insoluble material was removed by centrifugation and the supernatant used as the source of MMP. Collagenase enzyme levels were measured by hydrolysis of [^{3}H]labeled type I fibrillar collagen (Hu *et al*, 1978) after activation for 90 min with 1 mM aminophenyl mercuric acetate. Western blot analysis with antibodies to interstitial collagenase (MMP-1) was performed as described (Fisher *et al*, 1996, 1997). Gelatinase levels (MMP-2; 72 kDa gelatinase and MMP-9; 92 kDa gelatinase) were measured by gelatin zymography (Mulligan *et al*, 1993) and quantitated by scanning laser densitometry. Although we routinely assessed total enzyme levels, active forms of the MMP were always present along with precursor forms. These could be seen in the western blots for MMP-1 and in the zymograms used to assess MMP-2 and MMP-9. Active enzyme forms ranged from less than 10% of the total in some specimens to greater than 75% in others. There were no consistent age-related differences in the percentage of enzyme in the active form. Likewise, there was no consistent effect of retinol treatment on the percentage of enzyme in the active form.

Collagen synthesis Type I procollagen (α1 chain) protein levels were assessed by western blot analysis and by immunohistology as described (Talwar *et al*, 1995). Type III procollagen immunohistology (α1 chain) was performed as described (Griffiths *et al*, 1993) using an antibody from Chemicon International (Temecula, CA). Total collagen biosynthesis by fresh skin samples was assessed by incorporation of [^{14}C]proline into pepsin-resistant, trichloroacetic acid (TCA)-precipitatable material, as described previously (Sykes 1976; Sykes *et al*, 1976; Varani *et al*, 1990). Skin samples that had been freeze-thawed prior to incubation with [^{14}C]proline (to disrupt cells and thereby prevent collagen biosynthesis) served as a control for nonspecific label incorporation. To measure type I procollagen biosynthesis specifically, fresh skin samples were incubated for 24 h in keratinocyte basal medium (Clonetics, Walkersville, MD), supplemented with Ca^{2+} to a final concentration of 1.4 mM. At the end of the incubation period, media were collected and analyzed for type I procollagen protein by enzyme-linked immunosorbent assay (ELISA) (PanVera, Madison, WI).

***In situ* detection of type I procollagen (α1) mRNA** Type I procollagen (α1) gene expression in skin specimens was assessed by *in situ* hybridization. Frozen sections were hybridized with digoxigenin-labeled anti-sense and sense type I procollagen α1 cRNA probes as described previously (Kang *et al*, 1995).

Detection of type III procollagen (α1) mRNA by reverse transcription–polymerase chain reaction (reverse transcription–PCR) Reverse transcription–PCR was used to assess type III procollagen gene expression. Total RNA was isolated from skin

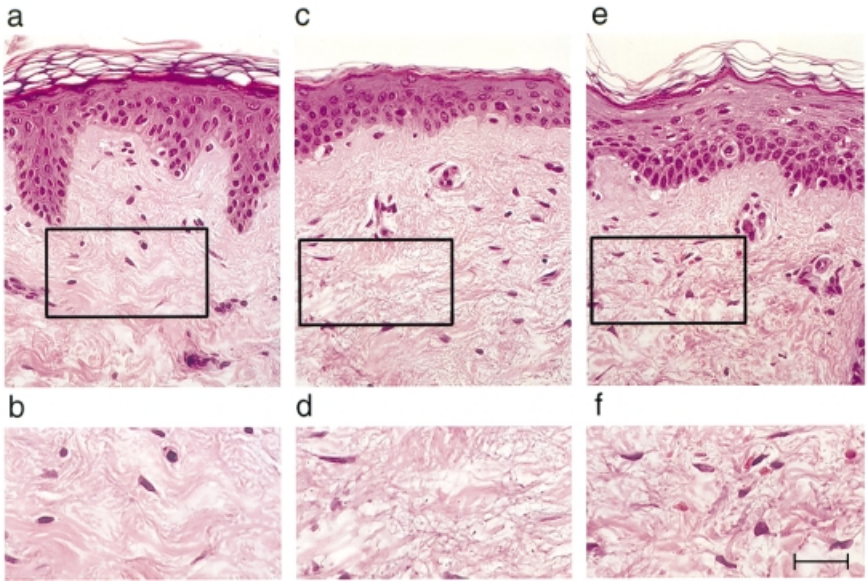


Figure 2. Histologic features of skin connective tissue are altered in aged skin. Representative histology of skin connective tissue seen in sun-protected skin from a young person (22 y old; *a*, *b*) and from 7 d vehicle-treated (*c*, *d*) and retinol-treated (*e*, *f*) skin from an aged person (86 y old). Formalin-fixed skin sections were stained with hematoxylin and eosin. *Scale bar*: (*a*, *c*, *e*) 100 μm; (*b*, *d*, *f*) 50 μm.

