Reduced Fibroblast Interaction with Intact Collagen as a Mechanism for Depressed Collagen Synthesis in Photodamaged Skin

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This report provides evidence from a number of different approaches (i.e., comparison of cell shape in 1-μm sections of photodamaged versus healthy skin at the light microscopic level; comparison of cell shape and apposition to collagen fibrils in ultrathin sections of the same tissues examined by transmission electron microscopy, and fluorescence staining for adhesion site protein expression and actin filament architecture in frozen tissue sections) that dermal cells in healthy skin are attached to collagen fibrils over a large part of the cell border, have a flattened/spread (two-dimensional) appearance and have abundant actin in their cytoplasm. In contrast, cells in photodamaged skin are often in contact with fragmented collagen or amorphous debris rather than intact collagen, have a collapsed/elongated shape, and have a lower amount of actin. Collagen synthesis is reduced in severely photodamaged skin relative to collagen synthesis in corresponding sun-protected skin (N Engl J Med 329:530, 1993). We hypothesize that fibroblasts in severely damaged skin have less interaction with intact collagen and as a result experience a reduction in mechanical tension. Decreased collagen synthesis is (presumed to be) the result.

Key words: actin filament/integrin/collagen fibrils/fibroblasts/focal adhesion kinase/mechanical tension/photodamage/skin/vinculin


Extensive degradation of fibrillar (types I and III) collagen is a characteristic feature of severely photodamaged skin (Smith et al, 1962; Lavker, 1979; Marks, 1992; West, 1994; Lavker, 1995), and is thought to underlie the course, rough, wrinkled appearance of chronically sun-exposed skin. Collagen-degrading matrix metalloproteinases (MMP) are upregulated in skin by exposure to ultraviolet (UV) irradiation (Fisher et al, 1996, 1997). Repeated induction of these enzymes by exposure to solar irradiation over years or decades is likely responsible for producing collagen fragmentation in chronically sun-exposed skin. Although destruction of existing collagen is, undoubtedly, central to the problem, failure to replace damaged collagen with newly synthesized material is also a contributing factor. There is a sustained downregulation in collagen synthesis in photodamaged skin relative to what occurs in healthy sun-protected skin (Griffiths et al, 1993; Talwar et al, 1995). Interestingly, although upregulation of collagen-degrading MMP occurs episodically over years or decades with UV-exposure, the sustained loss of collagen synthetic capacity is a late event seen in severely damaged skin.

The mechanism(s) underlying sustained reduction in collagen synthesis in severely photodamaged skin has/have not been fully delineated. As a way to begin addressing this issue, we assessed production of type I procollagen by human dermal fibroblasts in three-dimensional collagen lattice cultures under control conditions and following partial degradation of the collagen fibrils with interstitial collagenase (MMP-1) (Varani et al, 2001, 2002; Fligiel et al, 2003). Procollagen synthesis was reduced (up to 80% reduction) on the partially degraded matrix relative to that observed on intact collagen. On intact collagen, fibroblast attachment was followed by spreading of attached cells whereas on partially degraded collagen, cells did not fully spread after attachment. Based on this, we hypothesized that failure to synthesize type I procollagen in partially degraded collagen reflected a failure to generate sufficient mechanical tension (evidenced by failure of the cells to spread fully) for optimal synthetic activity. This is consistent with a variety of in vitro studies showing a relationship between cell spreading/mechanical tension and collagen synthetic activity. Specifically, a number of past studies have compared collagen production by fibroblasts in collagen gels versus the same cells on plastic or have

Abbreviations: DMEM–FBS, Dulbecco’s modified minimum essential medium–fetal bovine serum; DPBS, Dulbecco’s phosphate-buffered saline; FITC, fluorescein isothiocyanate; KBM, keratinocyte basal medium; MMP, matrix metalloproteinase; MMP-1, interstitial collagenase; SDS-PAGE, sodium dodecylsulfate-polyacrylamide gel electrophoresis; TEM, transmission electron microscopy; UV, ultraviolet

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compared collagen production by cells in stressed collagen versus cells in relaxed (contracted) collagen gels (Lambert et al, 1992; Geesin et al, 1993; Clark et al, 1995; Chiquet 1999; Kessler et al, 2001; Le et al, 2002; Fluck et al, 2003). In aggregate, these studies have demonstrated that the more rigid the substrate—i.e., plastic > stressed collagen > relaxed collagen—the greater the synthetic activity.

