

Decreased Collagen Production in Chronologically Aged Skin

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This study investigates if collagen production is decreased with age, and if so, what causes this decrease in collagen.

Conclusion

Collagen synthesis is lower in aged skin due to lower amounts of fibroblasts (collagen synthesizing cells) and their diminished ability to synthesize collagen.

Collagen fibers in elderly skin become fragmented and less linear.

Fibroblast size decreases with age, with most of the size decreases occurring by decreases in cytosol diminishment.

The proposed mechanism for aging skin, beyond lower innate collagen synthesis, is decreased fibroblast ability to attach the extracellular matrix and be mechanically stimulated to produce more collagen, and instead, increases proteinase production and secretion.

Amendments

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Cell Injury, Repair, Aging and Apoptosis

Decreased Collagen Production in Chronologically Aged Skin

Roles of Age-Dependent Alteration in Fibroblast Function and Defective Mechanical Stimulation

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Reduced synthesis of collagen types I and III is characteristic of chronologically aged skin. The present report provides evidence that both cellular fibroblast aging and defective mechanical stimulation in the aged tissue contribute to reduced collagen synthesis. The reduction in collagen synthesis due to fibroblast aging was demonstrated by a lower *in vitro* production of type I procollagen by dermal fibroblasts isolated from skin of young (18 to 29 years) versus old (80+ years) individuals (82 ± 16 versus 56 ± 8 ng/ml; *P* < 0.05). A reduction in mechanical stimulation in chronologically aged skin was inferred from morphological, ultrastructural, and fluorescence microscopic studies. These studies, comparing dermal sections from young and old individuals, demonstrated a greater percentage of the cell surface attached to collagen fibers (78 ± 6 versus 58 ± 8%; *P* < 0.01) and more extensive cell spreading (1.0 ± 0.3 vs. 0.5 ± 0.3; *P* < 0.05) in young skin compared with old skin. These features are consistent with a lower level of mechanical stimulation on the cells in old versus young skin. Based on the findings presented here, we conclude that reduced collagen synthesis in chronologically aged skin reflects at least two different underlying mechanisms: cellular fibroblast aging and a lower level of mechanical stimulation. (*Am J Pathol* 2006; 168:1861–1868; DOI: 10.2353/ajpath.2006.051302)

Reduction of fibrillar (types I and III) collagen is a characteristic feature of chronologically aged skin and is en-

hanced in photodamage. This has been well described using histological and ultrastructural approaches in the past,^{1–6} and our own recent studies have documented this biochemically in both chronological aging⁸ and photoaging.⁷ Collagen-degrading matrix metalloproteinases (MMPs) are up-regulated in skin by UV radiation.^{8,9} Repeated induction of these enzymes by exposure to solar radiation over years or decades is likely responsible for producing collagen fragmentation in sun-damaged skin. During natural or chronological aging of the skin, the same MMPs that are up-regulated acutely in response to UV radiation are gradually increased. This has been observed in monolayer culture with cells obtained from old versus young subjects,^{10–14} and our own studies have shown gradual up-regulation of MMPs in intact (old versus young) skin.¹⁵

Although destruction of existing collagen is, undoubtedly, central to the deleterious changes observed in aged/photoaged skin, failure to replace damaged collagen with newly synthesized material is also critical to the overall pathophysiology. There is a sustained down-regulation in collagen synthesis in photodamaged skin relative to what occurs in healthy sun-protected skin¹⁶ and in chronologically aged, sun-protected skin compared with what is seen in young skin.¹⁵

Mechanisms underlying the loss of collagen synthesis in photodamaged skin and chronologically aged skin have not been fully delineated. In a recent series of studies, we demonstrated that in severely photodamaged skin, the presence of fragmented collagen in the dermis inhibited collagen synthesis. Based on results from a variety of *in vitro* and *in vivo* approaches, we

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MMPs are enzymes that degrade proteins (like collagen).

The sun radiation increases the expression/formation of these enzymes (MMPs)

In aged skin, this MMP expression is further increased by sun radiation.

Not only is the increased degradation of skin collagen deleterious to skin, but the speed at which it is replaced/synthesized could also play a role.

concluded that damaged collagen did not support a level of mechanical tension on resident fibroblasts necessary for efficient collagen synthesis.^{6,7,17,18} Whether a similar mechanism contributes to decreased collagen production in chronologically aged skin, and if so, to what extent it is responsible for the overall collagen reduction observed in aged skin, is not known. These issues are addressed in the present study.