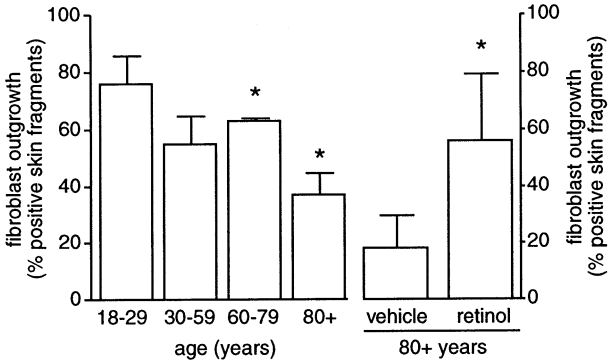


Figure 3. Fibroblast growth potential is reduced with increasing age and is increased with retinol treatment. Freshly obtained skin samples from persons of varying age were cut into small pieces (15–20 pieces/skin sample) and placed in culture to allow outgrowth of fibroblasts from the tissue. Skin from persons in the four age groups (n = 12, 11, 12, and 10, respectively) was analyzed. Data are presented as the percentage of skin pieces from which fibroblasts were isolated. *$p < 0.05$ *versus* 18–29 y old group. Fibroblast outgrowth was also determined in skin samples from 17 persons 80+ y of age who were treated with 1% retinol and its vehicle for 7 d. *$p < 0.05$ *versus* vehicle-treated skin. Values are mean ± SEM.

specimens using Trizol (Life Technologies BRL, Grand Island, NY) followed by extractions with chloroform and isopropanol. First strand cDNA was synthesized using oligo (dT) primers (cDNA Cycle Kit; Invitrogen, Carlsbad, CA). Sequence-specific primers [3′CGAGTA-GGAGCAGTTGGAGG and 5′GCAGGGAA-CAACTTGATGGT] were used to amplify human type III procollagen (α1). Glyceraldehyde-3 phosphatate dehydrogenase [3′CTGCTTCAC-CACCTTCTTGA and 5′TCACCATCTT-CCAGGAGCG] was amplified as an internal control. PCR products were separated on ethidium bromide-impregnated agarose gels and the bands visualized by UV light (Murata *et al*, 1997). Laser densitometry was used for quantitation.

Statistical analysis Data were evaluated using analysis of variance followed by paired group comparisons for studies involving individuals in different age groups, and using paired t tests for comparisons between vehicle-treated and retinol-treated skin. All differences were two-tailed (Woodson, 1987). $p \leq 0.05$ were considered statistically different.

RESULTS

Connective tissue alterations increase with increasing age We assessed the degree of connective tissue damage in sun-protected skin from individuals in each of four age groups (18–29, 30–59, 60–79, and 80+ y). With increasing age, there was progressive loss of dermal fibroblasts (**Fig 1*a***), and increased dermal connective tissue abnormalities, as indicated by increased space between connective tissue fiber bundles, increased thinning of connective tissue fiber bundles, increased disorganization of fiber

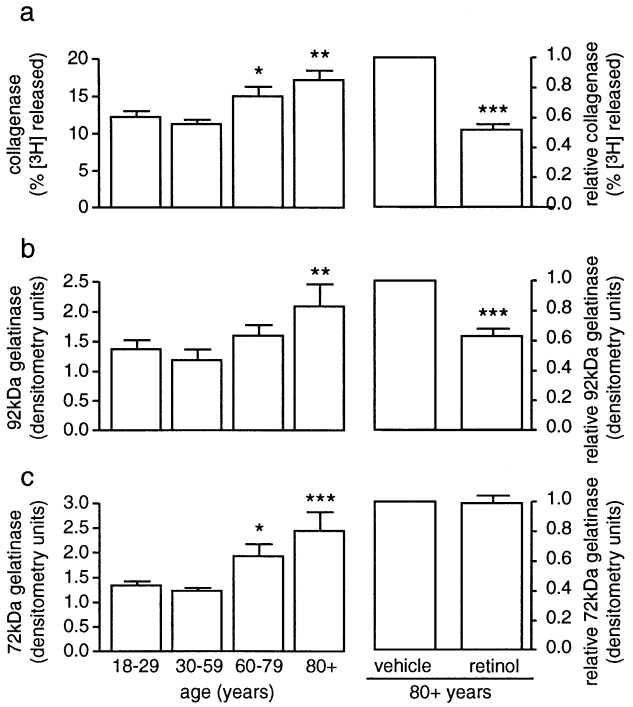


Figure 4. MMP levels are increased in skin with increasing age and partially reduced with retinol treatment. Skin samples of persons of varying age were analyzed for expression of three MMP: (*a*) MMP-1 (interstitial collagenase) (n = 10 persons per age group); (*b*) MMP-9 (92 kDa gelatinase); and (*c*) MMP-2 (72 kDa gelatinase) (n = 10, 11, 8, and 8 persons per age group). Values are mean ± SEM. *$p < 0.05$ *versus* 18–29 y old group. **$p < 0.01$ *versus* 18–29 y old age group. ***$p < 0.001$ *versus* 18–29 y old age group. Skin samples from 16 persons 80+ y of age who were treated with 1% retinol and vehicle for 7 d were also analyzed for the three MMP. Values are mean ± SEM. Values for the vehicle-treated 80+ y old individuals were normalized to 1.0 and the other values expressed relative to the normalized values. ***$p < 0.001$, retinol *versus* vehicle-treated skin.