Taken together with these past studies, our data suggest that reduced procollagen production by fibroblasts on damaged collagen in vitro is associated with a failure to fully spread on the damaged matrix (and presumably reflecting a reduction in mechanical force acting across the cells). Since collagen fragmentation, loss of intact collagen and reduced collagen synthesis all characterize severely photodamaged skin in vivo (Smith et al, 1962; Lavker, 1979; Marks, 1992; Griffiths et al, 1993; Lavker, 1995; Talwar et al, 1995; Varani et al, 2001), the critical issue is whether in vivo there is a similar reduction in attachment to intact collagen fibrils and a concomitant reduction in mechanical tension on resident fibroblasts in badly photodamaged skin, and whether this accounts for decreased collagen production. Efforts to begin addressing these issues are described in this report.

Results

Adhesion site protein expression and actin filament architecture in fibroblasts maintained on intact collagen and partially degraded collagen in vitro Dermal fibroblasts were plated on intact or collagenase-fragmented collagen. Slides without collagen served as control. Consistent with past studies (Varani et al, 2002), fibroblasts rapidly attached to the uncoated slides as well as to the intact collagen substrate and the partially degraded substrate. Cell spreading occurred on the uncoated slides and on the intact collagen, but the cells failed to completely spread on the damaged matrix. Differences in spreading could be seen as early as 15 min after attachment and were still evident after 18 h. Differences in cell spreading on the three substrates were associated with differences in adhesion site protein expression and differences in actin filament architecture (Fig 1). When cells on uncoated slides were examined by confocal fluorescence microscopy (18 h after seeding), adhesion sites were defined in the well-spread cells by intense, focal staining for vinculin (green fluorescence in panel A). Actin filaments (red fluorescence) terminated in the attachment sites. On intact collagen, the staining patterns for vinculin and actin filaments were similar to those seen on uncoated slides. Specifically, there was localized vinculin staining (green fluorescence) around the cell periphery (arrowheads) at sites where the cell appeared to be in contact with the substratum. Actin filaments (red fluorescence) terminated at these sites (panel B). The inset in panel B confirms that actin filaments were associated with vinculin (arrowheads). The staining patterns for both vinculin and actin were much different in the cells maintained on partially degraded collagen. On the partially degraded collagen substrate, vinculin (green fluorescence) was diffusely present over the whole cell surface and actin (red fluorescence) was observed diffusely at the cell boundary. No actin filaments were visualized in these cells (panel C).

In subsequent studies, an antibody to β1 integrin was used in place of the antibody to vinculin. Like vinculin, β1 integrin is found in adhesion sites, but unlike vinculin, is
part of the molecular complex that directly interacts with extracellular collagen. Results virtually identical to those obtained with vinculin were obtained. Specifically, β1 integrin (green fluorescence) staining was present at discreet focal sites in the cells maintained on intact collagen (arrowheads) and actin filaments (red fluorescence) terminated in these sites (panel D). (Note: the red fluorescence in this image was so intense that where it overlay the green fluorescence of the β1 integrin, the appearance was yellow). Similarly, in cells attached to partially degraded collagen, staining for β1 integrin produced a uniform green fluorescence over the whole cell surface. There were no visible actin filaments in most of the cells. An occasional thin actin filament could be seen running parallel to the long axis of the cell in a few cells, and actin staining was visible at sites where the cell appeared to be in contact with the substratum (arrowheads) (panel E). Finally, a third antibody—to FAK—was also examined. As with vinculin and β1 integrin, antibody to FAK was localized to discreet sites along the cell surface in cells incubated on uncoated slides or on intact collagen. In contrast, only a faint, diffuse fluorescence was observed in cells on damaged collagen (not shown).