Materials and Methods

Skin Biopsies

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For this study, individuals between the ages of 18 and 29 years (young cohort) and individuals 80 years or older (old cohort) were recruited. Replicate 2- and/or 4-mm punch biopsies of sun-protected hip skin were obtained from each individual. The 4-mm punches were used for fluorescence microscopic and ultrastructural analysis and for assessment of type I procollagen levels. On arrival in the laboratory, one of the 4-mm biopsies was immediately frozen in optimal cutting temperature medium (OCT), and the other piece was fixed in glutaraldehyde. The 2-mm punch biopsies were used for routine histology at the light microscopic level and as a tissue source for the isolation of dermal fibroblasts. For routine histology, the biopsies were fixed in 10% buffered formalin. Fibroblast isolation was accomplished as indicated below. All procedures involving human subjects were approved by the Institutional Review Board, and biopsies were obtained after receiving informed consent.

Human Dermal Fibroblasts in Monolayer Culture

Fibroblasts were isolated from skin biopsies as described previously.¹⁵ Briefly, the biopsy was minced with a scissors and forceps, and tissue fragments were transferred to individual wells of a 24-well dish. Dulbecco's modified minimal essential medium supplemented with nonessential amino acids and 10% fetal bovine serum (DMEM-FBS) was used as culture medium. Only a minimal amount of medium was included so that tissue pieces would adhere to the plastic surface. The dishes were maintained at 37°C in an atmosphere of 95% air and 5% CO₂. Eventually, fibroblast proliferation around the edge of some of the tissue fragments was observed. When cells in sufficient number were available, they were harvested with trypsin/ethylenediamine tetraacetic acid (EDTA) and grown in monolayer culture using DMEM-FBS. Cells were subcultured by exposure to trypsin/EDTA one or two times and then used in experiments.

Type I Procollagen

Serial frozen sections were prepared from OCT-embedded skin biopsies as follows: 7, 200, and 7 μm. After hematoxylin and eosin staining, the areas of the sections from both ends of the 200-μm sample were measured using Image ProPlus software to calculate the volume of the 200-μm sample. Soluble protein extracts were pre-

pared from the 200-μm samples, which were homogenized in ice-cold extraction buffer (50 mmol/L Tris-HCl, pH 7.4, 0.15 mol/L NaCl, 1% Triton X-100, and protease inhibitors [Complete Mini, Hoffmann-LaRoche, Nutley, NJ]), and vortexed in the presence of glass beads (Bio-spec, Bartlesville, OK). After centrifugation for 10 minutes at 10,000 × g and 4°C, supernatants were assayed for procollagen I using a commercial enzyme-linked immunosorbent assay kit (Panvera, Madison, WI) as described previously.¹⁵ The procollagen assay uses an antibody to the C-terminal propeptide region that is part of the collagen molecule as it is synthesized and secreted (before being proteolytically cleaved). As such, this assay is a measure of newly synthesized collagen. Type I procollagen concentrations were normalized to the volume of tissue used for the preparation of each sample.

Type I procollagen production was also assessed in dermal fibroblasts in monolayer culture. Fibroblasts were seeded at 4 × 10⁴ cells per well in 24-well plates using DMEM-FBS as culture medium. The cells were allowed to attach overnight. The next day, they were washed and then incubated in keratinocyte growth medium (Cambrex Bioscience, Walkersville, MD) supplemented with 1.4 mmol/L Ca²⁺. Keratinocyte growth medium is a serum-free, low-Ca²⁺ modification of MCDB-153 medium supplemented with epidermal growth factor (EGF), insulin, hydrocortisone, and pituitary extract. Cell numbers were determined 2 days later by releasing the cells with trypsin/EDTA and enumerating them using a particle counter (Coulter Electronics, Hiialeah, FL). At the time of harvest, the serum-free culture fluids were collected and assessed for type I procollagen using the same enzyme-linked immunosorbent assay procedure and then normalized to cell number.

Immunostaining and Confocal Microscopy

A mouse monoclonal antibody to vinculin was obtained from Chemicon (Temecula, CA). The primary antibody was visualized with rabbit anti-mouse IgG antibody bound to Alexa Fluor 488 (Invitrogen, Carlsbad, CA) and further amplified with Alexa Fluor 488 goat anti-rabbit IgG. Alexa Fluor 546-phalloidin (Invitrogen) was used as a probe for actin. Nuclei were counterstained with the nuclear dye 4',6-diamidino-2-phenylindole, dihydrochloride (DAPI) (Prolong Gold; Invitrogen). (Note that Alexa Fluor 488 is spectrally similar to fluorescein, whereas Alexa Fluor 546 is spectrally similar to rhodamine.)