bundles and increased depth to which disorganization extended (**Fig 1*b***). Dermal cellularity and connective tissue features were similar in the two youngest age groups (18–29 and 30–59). Persons aged 60–79 and 80+ y had significantly reduced dermal cellularity and increased connective tissue abnormalities, compared with persons 18–29 y ($p < 0.05$ for both parameters in the 60–79 y old group and $p < 0.001$ and 0.05 for the same parameters in the 80+ y old group). Topical treatment of sun-protected skin of 80+ y old individuals with retinol for 7 d increased dermal cellularity approximately 25% ($p = 0.009$; $n = 17$) (**Fig 1*a***). Staining of vehicle-treated and retinol-treated skin from 80+ y old individuals with a monoclonal antibody to the proliferation-associated antigen, Ki-67, revealed a significantly higher number of reactive cells in the retinol-treated skin than in skin treated with vehicle alone (20 ± 3 cells in retinol-treated skin *versus* 3 ± 1 cells in vehicle-treated skin ($p < 0.01$; $n = 5$). Although retinol treatment for as little as 7 d induced measurable changes in the dermal fibroblast population, this short-term retinol treatment did not alter age-associated connective tissue abnormalities (**Fig 1*b***). **Figure 2** shows the typical histologic appearance of dermal connective tissue in sun-protected skin of a 22 y old individual, and vehicle-treated and retinol-treated sun-protected skin of an 86 y old individual. Reduced numbers of fibroblasts and alterations in connective tissue structure are apparent in the aged (vehicle-treated) skin relative to the young skin. Also apparent is the increased number of fibroblasts in the aged retinol-treated skin.

Fibroblast growth potential decreases with increasing age and is stimulated with retinol Fibroblast outgrowth from skin fragments was used as a measure of fibroblast growth potential within the tissue (**Fig 3**). Fibroblast outgrowth declined with increasing age. In the 18–29 y old group, fibroblasts were isolated from 182 of 240 tissue fragments ($n = 12$ subjects, 76%). The percentage of tissue fragments from which fibroblasts were obtained decreased with donor age until in the 80+ y old group, fibroblasts were isolated from only 76 of 200 tissue fragments ($n = 10$ subjects, 38%) ($p < 0.05$ compared with the 18–29 y old group). Treatment of 80+ y old individuals with retinol for 7 d increased fibroblast outgrowth greater than 3-fold (46 of 255 tissue fragments or 18% in the vehicle-treated group *versus* 144 of 255 tissue fragments or 56% in the retinol-treated group) ($p < 0.05$; $n = 17$).

MMP levels increase with increasing age and are reduced by retinol We next assessed levels of three connective tissue-degrading MMP, including MMP-1 (interstitial collagenase), MMP-9 (92 kDa gelatinase) and MMP-2 (72 kDa gelatinase), in sun-protected skin as a function of age. All three MMP were elevated in the 80+ y old group, compared with the 18–29 y old age group (approximately 40, 52, and 82% for MMP-1, MMP-9, and MMP-2, respectively) ($p < 0.01$, 0.05, and 0.001) (**Fig 4**). The three MMP were also elevated in the 60–79 y old group (23, 20, and 44%, respectively). Western blot analysis performed on skin samples from a separate group of 18–29 and 80+ y old individuals revealed that MMP-1 protein levels were increased by approximately 40% in skin samples from persons 80+ y of age ($p < 0.02$, $n = 16$), compared with persons 18–29 y of age (**Fig 5**).

MMP-1, MMP-2, and MMP-9 levels were also assessed in retinol-treated and vehicle-treated skin from 80+ y old individuals. Retinol treatment reduced MMP-1 and MMP-9 levels to levels seen in persons 18–29 y old ($p < 0.001$; $n = 16$). In contrast, retinol treatment had no effect on the elevated MMP-2 level in skin of persons in the 80+ y old group (**Fig 4**).

Type I and type III procollagen expression are decreased in aged skin Type I procollagen (α1 chain) protein levels were assessed by western blot analysis in skin samples from persons 18–29 y of age and 80+ y of age. Type I procollagen expression was decreased by 52% in aged skin, compared with skin from younger persons ($n = 16$, $p = 0.022$) (**Fig 6*a***). Immunohistology of type I procollagen revealed prominent extracellular staining in the dermis, adjacent to the dermoepidermal junction, in skin of persons 18–29 y of age. This staining was substantially reduced in skin of persons 80+ y of age (**Fig 6*b***). We also performed immunohistology of type III procollagen (α1 chain). In skin from young persons, type III procollagen was found associated with

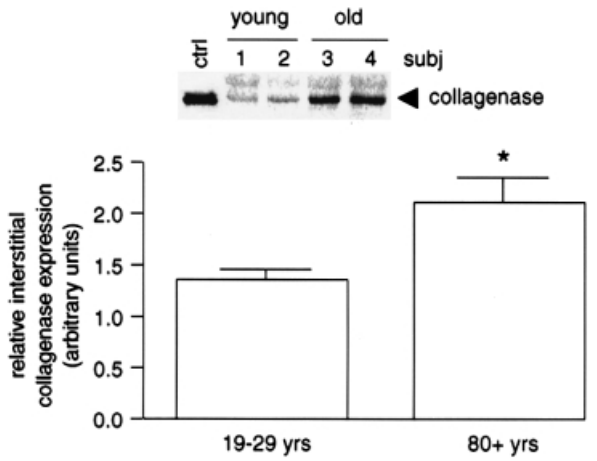


Figure 5. MMP-1 protein level is increased in skin from 80 + y old individuals compared with 18–29 y old individuals. MMP-1 protein levels were assessed by western blot analysis in 16 individuals between 18 and 29 y of age and in 16 individuals 80+ y of age. Values are mean ± SEM. *$p < 0.02$ *versus* 18–29 y old individuals.