In a final set of experiments, an antibody to smooth muscle α-actin was used in place of phalloidin to confirm that phalloidin staining of the visible stress fibers was actually due to the presence of the actin filaments. As expected, results with antibody to smooth muscle α-actin (Fig 2) were virtually identical to results obtained with phalloidin (Fig 1B and D). That is, actin filaments were present in the cells attached to the intact collagen (demonstrated by the presence of visible stress fibers after staining). In contrast, cells attached to the enzyme-treated (partially degraded) collagen demonstrated actin staining at the periphery of the cell (Fig 2) at what appeared to be sites of attachment to the collagen; however, actin filaments were not observed (Fig 2).

**Fibroblast cell shape in vivo in healthy skin and severely photodamaged skin: light microscopic studies** The *in vitro* studies described above provide a “framework” for understanding the relationship between collagen structure, cell attachment/spreading and cytoskeletal architecture. The critical question is whether the *in vitro* findings have relevance to what occurs *in vivo* in photo-damaged skin. To address this issue, samples of severely photodamaged skin and samples of healthy sun-protected skin were prepared as described in the Materials and Methods section and examined under light microscopy. The results of this study are shown in Fig 3, where it can be seen that cells in tissue sections from sun-protected skin were much more extensively flattened/spread than were cells in photodamaged skin. The cells with a flattened/spread appearance were always surrounded by intact collagen. In severely photodamaged skin, many cells expressed a collapsed/elongated shape. Cells in photodamaged skin were often in contact with amorphous acellular material, which in some cases appeared to be the remnants of collagen fibrils but in other cases consisted of elastotic material or was completely unidentifiable. Still other cells were not in close proximity to any structural element. Cell flattening/spreading (specifically, the two-dimensional surface area occupied by the cell in the plane of focus) was quantified using NIH Image software.

**Fibroblast cell shape in vivo in healthy skin and severely photodamaged skin: Electron microscopy** Light microscopy studies provide convincing evidence that in healthy skin, interstitial cells are embedded in a collagen matrix whereas in areas of severe photodamage, there appears to be little or no intact collagen surrounding many of the interstitial cells. To provide confirmation of these light microscopic observations as well as to obtain a high-definition assessment of the cell–matrix interface in healthy and photodamaged skin, TEM was used. Consistent with past studies (Varani *et al*, 2001; Fligel *et al*, 2003), TEM demonstrated the presence of large collagen fibril bundles throughout the dermis of healthy, sun-protected skin. In contrast, damaged skin was characterized by large areas that were seemingly devoid of structural components. In other areas, individual, disorganized collagen fibrils were present as was a large amount of amorphous material. In this study, ultrastructural features of resident fibroblasts in sun-protected skin were compared with ultrastructural
features of cells in photodamaged skin. To summarize, cells in healthy, sun-protected skin were in intimate contact with individual collagen fibrils or with large collagen fibril bundles (Fig 4). Many of the cells had a flattened/spread appearance (low length to width ratio) and numerous cellular processes were apparent in these cells. The cellular processes were sites of contact with the surrounding collagen. In contrast, rather than being in contact with intact collagen, many of the fibroblasts in photodamaged skin were in contact with amorphous material or not attached to any structural element (Fig 4). Many of these cells demonstrated a collapsed/elongated shape (high length to width cytoplasmic ratio) with very little cytoplasm in the plane of focus. These cells were often not in contact with any intact collagen fibrils. These ultrastructural features were quantified (Fig 4) as indicated in the Materials and Methods section.

Although there were significant differences between severely photodamaged skin, on the one hand, and healthy,
sun-protected skin on the other, the quantitative data presented in Figs 3 and 4 demonstrate variability within both groups as well. This reflects, at least in part, variability in the collagenous matrix from site to site. Even in the most damaged skin, there were areas where the collagen matrix appeared normal at the TEM level. When cellular process formation (data in Fig 4B) was examined for correlation with the percentage of cell boundary in contact with intact collagen (data in Fig 4A), the two variables were strongly correlated ($r = 0.67$; $p = 0.018$). Cell shape (data in Fig 4C) was also correlated (inversely) with cell–collagen interaction, but the correlation did not reach statistical significance ($r = -0.44$; $p = 0.15$). In summary, where intact collagen was observed in the tissue and where fibroblasts were in close proximity to the collagen (regardless of whether the tissue was from a sample that was overall healthy or photodamaged), cellular phenotype was similar. Likewise, where there was a lack of intact collagen, resident cells had a similar phenotype (i.e., different from that of cells in contact with intact collagen).