OCT-embedded frozen skin biopsies from young and old individuals were used for staining. Briefly, the frozen tissue sections were fixed with 4% formaldehyde for 20 minutes. After fixation, the tissue sections were washed twice with wash buffer (0.05% Tween 20 in Dulbecco's phosphate-buffered saline), followed by permeabilization with 0.1% Triton X-100 for 10 minutes. Tissue sections were again washed and then exposed to a blocking solution consisting of 1% bovine serum albumin in Dulbecco's phosphate-buffered saline for 30 minutes. Next, the sections were treated with anti-vinculin antibody in blocking solution for 1 hour. After

1. Young (18-29 years) were compared against an Old (80+ years) group by taking skin biopsies

three subsequent washing steps (5 minutes each), each sample was treated with Alexa Fluor 488-conjugated secondary antibody in blocking solution and incubated for 45 minutes. Sections were simultaneously stained for actin expression using Alexa Fluor 546-phalloidin along with the secondary antibody. After three additional washing steps (5 minutes each), tissue sections were treated for 30 minutes with the amplification antibody (Alexa Fluor 488 goat anti-rabbit IgG). This was followed by two additional washing steps and treatment with DAPI (nuclei counterstain) for 3 minutes. After three final washing steps, tissue sections were rinsed once with water. Coverslips were mounted onto the microscope slides with Prolong Anti-Fade (Invitrogen). Stained tissue sections were examined by fluorescence microscopy. Microscopy was performed on a Zeiss LSM 510 confocal microscope using a 63x (C-Apochr) water immersion objective lens (numerical aperture (NA) = 1.2). Laser excitation wavelengths included 364, 488, and 543 nm scanned in sequence by the line method.

Light Microscopic and Transmission Electron Microscopic Studies

Skin biopsies were fixed overnight in 2% glutaraldehyde in 0.1 mmol/L cacodylate buffer (Sigma, St. Louis, MO) at pH 7.4. Glutaraldehyde-fixed specimens were treated with 2% osmium tetroxide buffered in 0.1 mmol/L cacodylate buffer. Specimens were dehydrated with graded ethanol to 2 x 100% ethanol and 2x propylene oxide (EM Sciences). The samples were embedded in pure epon resin. One-micrometer tissue sections were cut, stained with toluidine blue, and examined at the light microscopic level. Surface area occupied by individual cells was assessed quantitatively using NIH Image software on 1-µm sections obtained from glutaraldehyde-fixed, plastic-embedded tissue.

The same tissue sections used for quantification of surface area at the light microscopic level were also used to identify areas of interest for transmission electron microscopy. Ultrathin sections were cut from areas of interest, stained with lead citrate and uranyl acetate (both from EM Sciences), and observed using a Phillips 400 transmission electron microscope. Photographs were made from several areas of each specimen. Using the high-resolution photographs, interstitial cells were quantitatively evaluated for percentage of the cell boundary in contact with individual collagen fibrils or collagen fibril bundles. Although it is difficult to identify interstitial fibroblasts with 100% accuracy, obvious contaminants (mast cells, cells in vascular structures, glandular epithelial cells, and red blood cells) were not evaluated.

Statistical Analysis

Proliferation, type I procollagen production, two-dimensional surface area measurements and collagen-attachment data were compared between young and old skin. The data were analyzed using Student's two-sample t-

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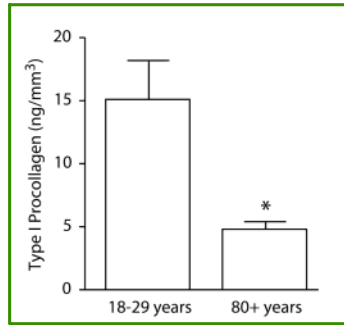


Figure 1. Type I procollagen production in young and old skin. Values shown are averages ± SEM, based on six young and six old individuals. Statistical significance of the differences between young and old skin was determined using Student's t-test. *Significance at the *P* < 0.05 level.

test (Microsoft Excel and SAS analytic software). Summary data are expressed as means ± SEM. All *P* values are two-tailed.