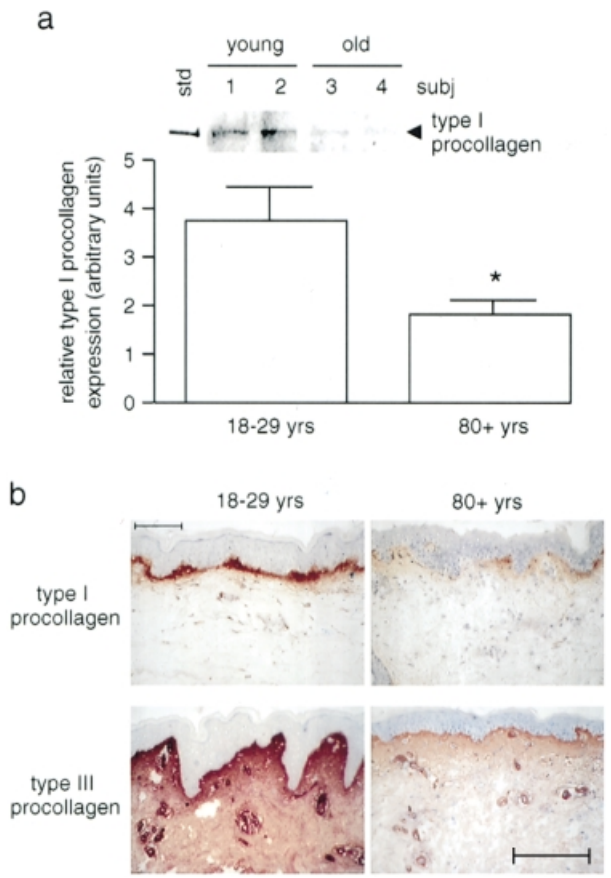


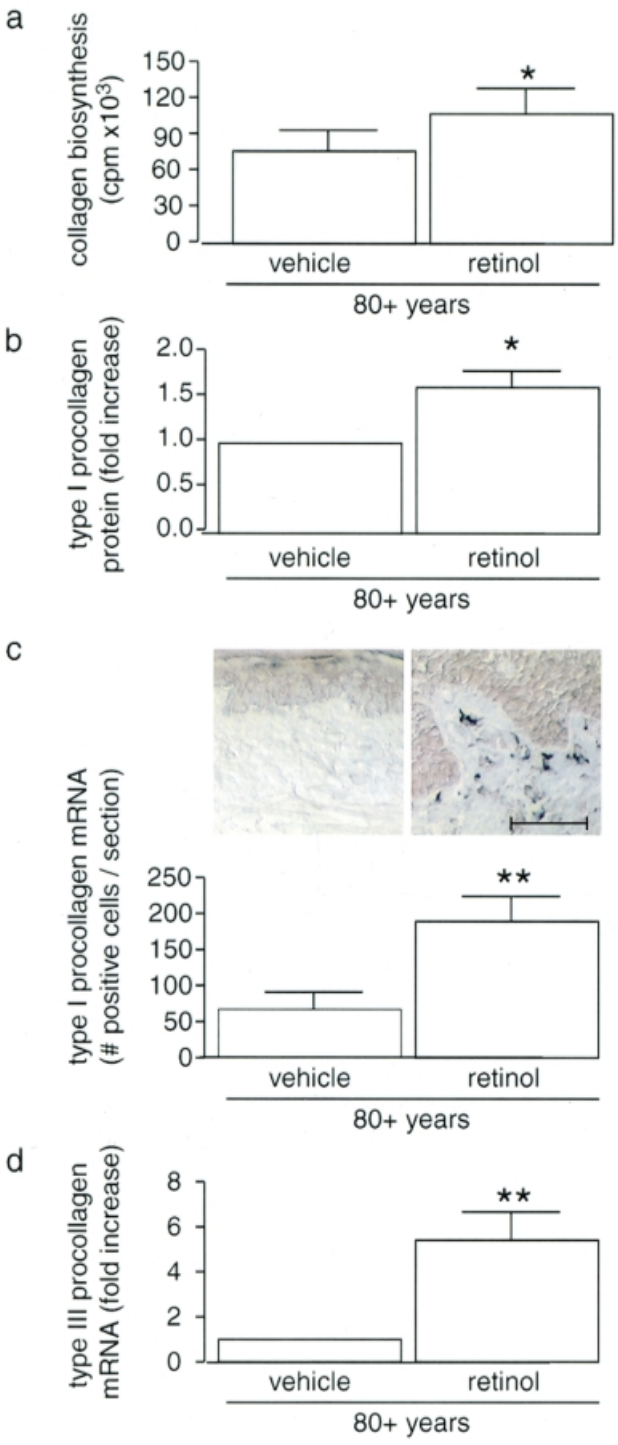
Figure 6. Type I and type III procollagen levels are decreased in aged skin. (*a*) Type I procollagen (α1 chain) levels in skin samples from 18 to 29 y old persons and 80+ y old persons ($n = 16$ per age group) were determined by western blot analysis. Values are mean ± SEM. *$p < 0.001$ *versus* 18–29 y old individuals. (*b*) Representative immunohistology of type I procollagen (α1 chain) and type III procollagen (α1 chain) in skin from young and aged persons. *Scale bar*: 200 μm.

mature collagen fibers throughout the dermis. This dermal staining was considerably reduced in the skin of aged persons (**Fig 6*b***).