**Adhesion site protein expression and actin filament architecture in healthy skin and severely photodamaged skin** In a final set of experiments, frozen sections were prepared from severely photodamaged forearm skin and sun-protected hip skin from six individuals. Tissue sections were stained with the same combinations of antibodies used with fibroblasts in vitro and examined by confocal fluorescence microscopy. Figure 5 demonstrates cells stained with antibodies to vinculin (green fluorescence) and with phalloidin (for actin filament architecture; red fluorescence). In healthy skin, vinculin-stained cells (identified by the presence of DAPI-stained nuclei) demonstrated intense, focal green fluorescence over a wide area out from the nucleus. In these cells, vinculin was often closely associated with collagen bundles, which were evident in the tissue sections by their dull orange fluorescence. In contrast, cells in badly damaged skin showed much less green fluorescence. Apposition of cells and collagen bundles was much less apparent.

Concomitant with staining for vinculin, the same tissue sections were stained with phalloidin. Actin filaments were not visualized by this method in any of the tissue sections examined—either from healthy skin or from photodamaged skin. Furthermore, we were unable to clearly identify phalloidin staining in the cells. Therefore, experiments were conducted in which fluorescein isothiocyanate (FITC)-phalloidin was used in place of Alexa Fluor 546-phalloidin. (Note: the green fluorescence associated with fluorescein is much more intense than the red (rhodamine) stain.) Consistent with data from Alexa Fluor 546-phalloidin-stained tissue sections, there was no evidence of actin filaments in cells from either healthy or damaged skin. When FITC-phalloidin was used as a probe, however, cells in healthy skin demonstrated intense green fluorescence throughout the cytoplasm. Much less fluorescence was observed in cells from photodamaged skin (Fig 6).

In a final set of experiments, tissue sections from healthy skin and photodamaged skin were examined by confocal microscopy after staining with an antibody to β1 integrin or with an antibody to FAK. These antibodies produced staining patterns similar to the pattern observed with vinculin. That is, there was intense focal staining in cells from healthy skin, but less widespread staining in cells from damaged skin (not shown).
Discussion

Based on past observations demonstrating a relationship between mechanical tension on fibroblasts in vitro and collagen synthetic capacity (Lambert et al, 1992; Geesin et al, 1993; Clark et al, 1995; Chiquet, 1999; Kessler et al, 2001; Le et al, 2002; Fluck et al, 2003), we hypothesized that the lack of collagen synthesis in severely photodamaged skin (Griffiths et al, 1993; Talwar et al, 1995) results, at least in part, from extensive damage to the collagenous matrix in the sun-exposed skin, and the inability of the damaged matrix to support a level of mechanical tension in resident fibroblasts that is sufficient for collagen synthesis. Although it is impossible to reliably measure mechanical stress on individual cells in vivo, we have in this study, analyzed a number of surrogate markers of mechanical tension in cells of healthy skin and cells of severely photodamaged skin. The markers include (i) two-dimensional cell shape in 1-μm sections from plastic-embedded tissue; (ii) cell shape, cellular process formation, and contact with collagen at the TEM level; and (iii) immunostaining patterns using probes for adhesion site proteins and actin filament architecture. All of these surrogate markers were differentially expressed in severely photodamaged skin relative to what was seen in healthy skin. These findings provide a strong (albeit, indirect) indication that cells in photodamaged skin are under less mechanical tension than cells in healthy sun-protected skin. Figure 7 is an attempt to show how the shape of a three-dimensional cell (visualized in two dimensions) is determined by its interaction with intact collagen and how the shape changes as attachment to an anchored support is lost. The change in cell shape from flattened to elongated reflects, presumably, a loss of isometric mechanical tension that is provided by the cell contracting against the rigid extracellular matrix in healthy skin. The data presented here, thus, support our hypothesis that the severely damaged matrix in photoaged skin acts to prevent cells that are intrinsically capable of expressing the collagen-synthetic phenotype from doing so.