Results

Type I Procollagen Synthesis in Skin from Young and Old Individuals

In the first series of experiments, type I procollagen content was measured in skin from young and old individuals. Type I procollagen content, a marker of ongoing collagen synthesis, was decreased by 66% in old skin versus young skin (Figure 1).

Type I Procollagen Synthesis by Fibroblast Isolates from Young and Old Skin

Next, conditioned medium from cultures of young and old fibroblasts was assessed for type I procollagen production. Isolates from the cohort of young individuals synthesized more type I procollagen than did the fibroblast isolates from the cohort of old individuals (Figure 2). Cells isolated from young skin also proliferated to a greater extent (Figure 2).

Collagen Structure, Fibroblast-Collagen Interactions, and Cell Shape in Vivo: Comparison of Young and Old Skin

A series of related histological, ultrastructural, and fluorescence microscopic analyses were performed to determine the relationship between collagen structure, cell shape and adhesion site protein distribution. Differences in fiber bundle content were observed between young and old skin. Fiber bundles were thicker and there was

2. This first piece of data shows the average amount of procollagen measured from biopsies taken from the young group and the old group (6 biopsies for each group). This is a sensitive measure of newly synthesized collagen, because it has not been cleaved to its mature/final form (eliminating the "pro" from the name).

Take Away: Collagen production is decreased in the elderly individuals

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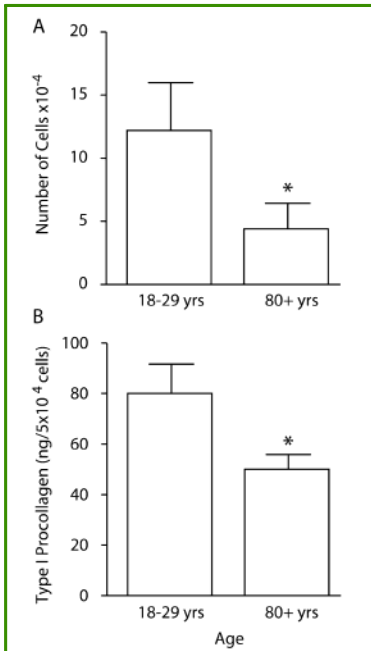


Figure 2. Cellular proliferation and type I procollagen production in mono-layer culture. Proliferation (A) and type I procollagen production (B) by fibroblasts isolated from young and old skin. Values shown are averages \pm SEM, based on 26 fibroblast isolates from eight young individuals and 37 isolates from eight old individuals. Statistical significance of the differences between isolates from young and old skin was determined using Student's *t* test. *Significance at the $P < 0.05$ level.

less open space within and between bundles in the papillary dermis of the 18- to 29-year-old individuals than in the corresponding tissue from the 80+-year-old subjects (Figure 3). In sections of young skin, interstitial cells could be seen oriented in the plane of the collagen polymer (Figure 3, inset). In old skin, the papillary dermis was characterized by the presence of open space interspersed with criss-crossing, tangled, thin fibers. There was little evidence of fiber bundle orientation. Open space around interstitial cells was apparent (Figure 3B, arrows), and there was little evidence of cell orientation (Figure 3, inset).

Cell shape (two-dimensional cross-sectional area of cells in 1- μm -thick sections from plastic embedded tissue) was quantitatively examined. Cells from young indi-

viduals were more spread than the corresponding cells from old individuals (Figure 4). The quantitative data shown in Figure 4 were obtained from sections of superficial (papillary) dermis. When the same analyses were done in the deeper layers of the (reticular) dermis, results were similar (not shown).

Ultrastructural features of the matrix were compared in sections from young and old skin. Overall, there were no major differences in the appearance of the collagen polymers that could be used to distinguish young and old skin. There were, however, areas in sections of old skin where the density of collagen was reduced. Such areas were primarily (though not always) confined to the papillary dermis. Occasional unstriated fibers were present in some of the sections from the old skin samples, but we never detected the elastic material characteristic of badly sun-damaged skin.^{2,3,5}

As part of the analysis, the interaction between interstitial cells and the surrounding collagen was examined. For these studies, we avoided mast cells, epithelial cells in glandular structures, cells associated with the microvasculature and any inflammatory cell that might be present. Thus, a majority of the cells characterized were interstitial fibroblasts. Figure 5 demonstrates and quantifies the interaction of these cells with the surrounding collagen in sections from young and old individuals. In skin sections from the 18- to 29-year-old cohort, cells were in contact with intact collagen fibrils over a greater proportion of their surface (two-dimensional image) than were cells in sections from old skin.