Collagen synthesis in skin of 80+ y old individuals is stimulated by retinol As described above, sun-protected skin of persons in the 80+ y old age group contains reduced numbers of fibroblasts, with diminished growth potential, increased MMP levels, and reduced expression of type I and III procollagen. These properties would be expected to cause a deficit in connective tissue collagen. The finding that 7 d retinol treatment substantially restored fibroblast numbers and growth potential, and reduced MMP levels suggests that retinol might increase collagen content of aged skin. We therefore assessed collagen biosynthesis in vehicle-treated and retinol-treated skin from 80+ y old individuals by three methods: (i) *ex vivo* incorporation of [^{14}C]proline into total collagen; (ii) *ex vivo* secretion of type I procollagen protein; and (iii) *in vivo* expression of type I and type III procollagen mRNA. As shown in **Fig 7(*a*)**, collagen biosynthesis, measured by [^{14}C]proline incorporation, was increased 1.4-fold in skin treated with retinol relative to skin treated with vehicle ($p = 0.03$; $n = 9$). Analysis of the radioactive collagen fraction by sodium dodecylsulfate–polyacrylamide gel electrophoresis revealed that the α1 and α2 chains of type I collagen accounted for the majority of the [^{14}C]proline incorporation (not shown).

We also utilized an ELISA procedure to measure production of type I procollagen by retinol-treated and vehicle-treated skin samples during a 24 h period. Skin samples from 80+ y old persons that had been treated *in vivo* with retinol for 7 d produced 65% more type I procollagen than matched vehicle-treated skin ($p < 0.05$; $n = 9$) (**Fig 7*b***). This increase is consistent with the increase in collagen synthesis measured by [^{14}C]proline incorporation (**Fig 7*a***).

In addition, we examined type I procollgen (α1 chain) gene expression by *in situ* hybridization in retinol-treated and vehicle-treated skin of 80+ y old persons. The number of cells expressing detectable levels of type I procollagen mRNA was approximately



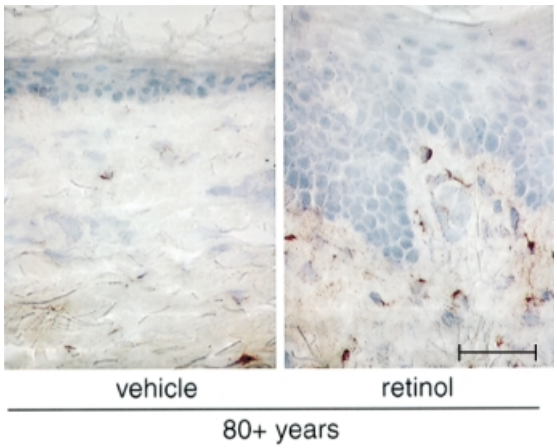


Figure 8. Retinol treatment increases type I procollagen expression in fibroblasts from aged skin. Representative immunohistologic staining of type I procollagen (α1 chain) in vehicle-treated and retinol-treated skin from an 86 y old person. Tissue was frozen in OCT and stained by the immunoperoxidase method; *scale bar*: 100 μm. Data are representative of $n = 6$.

Figure 7. Retinol treatment increases collagen synthesis in aged (80 + y) skin. (*a*) Freshly obtained skin samples from nine persons treated with 1% retinol and its vehicle for 7 d were incubated for 24 h with [^{14}C]proline. The amount of radioactivity (counts per minute, cpm) incorporated into the collagen fraction was determined. Values are mean ± SEM. *$p < 0.05$ *versus* vehicle-treated skin. (*b*) Freshly obtained skin samples from nine persons treated with 1% retinol and its vehicle for 7 d were incubated in culture medium for 24 h. The amount of type I procollagen secreted into the medium was determined by ELISA. Values from vehicle-treated skin were assigned a number of 1.0 and the values from corresponding retinol-treated skin normalized to this value. Values are mean ± SEM. *$p < 0.05$ *versus* vehicle-treated skin. (*c*) Type I procollagen α1 gene expression was measured by *in situ* hybridization in frozen skin sections from seven persons treated with 1% retinol and its vehicle for 7 d. The number of cells expressing detectable type I procollagen α1 mRNA in three sections from each skin sample were counted. **$p < 0.01$ *versus* vehicle-treated skin. (*Insert*: Vehicle-treated and retinol-treated skin from an 87 y old person; *scale bar*: 150 μm. (*d*) Type III procollagen α1 gene expression was measured in skin samples from seven persons treated with 1% retinol and its vehicle for 7 d by reverse transcription–PCR. The values of the vehicle-treated skin were assigned a number of 1.0, and values from corresponding retinol-treated skin normalized to this value. Values are mean ± SEM. **$p < 0.01$ *versus* vehicle-treated skin.