How alterations in mechanical tension affect cellular function is not fully understood, but data from in vitro studies provide insight (reviewed in Chiquet, 1999; Grinnell, 2000; Geiger et al, 2001; Grinnell, 2003; Silver et al, 2003). To summarize, a variety of approaches have demonstrated that fibroblast interaction with three-dimensional collagen matrices results in cell attachment to the substrate through specific cell surface integrin receptors (primarily α1β1 and α2β1) (Dartsch and Hammerle, 1986; Iba and Sumpio, 1991; Langholz et al, 1995; Knight et al, 1999; Xu et al, 2000). Attachment of the cells is followed by activation of protein kinases (i.e., focal adhesion kinase and integrin-linked kinase) at the adhesion site (Burr ridge et al, 1992; Kornberg et al, 1992; Volberg et al, 2001). Phosphorylation of cytosolic proteins by these kinases allows the proteins to be incorporated into the forming focal adhesion, and this provides an anchoring site for cytosolic actin (Geiger et al, 2001; Brakebusch and Fassler, 2003), which then polymerizes to form the actin cytoskeleton. Contraction of the cell’s actin–myosin-based cytoskeleton against the rigid structural support provided by the collagen lattice generates mechanical stress on the cell and results in shape change from rounded to spread (Tomasek et al, 2002; Silver et al, 2003).

Under conditions of mechanical stress, signals emanating from interaction of soluble factors with their cell-surface receptors (as well as perhaps, signals generated through the adhesion receptors themselves) are effectively transduced to the nucleus through the mitogen-activated protein kinase pathways (Lee et al, 2000; Rosenfeldt and Grinnell, 2000; Alpin et al, 2001; Fluck et al, 2003) and the transforming growth factor–β pathway (Desmouliere and Gabbiani, 1996). Genes for the interstitial collagen (among others) are transcribed whereas genes for matrix-degrading MMP are repressed (Lambert et al, 1992; Kessler et al, 2001; Le et al, 2002; Fluck et al, 2003). Under conditions in which mechanical tension on the cells is reduced, focal adhesion contacts dissolve and actin filaments depolymerize. As the cell loses its contact points with the matrix, it concomitantly looses its flattened shape and becomes spindle shaped or rounded. In this “mechanically relaxed” state, gene transcription is altered. Collagen synthesis is downregulated and production of matrix-degrading MMP is increased (Kessler et al, 2001; Le et al, 2002; Fluck et al, 2003). An unresolved question is whether the lack of mechanical stress directly hinders efficient signal transduction or whether under conditions of low mechanical stress, interaction of cell surface receptors with their ligands does
not occur efficiently. Additional studies will be needed to resolve this issue.

Although the focus of this study is on collagen damage and altered fibroblast function in photodamaged skin, a reduction in the amount of intact collagen in the skin and the presence of fragmented collagen are also characteristic of chronologically aged skin (Fligiel et al, 2003), although the overall damage to the connective tissue tends not to be as severe in normal aging as in photaging (Smith et al, 1962; Lavker, 1979, Marks, 1992; Lavker, 1995). Likewise, elaboration of both types I and III collagen is reduced in naturally aged skin (Varani et al, 2000) as well as in photoaged skin (Griffiths et al, 1993; Talwar et al, 1995). Thus, although we utilized photodamaged forearm skin in this study, the findings may also be applicable to skin damage occurring as a result of the natural aging process. Our suggestion, based on this data and past findings, is that the loss of collagen synthetic capacity in chronologically aged skin relative to that of young skin (Varani et al, 2000) reflects both an intrinsic decrease in collagen synthetic capacity by the aged fibroblasts (Millis et al, 1989, 1992; Burke et al, 1994; Bizot-Foulon et al, 1995) and interaction with damaged matrix.

In summary, collagen synthetic capacity is low in severely photodamaged skin relative to collagen synthetic capacity in corresponding sun-protected skin. Capacity to synthesize collagen is also low in aged (sun-protected) skin relative to that in healthy young skin. We hypothesize, based on the findings presented here, and by analogy with in vitro models, that fibroblasts in severely damaged skin (either photoaged, naturally aged or both) experience a loss of mechanical tension as a result of decreased interaction with intact collagen. Decreased collagen synthesis is presumed to reflect decreased mechanical tension.