Adhesion Site Protein Expression: Comparison of Cells in Young and Old Skin

In a final set of experiments, anti-vinculin antibody was used to identify focal adhesion sites by fluorescence microscopy (Figure 6). In cells from both young and old skin, anti-vinculin staining was evident. Staining in skin sections from young individuals was associated with fiber bundles, which were evident by their dull orange appearance in the stained sections. In sections of old skin, there were fewer focal adhesions, and much of the staining was closely associated with nuclei (blue color). The differential pattern of adhesion site protein expression shown in Figure 6 correlated with differences in cell spreading, as observed at the light microscopic level (Figure 4), and with cell-collagen interactions, as observed at the transmission electron microscopic level (Figure 5). Alternatively, nuclear-associated fluorescence could indicate an intracellular (presumably, functionally inactive) pool of vinculin.

Discussion

There is a large body of literature demonstrating a relationship between mechanical tension on cells *in vitro* and biological responses of cells to stress.¹⁹⁻²³ When there is a sufficient level of mechanical tension on fibroblasts, production of collagen and other components of the ex-

3. In the second piece of data, the researchers are showing two things after plating (putting cells on a cell culture plate) the cells from another skin biopsy of both groups (young vs old): A) The number of fibroblasts (collagen synthesizing cells) in the skin, and B) The amount of collagen produced by these cells.

Take Away: The researchers controlled for the number of cells plated initially and removed any collagen from the media (liquid feeding the cells) and let the cells alone for a set amount of time, indicating higher proliferation of fibroblasts (cell division) with the younger skin vs older skin (A). They also found that the amount of collagen synthesized was higher in the younger cells by measuring the initially collagen free media for collagen (whatever is present has been synthesized) (B).

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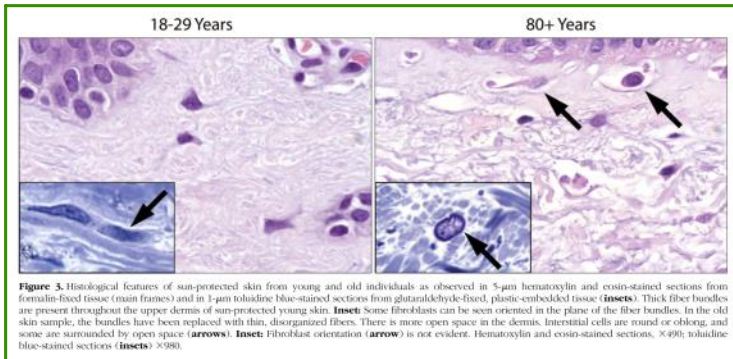


Figure 3. Histological features of sun-protected skin from young and old individuals as observed in 5- μ m hematoxylin and eosin-stained sections from formalin-fixed tissue (main frames) and in 1- μ m toluidine blue-stained sections from glutaraldehyde-fixed, plastic-embedded tissue (insets). Thick fiber bundles are present throughout the upper dermis of sun-protected young skin. Inset: Some fibroblasts can be seen oriented in the plane of the fiber bundles. In the old skin sample, the bundles have been replaced with thin, disorganized fibers. There is more open space in the dermis. Interstitial cells are round or oblong, and some are surrounded by open space (arrows). Inset: Fibroblast orientation (arrow) is not evident. Hematoxylin and eosin-stained sections, $\times 400$; toluidine blue-stained sections (insets) $\times 980$.

tracellular matrix is high. When tension is reduced, matrix production falls, and elaboration of matrix-degrading enzymes is concomitantly stimulated.²⁴⁻³² Lapiere and colleagues³¹ directly measured mechanical forces generated by fibroblasts in three-dimensional collagen lattices. In a series of studies, they demonstrated that the reduction in collagen synthesis occurring as a consequence of reduced mechanical tension reflected decreased transcription of genes for interstitial collagens, effects on enzymes involved in the posttranslational processing of procollagen peptides, and increased elaboration of collagen-degrading MMPs. It was shown, furthermore, that multiple signaling pathways were responsible for altered gene transcription.^{32,33} Of interest, changes in MMP production resulting from a loss of mechanical tension could be clearly separated from changes resulting from interleukin-1 stimulation.³⁴