2.8-fold higher in retinol-treated skin, compared with vehicle-treated skin ($p = 0.003$; $n = 7$) (**Fig 7*c***). The majority of fibroblasts that were positive for type I procollagen mRNA were in the upper dermis, immediately below the dermoepidermal junction (**Fig 7*c***, *insert*). Consistent with these findings, type I procollagen was detectable in the dermal fibroblasts from retinol-treated skin, whereas staining for type I procollagen was not evident in vehicle-treated tissue from the same individuals (**Fig 8**).

Finally, we utilized semiquantitative reverse transcription–PCR to assess type III procollagen mRNA levels in retinol-treated and vehicle-treated skin of 80+ y old persons. Type III procollagen mRNA levels were 5-fold higher in retinol-treated skin samples, compared with vehicle-treated skin ($p = 0.0015$; $n = 7$) (**Fig 7*d***).

DISCUSSION

A number of changes occur in the structure of skin connective tissue as a consequence of the natural aging process. Age-related changes include a decrease in the number of interstitial fibroblasts, a thinning of connective tissue (collagen) fiber bundles, an increase in space between connective tissue fiber bundles, and an increase in histologically observable connective tissue disorganization. These changes are evident in many of the individuals between the ages of 60 and 79 y, and are present in virtually every individual 80 y old or older. It is thought that these alterations in the dermal connective tissue are largely responsible for the thin, fragile, and finely wrinkled quality of naturally aged skin (Lovell *et al*, 1987; Miyahara *et al*, 1992).

What accounts for these changes in the structure of sun-protected skin during chronologic aging? This study provides direct evidence that there is reduced fibroblast proliferation (as indicated in the *ex vivo* growth assay), increased MMP expression (as assessed by collagen degradation, gelatin zymography, and western blotting) and reduced elaboration of new collagen (mRNA and protein). Previous studies using cells in monolayer cultures have demonstrated reduced proliferative capacity of dermal fibroblasts with age (Plisko and Gilchrest, 1983; Gilchrest, 1983; Stanulis-Praeger and Gilchrest, 1986; Sauder *et al*, 1988). Other *in vitro* studies have shown that MMP expression by skin cells increases as they age (Millis *et al*, 1989, 1992; West *et al*, 1989; Burke *et al*, 1994; Bizot-Foulon *et al*, 1995; Ricciarelli *et al*, 1999), and still other *in vitro* studies have demonstrated decreased collagen synthesis in aged fibroblasts (Johnson *et al*, 1986; Gregory *et al*, 1986; Mays *et al*, 1990; Furth, 1991).

This study also demonstrates that these changes observed in aged skin may be partially reversed by treatment with topical retinol (vitamin A). Treatment of aged, sun-protected skin with 1% retinol for only 7 d resulted in increased numbers of fibroblasts in the skin. In parallel, topical treatment with 1% retinol for 7 d increased fibroblast growth from tissue specimens, reduced collagenase, and gelatinase expression in these same tissues and stimulated new collagen synthesis.

How retinol acts to reverse age-associated changes in aged skin *in vivo* is not fully understood. With regard to MMP expression, we recently demonstrated that UV irradiation upregulates transcription factor AP-1 and its target genes, MMP-1 and MMP-9, in human skin *in vivo* (Fisher *et al*, 1996, 1997). MMP-2 gene expression is not regulated by AP-1 and is not induced by UV in human skin. *All-trans* retinoic acid, which is formed from retinol in human skin (Kang *et al*, 1995), was found to inhibit AP-1 activation as well as MMP-1 and MMP-9 gene expression (Fisher *et al*, 1996, 1997). Expression of MMP-2 was not inhibited by *all-trans* retinoic acid in photoaged skin (Fisher *et al*, 1996), nor by retinol in naturally aged skin (this study), consistent with the fact that its promoter lacks an AP-1 site. Elevated levels of MMP-1 and MMP-9 in aged skin *in vivo* and the reduction in both activities by retinol are consistent with AP-1 activation during aging and with retinol repression of AP-1 activation. In photoaged skin, oxidant stress is thought to play a part in the signaling events that lead to MMP upregulation (Brenneisen *et al*, 1998). Oxidant stress in the natural aging process (Sohal and Weindruch, 1996) may, likewise, lead to MMP induction.

MMP inhibition by topical retinol could suppress degradation of newly synthesized procollagen, and thereby lead to enhanced procollagen expression. Alternatively, new collagen biosynthesis may be directly stimulated by retinol. Whereas [^{14}C]proline incorporation experiments and ELISA results cannot distinguish between decreased breakdown and increased synthesis of collagen, the existence of elevated mRNAs for both type I and type III procollagen in retinol-treated skin strongly argues that new collagen synthesis is at least partially responsible for the enhanced procollagen levels. This increased procollagen production could occur as a result of fibroblast activation. Past studies have shown that treatment of human skin in organ culture with *all-trans* retinoic acid increases overall protein synthesis and the production of several components of the extracellular matrix (Varani *et al*, 1993, 1994a). As another possibility, retinol-induced proliferation of fibroblasts in aged skin could result in increased collagen production simply as a secondary consequence of the presence of additional collagen-producing cells. Ultimately, these possibilities are not mutually exclusive.