**Materials and Methods**

**Skin biopsies** Eleven individuals with severe photodamage on the forearms were recruited for this study (age range 50–88 y with only one individual older than 80 y). Duplicate 4-mm full-thickness punch biopsies from the severely photodamaged forearm skin were obtained from each individual. Fourmillimeter punch biopsies of sun-protected hip skin were obtained from the same individuals. Biopsies of hip skin were obtained from a third cohort of young (18–29-y-old) individuals. Upon arrival in the laboratory, the biopsies were cut into two pieces. One piece was fixed in glutaraldehyde, and the other piece was frozen in OCT. For the studies shown in Fig 3, the data are based on healthy skin from six individuals and photodamaged skin from five individuals. The studies shown in Fig 4 are based on healthy skin from seven individuals and photodamaged skin from five individuals. The studies depicted in Figs 5 and 6 are representative of results with healthy skin and photodamaged skin from six individuals each. All procedures involving human subjects were approved by the Institutional Review Board and biopsies were obtained after receiving informed consent. (Note: In this study we chose to compare photodamaged forearm skin with hip skin rather than underarm skin because underarm skin tends to be more variable (Varani et al, 2001) and because biopsies from the hip are better tolerated by volunteers.)

**Human dermal fibroblasts in monolayer culture** Normal human dermal fibroblasts were isolated from skin biopsies as described previously (Varani et al, 1994) and grown in monolayer culture using Dulbecco’s modified minimal essential medium supplemented with non-essential amino acids and 10% fetal bovine serum (DMEM–FBS) as culture medium. Fibroblasts were maintained at 37°C in an atmosphere of 95% air and 5% CO2. Cells were sub-cultured by exposure to trypsin/EDTA and used at passage 2–5.

**Intersitial collagenase (MMP-1)** Human MMP-1 was obtained from Calbiochem (San Diego, California). The enzyme was purified from human rheumatoid synovial fibroblasts as the naturally occurring proenzyme form. The MMP-1 preparation appeared as a doublet at 52 and 57 kDa in β-casein zymography and was reactive with rabbit polyclonal anti-MMP-1 antibodies by western blotting. Activation of the proenzyme was accomplished by exposure to 1 μg of crystalline trypsin for 5 min at 37°C following by 10 μg of soybean trypsin inhibitor.

**Maintenance of fibroblasts on intact or partially degraded collagen in vitro** Rat tail collagen (4.7 mg per mL in 1 N HCl) (BD Biosciences, Bedford, Massachusetts) was diluted to 1 mg per mL in 0.1 N acetic acid and neutralized. Into each chamber of a four-chamber glass Lab-Tek II chamber slide (Nalge Nunc International, Naperville, Illinois) was deposited 0.5 mL of the neutralized collagen solution.

Degradation of the collagen was achieved by exposing the collagen to 200 ng of activated MMP-1 for 5 h at 37°C. Collagen exposed to buffer alone served as a control. At the end of the incubation period, the presence of collagen fragments released into the incubation buffer was assessed by resolution in SDS-PAGE (8.5% gel) and staining with Brilliant Blue dye. Intact and MMP-1-degraded collagen was then seeded with 8 × 10^4 human dermal fibroblasts. Culture medium consisted of serum-free, Ca^2+ -supplemented (1.4 mM Ca^2+), final concentration) keratinocyte basal medium (KBM) (Cambrex Biologicals, Walkersville, Maryland). Cells were incubated for 18 h. At the end of the incubation period, slides were fixed in 4% paraformaldehyde and stained as described below.

**Immunostaining** Mouse monoclonal antibodies to vinculin, β1 integrin and focal adhesion kinase (FAK) were obtained from Chemicon (Temicula, California). A monoclonal anti-α-actin antibody was purchased from Dako (Carpenteria, California). Primary antibodies were visualized with rabbit anti-mouse IgG antibodies bound to Alexa Fluor 488 (Molecular Probes, Eugene, Oregon) and further amplified with Alexa Fluor 488 goat anti-rabbit IgG. Nuclei were counterstained with the nuclear dye DAPI (Chemicon). In some experiments, Alexa Fluor 546 phallolidin (Molecular Probes) or fluorescein (FITC)-phallolidin (Molecular Probes) was used. (Note: Alexa Fluor 488 is spectrally similar to fluorescein, whereas Alexa fluor 546 is spectrally similar to tetramethylrhodamine.)