Consistent with these past observations, studies from our laboratory demonstrated that when human dermal fibroblasts were maintained on native three-dimensional collagen lattices, these cells produced type I procollagen. When the collagen in the lattice was fragmented by exposure to MMP-1 (interstitial collagenase) or to enzymes elaborated by UV-exposed skin (primarily MMP-1), a fall-off in collagen production occurred. Concomitant with the decrease in collagen production on the fragmented collagen lattices was a reduction in mechanical tension as evidenced by reduced cell spreading, decreased focal adhesions, and dissolution of actin stress fibers.^{6,7,17,18} Parallel studies showed that in severely photodamaged skin (with its extensive collagen degradation), resident fibroblasts were characterized by similar morphological, ultrastructural, and fluorescence microscopic findings as observed *in vitro* on fragmented collagen.¹⁸ Our interpretation of these findings is that degradation of collagen in severe photodamage produces an environment that is unable to support a level of

mechanical tension required for efficient collagen-synthetic activity.

Collagen fragmentation, a reduction in total collagen, and decreased cell-collagen fiber interactions also characterize chronologically aged skin.¹⁵⁻³⁵ The enzymes responsible for collagen degradation increase gradually over time in the skin.¹³ Collagen synthesis may also decline gradually, but the fall-off in new collagen production is most evident when skin damage is clinically evident.¹⁵ The present study was undertaken to determine what factors contribute to the loss of collagen-synthetic activity in chronological aging. Based on the results presented here, we suggest at least two mechanisms. *In vitro* studies indicate that reduced collagen synthesis in old skin reflects, at least in part, an age-related reduction in collagen-synthetic activity in the resident population of fibroblasts. Fibroblasts obtained from sun-protected skin of young adults (18 to 29 years of age) synthesized an average of 82 ng of type I procollagen per 5×10^4 cells, whereas cells from old individuals (80+ years of age) synthesized 56 ng per 5×10^4 cells under identical *in vitro* conditions. Coupled with this is the fact that there are fewer interstitial fibroblasts in aged skin compared with young skin,¹⁵ contributing to reduced growth capacity (Figure 2). Thus, even when all environmental factors that may contribute to differences *in vivo* are removed, there is still an age-dependent difference in collagen-synthetic capacity that explains at least part of the previously documented reduction in collagen content of aged skin.⁶

If a decrease in collagen synthetic capacity occurs as a function of fibroblast (cellular) aging, then what role does a reduction in mechanical tension play? Although it may be difficult to precisely estimate the percentage of the overall decrease in collagen production (in old skin relative to young skin) accounted for by decreased mechanical tension, the following analysis serves as a basis for comparison. Our past studies¹⁵ indicate that collagen

4. The researchers have taken simple images of the biopsied skin samples taken from the participants (young and old) and stained them so that the darker stain shows the fibroblasts and the lighter pink stain shows the extracellular matrix (in the large images), which is to what the cells anchor/attach themselves and is also partly made up of collagen fibers. In the smaller images, the darker stain is still the cells and the lighter blue stain around the dark stain are the collagen fibers, zoomed in.

Take Away: While it is more subjective than objective, this shows that younger skin has elongated cells tracking along long, wide collagen fibers while the older skin has smaller, roundish cells not tracking with elongated collagen fibers as the collagen is fragmented and separated by open space.

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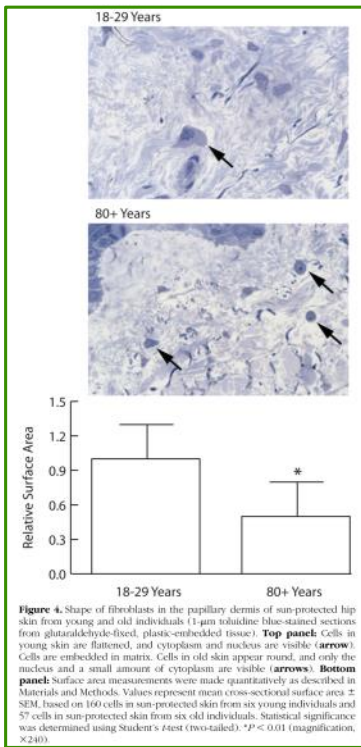


Figure 4. Shape of fibroblasts in the papillary dermis of sun-protected hip skin from young and old individuals (1- μ m toluidine blue-stained sections from glutaraldehyde-fixed, plastic-embedded tissue). **Top panel:** Cells in young skin are flattened, and cytoplasm and nucleus are visible (arrow). Cells are embedded in matrix. Cells in old skin appear round, and only the nucleus and a small amount of cytoplasm are visible (arrows). **Bottom panel:** Surface area measurements were made quantitatively as described in Materials and Methods. Values represent mean cross-sectional surface area \pm SEM, based on 160 cells in sun-protected skin from six young individuals and 57 cells in sun-protected skin from six old individuals. Statistical significance was determined using Student's *t*-test (two-tailed). **P* < 0.01 (magnification, $\times 240$).