The findings presented here are of interest from a number of standpoints. First, they indicate that there is significant overlap in the pathophysiology of natural skin aging and sun-induced premature skin aging (photoaging), although the etiologies are different. Equally important, our findings suggest that naturally aged, sun-protected skin and photoaged skin respond to topical retinoid treatment in an analogous manner. It has been established that topical retinoid treatment can partially reverse some of the clinical and histologic features associated with sun-induced premature skin aging (Kligman *et al*, 1986; Weiss *et al*, 1988). Recently, it has been shown in a small, clinical study that topical retinoid treatment can also improve the clinical appearance of aged, sun-protected skin. Although epidermal changes were the major focus of this study, cytologic evidence of fibroblast activation in the retinoid-treated skin was noted (Kligman *et al*, 1993). The ability of retinoids to induce the repair of connective tissue damage in naturally aged skin suggests that topical retinoid treatment might be useful for treating and preventing the thin, fragile skin of aged persons. If this proves to be the case, retinol (and other retinoids) may be beneficial for long-term use in aged populations. This will, of course, need to be established in controlled clinical studies where drug dosage, formulation, and application conditions are all carefully considered and evaluated.

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REFERENCES

Bizot-Foulon V, Bouchard B, Hornebeck W, Dubertret L, Bertaux B: Uncoordinate expressions of type I and III collagens, collagenase and tissue inhibitor of matrix metalloproteinase 1 along the *in vitro* proliferative lifespan of human skin fibroblasts: regulation by *all-trans* retinoic acid. *Cell Biol Int* 19:129–135, 1995

Brenneisen P, Wenk J, Klotz LO, *et al*: Central role of ferrous/ferric iron in the ultraviolet B irradiation-mediated signaling pathway leading to increased interstitial collagenase (MMP-1) and stromelysin-1 (MMP-3) mRNA levels in cultured human dermal fibroblasts. *J Biol Chem* 273:5279–5287, 1998

Burke EM, Horton WE, Pearson JD, Crow TM, Martin GR: Altered transcriptional regulation of human interstitial collagenase in cultured skin fibroblasts from older donors. *Exp Gerontol* 29:37–53, 1994

Fisher GJ, Datta SC, Talwar HS, Wang ZQ, Varani J, Kang S, Voorhees JJ: The molecular basis of sun-induced premature skin ageing and retinoid antagonism. *Nature* 379:335–338, 1996

Fisher GJ, Wang Z-Q, Datta SC, Varani J, Kang S, Voorhees JJ: Pathophysiology of premature skin aging induced by ultraviolet light. *N Engl J Med* 337:1419–1428, 1997

Furth JJ: The steady-state levels of type I collagen mRNA are reduced in senescent fibroblasts. *J Gerontol* 46:B1224–B1225, 1991

Gilchrest BA: In vitro assessment of keratinocyte aging. *J Invest Dermatol* 81(Suppl. 1):184s–189s, 1983

Gregory C, Sephel BZ, Davidson JM: Elastin production in human skin fibroblast cultures and its decline with age. *J Invest Dermatol* 86:279–285, 1986

Griffiths CEM, Russman G, Majmudar G, Singer RS, Hamilton TA, Voorhees JJ: Restoration of collagen formation in photodamaged human skin by tretinoin (retinoic acid). *N Engl J Med* 329:530–534, 1993

Hu C-L, Crombie G, Franzblau C: A new assay for collagenolytic activity. *Anal Biochem* 88:638–645, 1978

Johnson BD, Page RC, Narayanan AS, Pieters HP: Effects of donor age on protein and collagen synthesis *in vitro* by human diploid fibroblasts. *Lab Invest* 55:490–496, 1986

Kang S, Duell EA, Fisher GJ, *et al*: Application of retinol to human *skin in* vivo induces epidermal hyperplasia and cellular retinoid-binding proteins characteristic of retinoic acid but without measurable retinoic acid levels or irritation. *J Invest Dermatol* 105:549–556, 1995

Key G, Becker MH, Baron B, Duchrow M, Schluter C, Flad HD, Gerdes J: New Ki-67-equivalent murine monoclonal antibodies (MIB1–3) generated against bacterially expressed parts of the Ki-67 cDNA containing three 62 base pair repetitive elements encoding for the Ki-67 epitope. *Lab Invest* 68:629–636, 1993

Kligman AM: Early destructive effects of sunlight on human skin. *JAMA* 210:2377–2380, 1969

Kligman AM, Grove GL, Hirose H, Leyden JJ: Topical tretinoin for photoaged skin. *J Am Acad Dermatol* 15:836–859, 1986

Kligman AM, Dogadkina D, Lavker RM: Effects of topical tretinoin on non-sun-exposed skin of the elderly. *J Am Acad Dermatol* 29:25–33, 1993

Lavker RM. Cutaneous aging: chronologic versus photoaging. In: Gilchrest BA (ed.). *Photoaging*. Cambridge, MA: Blackwell Science, 1995, pp 123–135

Lavker RM: Structural alterations in exposed and unexposed aged skin. *J Invest Dermatol* 73:559–566, 1979

Lovell CR, Smolenski KA, Duance VC, Light ND, Young S, Dysons M: Type I and III collagen content in normal human skin during ageing. *Br J Dermatol* 117:419–428, 1987