Immunostaining was done on fibroblasts grown on glass Lab Tek II chamber slides or on intact or partially degraded collagen as described below. Frozen sections of healthy skin or photodamaged skin were also stained. Briefly, cells on the uncoated chamber slides and frozen tissue sections were fixed with 4% formaldehyde for 20 min. Cells incubated on intact or partially degraded collagen were fixed for 1 h. After fixation, cells were washed 2 x with wash buffer (0.05% Tween-20 in Dulbecco’s phosphate-buffered saline (DPBS)), followed by permeabilization with 0.1% Triton X-100 for 10 min. Cells or tissue sections were again washed and then exposed to a blocking solution consisting of 1% BSA in DPBS for 30 min. Next, cells were treated with anti-vinculin, anti-β1 integrin or anti-FAK antibodies in blocking solution for 1 h. After three subsequent washing steps (5 min each), each sample was treated with Alexa Fluor 488-conjugated secondary antibody in blocking solution and incubated for 45 min. When cells were concomitantly stained for actin expression, they were incubated with Alexa Fluor 546-phallolidin simultaneously with the secondary antibody. Following three additional washing steps (5 min each), cells or tissue sections were treated for 30 min with an amplification antibody (Alexa Fluor 488 goat anti-rabbit IgG). This was followed by two additional washing steps and treatment with
Assessment of cell shape at the light microscopic and transmission electron microscopic levels in vivo

Skin biopsies were fixed overnight in 2% glutaraldehyde in 0.1 mM cacodylate buffer (Sigma, St Louis, Missouri) at pH 7.4. Glutaraldehyde-fixed specimens were treated with 2% osmium tetroxide buffered in 0.1 mM cacodylate buffer. Specimens were dehydrated with graded ethanol to 20.1 mM cacodylate buffer. Specimens were dehydrated with a mixture of 50% ethanol and 50% acetone and examined at the light microscopic level. Light microscopic examination was performed with a Nikon eclipse E600 microscope. The overall appearance of the collagenous matrix was evaluated. Using the high-resolution photographs, the overall appearance of the collagenous matrix was evaluated. 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 (TEM). Ultrathin sections were cut from areas of interest, stained with lead citrate and uranyl acetate (both from EM Chemicals, Gibbstown, NJ). 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. (Note: when skin samples are fixed in formalin and paraffin-embedded, it is possible to cut sections of 3–5 μm in thickness. These sections are too thick to detect much of the cell's cytoplasm in vivo. Only the nucleus can routinely be seen in these specimens. It is only the much thinner sections obtained from glutaraldehyde-fixed, plastic-embedded tissue that can be examined in this fashion.)

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 (TEM). Ultrathin sections were cut from areas of interest, stained with lead citrate and uranyl acetate (both from EM Chemicals) and observed in a Phillips 400 Transmission Electron Microscope (FBI Company, Hillsboro, OR). Photographs were made from several areas of each specimen. Using the high-resolution photographs, the overall appearance of the collagenous matrix was evaluated. Using the same photographs, interstitial cells were quantitatively evaluated for three parameters: overall shape (i.e., a ratio of cell length and width), the degree of cell process formation (scored on a 0 to +4 basis), and percentage of the cell boundary in close 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. Seven specimens of sun-protected skin and five specimens of forearmskin from individuals with severe photodamage were examined in this way. Multiple sections from several different areas of each specimen were examined.

Statistical analysis

Collagen contact, measured as the percentage of the cell border in close contact with the collagen matrix, was compared between sections of healthy and photodamaged skin. The data were analyzed with Student's two-sample t test. Cell shape, measured as the ratio of length and width, was also compared statistically with Student's two-sample t test between the healthy and photodamaged collagen groups. Similarly, cell process formation, which can be observed at the TEM level, was also analyzed with the two-sample t test to compare healthy versus photodamaged collagen groups. Correlations between cell shape, cellular processes, and the percentage of cell in contact with collagen were analyzed with Pearson's product-moment correlation method. Summary data are expressed as means ± SEM. All p-values are two tailed. The data were analyzed with Microsoft Excel and SAS analytic software.
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