production in sun-protected skin of old (80+ years) individuals is decreased by approximately 75% relative to production in corresponding skin of young (18 to 29 years) adults. Both Western blotting of skin extracts and immunostaining for type I procollagen are consistent in this regard. These past findings are also consistent with the direct measurements of type I procollagen in young and old skin presented here (ie, 68% reduction in old versus young skin; Figure 1). If the number of fibroblasts in skin from 80+-year-old individuals is reduced by approximately 35% relative to the number in skin of 18- to 29-year-old individuals as indicated by morphometric analysis¹⁹ and if type I procollagen synthesis is reduced by an average of 30% in fibroblasts from old skin as

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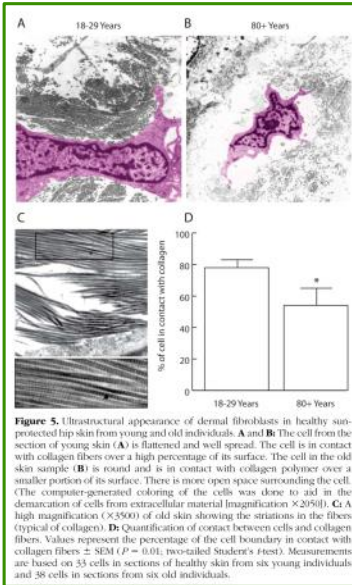


Figure 5. Ultrastructural appearance of dermal fibroblasts in healthy sun-protected hip skin from young and old individuals. **A and B:** The cell from the section of young skin (**A**) is flattened and well spread. The cell is in contact with collagen fibers over a high percentage of its surface. The cell in the old skin sample (**B**) is round and is in contact with collagen polymer over a smaller portion of its surface. There is more open space surrounding the cell. (The computer-generated coloring of the cells was done to aid in the demarcation of cells from extracellular material [magnification $\times 2090$]). **C:** A high magnification ($\times 3500$) of old skin showing the striations in the fibers (typical of collagen). **D:** Quantification of contact between cells and collagen fibers. Values represent the percentage of the cell boundary in contact with collagen fibers \pm SEM (*P* < 0.01, two-tailed Student's *t*-test). Measurements are based on 33 cells in sections of healthy skin from six young individuals and 38 cells in sections from six old individuals.

indicated in Figure 2 of the present study, then it is reasonable to suggest that age-dependent differences in fibroblast biosynthetic activity account for approximately 45% of the total decrease. Other factors (including loss of mechanical tension) account for the remaining 30%. This separation is shown schematically in Figure 7. How ultimate accuracy of the 45%/30% split is less important than the fact that while a loss of mechanical tension appears to be the major factor underlying decreased collagen synthesis in photodamaged skin,¹⁶ in chronologically aged skin, it is one of the two contributing mechanisms.

Of interest, age-dependent alterations in fibroblast biosynthetic activity and the reduction in external mechanical tension on cells in the dermis of aged skin may not be independent. The same reduction in mechanical tension that lowers collagen production by resident fibroblasts may also indirectly contribute to permanent alterations in fibroblast function. It is generally accepted that phenotypic changes seen in aged fibroblasts are largely mediated by oxygen radical damage.³⁵ This is potentially related to the issue at hand, because among the alterations that occur under conditions of reduced mechanical tension is increased oxidant stress as evidenced by

5. The researchers are staining for the cells and using microscope images - specifically for the nucleus of the cell (where DNA/genes are kept) and the cytosol (where all the other organelles of the cell are located - and, where collagen is synthesized/created). The arrows are pointing to cells and the dark color is the nucleus and the lighter color is the cytosol.

Take Away: The cells are markedly smaller (quantified as bar graphs) for the elderly skin sample and, although not directly quantified, the cytosol is diminished in size (can only see nucleus) in these cells.

6. In Figure 5, the researchers have taken images of single fibroblasts (computer generated purple colorization) and their surrounding extracellular matrix to visualize how much collagen is present and how much contact the cell has with the collagen.

Take Away: The older skin has smaller cells (confirming the previous data), but also has less collagen around it, leading to less cell to collagen interaction (quantified as bar graph).