Mays PK, McAnuly RI, Campa JS, Cambray AD, Laurent. GJ: Similar age-related alterations in collagen metabolism in rat tissues *in vivo* and fibroblasts *in vitro*. *Biochem Soc Trans* 18:957, 1990

Millis AJ, Sottile TJ, Hoyle M, Mann DM, Diemer V: Collagenase production by early and late passage cultures of human fibroblasts. *Exp Gerontol* 24:559–575, 1989

Millis AJ, Hoyle TM, McCue HM, Martini H: Differential expression of metalloproteinase and tissue inhibitor of metalloproteinase genes in aged human fibroblasts. *Exp Cell Res* 201:373–379, 1992

Miyahara T, Murai A, Tanaka T, Shiozawa S, Kameyama M: Age-related differences in human skin collagen: solubility in solvent, susceptibility to pepsin digestion and the spectrum of solubilized polymeric collagen molecules. *J Gerontol* 37:651–655, 1992

Mulligan MS, Desrochers PE, Chinnaiyan AE, Gibbs DF, Varani J, Johnson KJ, Weiss SJ: In vivo suppression of immune complex-induced alveolitis by secretory leukoproteinase inhibitor and tissue inhibitor of metalloproteinse-2. *Proc Natl Acad Sci USA* 90:11523–11527, 1993

Murata J, Ayukawa K, Ogasawara M, Fujii H, Saiki I: A melanocyte stimulating hormone blocks invasion of reconstituted basement membrane by murine B16 melanoma cells. *Invasion Metastasis* 17:82–93, 1997

Plisko A, Gilchrest BA: Growth factor responsiveness of cultured human fibroblasts declines with age. *J Gerontol* 38:513–518, 1983

Ricciarelli R, Maroni P, Ozer N, Zingg J-M, Azzi A: Age-dependent increase of collagenase expression can be reduced by α-tocopherol via protein kinase C inhibition. *Free Radic Biol Med* 27:729–737, 1999

Sauder DN, Stanulis-Praeger MM, Gilchrest BA: Autocrine growth stimulation of human keratinocytes by epidermal cell-derived thymocyte-activating factor: implications for skin aging. *Arch Dermatol Res* 280:71–76, 1988

Smith JG, Davidson EA, Clark WM: Alterations in human dermal connective tissue with age and chronic sun damage. *J Invest Dermatol* 39:347–356, 1962

Sohal RS, Weindruch R: Oxidative stress, caloric restriction and aging. *Science* 273:59–63, 1996

Stanulis-Praeger BM, Gilchrest BA: Growth factor responsiveness declines during adulthood for human skin-derived cells. *Mech Aging Dev* 35:185–198, 1986

Sykes BC. The separation of two soft tissue collagens by covalent chromatography. *FEBS Lett* 61:180–185, 1976

Sykes BC, Puddle B, Francis M, Smith R: The estimation of two collagens from human dermis by interrupted gel electrophoresis. *Biochem Biophys Res Commun* 72:1472–1480, 1976

Talwar H, Griffiths CEM, Fisher GJ, Hamilton TA, Voorhees JJ: Reduced type I and type III procollagens in photodamaged adult human skin. *J Invest Dermatol* 105:285–291, 1995

Varani J, Mitra RS, Gibbs D, Phan SH, Nickoloff BJ, Voorhees JJ: All-*trans* retinoic acid stimulates extracellular matrix production in growth-inhibited cultured human skin fibroblasts. *J Invest Dermatol* 794:717–723, 1990

Varani J, Larson BK, Perone P, Inman DR, Fligiel SEG, Voorhees JJ: All-*trans* retinoic acid and extracellular Ca2+ differentially influence extracellular matrix production by human skin in organ culture. *Am J Pathol* 142:1813–1822, 1993

Varani J, Perone P, Griffiths CEM, Inman DR, Fligiel SEG, Voorhees JJ: All-*trans* retinoic acid (RA) stimulates events in organ-cultured human skin that underlie repair. *J Clin Invest* 94:1747–1753, 1994a

Varani J, Perone P, Fligiel SEG, Inman DR, Voorhees JJ: *All-trans* retinoic acid preserves viability of fibroblasts and keratinocytes in full-thickness human skin and fibroblasts in isolated dermis in organ culture. *Arch Dermatol Res* 286:443–447, 1994b

Weiss JS, Ellis CN, Headington JT, Tincoff T, Hamilton TA, Voorhees JJ: Topical tretinoin improves photoaged skin: a double-blind, vehicle-controlled study. *JAMA* 259:527–232, 1988

Wenstrup RJ, Murad S, Pinnell SR. Collagen. In: Goldsmith LA (ed.). *Physiology Biochemistry and Molecular Biology of the Skin*. New York: Oxford University Press, 1991, pp 481–508

West MD: The cellular and molecular biology of skin aging. *Arch Dermatol* 130:87–92, 1994

West MD, Pereira-Smith O, Smith JR: Replicative senescence of human skin fibroblasts correlates with a loss of regulation and overexpression of collagenase activity. *Exp Cell Res* 184:138–147, 1989

Woodson RF: *Statistical Methods for the Analysis of Biomedical Data*. New York: John Wiley, 1987, pp 314–363