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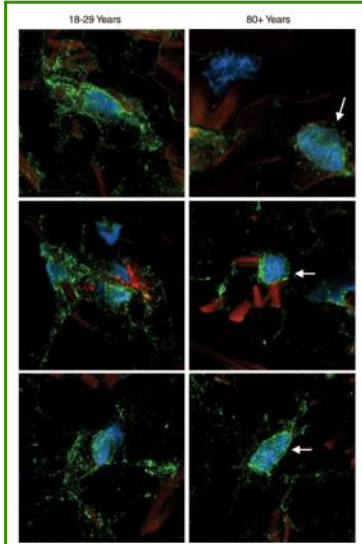


Figure 6. Adhesion-site protein expression in sections of healthy sun-protected hip skin from young and old individuals. Tissue sections (OCT-embedded frozen) were stained using antibody to vinculin and concomitantly with phalloidin (actin stain) and DAPI (nuclear stain) as described in Materials and Methods. After staining, cells were examined by confocal fluorescence microscopy. Cells are identified by their blue (DAPI)-stained nuclei. Bright green punctate fluorescence identifies vinculin. In the 18- to 29-year-old skin samples, vinculin can be seen at a distance from the nucleus, and in many areas, the vinculin appears to be in close apposition to collagen fibers. In the 80+-year-old skin, blue-stained nuclei are apparent, but there is less vinculin than in the young skin samples. Where intense focal staining is evident, it is surrounding the nucleus (**arrows**). Away from the nucleus, staining is more diffuse than seen in cells from young skin. The sections presented are representative of young and old sun-protected skin from six individuals, respectively. In both young and old skin, collagen fibers are apparent by their dull orange fluorescence (magnification, $\times 1200$).

increased levels of reactive oxygen species and altered expression of anti-oxidant enzymes (G.J. Fisher, unpublished observations). It might be inferred from this that environmental damage causally precedes changes in fibroblast function that are observed in the aged or senescent state.

The age-related decrease in collagen-synthetic activity may be, at least in part, reversible. It has been demonstrated that agents such as all-trans retinoic acid can stimulate collagen production in aged skin.^{16,36-38} Not surprisingly, topical retinoid use brings about an improvement in the appearance of aged skin³⁹ and photo-damaged skin.⁴⁰

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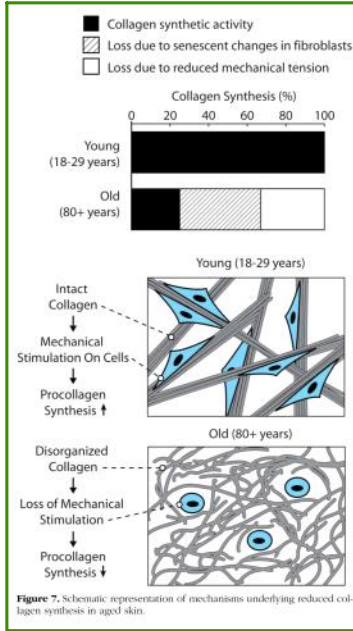


Figure 7. Schematic representation of mechanisms underlying reduced collagen synthesis in aged skin.

In summary, collagen synthetic capacity is low in aged (sun-protected) skin relative to that in healthy young sun-protected skin. Based on the findings presented here and by analogy with *in vitro* models and the findings of photoaging studies, we hypothesize that old fibroblasts have an age-dependent reduction in the capacity for collagen synthesis and simultaneously experience a loss of mechanical stimulation resulting from decreased intact collagen fibers.

Acknowledgments

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7. In Figure 6, the researchers are again looking at individual cells (or, at most, a few cells) by adding fluorescent antibodies/tags to particular components of the cell - blue for the nucleus (DNA), red for actin (a protein in the cytosol), and green for vinculin (a cell to extracellular anchoring protein). This allows for a more accurate investigation of 1. How much of each is present, and 2. Where each is present.

Take Away: The younger cells show more vinculin (cell to extracellular matrix adhesion) and more of it spread out, indicating that the larger cells allow for more spread extracellular connections.

8. The researchers propose this mechanism to explain the differences between the young and old skin. They believe that collagen synthesis decreases with age, because collagen fibers are disorganized, leading to less mechanical tension as the fibroblasts are not able to attach as much of the collagen, leading to less synthesis and their rounding (they are at least partly dependent on the long collagen fibers to create their/fibroblast elongated state).

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