Fibrillin-Rich Microfibrils are Reduced in Photoaged Skin. Distribution at the Dermal–Epidermal Junction

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Chronic sun exposure results in photoaged skin with deep coarse wrinkles and loss of elasticity. We have examined the distribution and abundance of fibrillin-rich microfibrils, key structural components of the elastic fiber network, in photoaged and photoprotected skin. Punch biopsies taken from photoaged forearm and from photoprotected hip and upper inner arm of 16 subjects with a clinical range of photoaging were examined for fibrillin-1 and fibrillin-2 expression and microfibril distribution. *In situ* hybridization revealed decreased fibrillin-1 mRNA but unchanged fibrillin-2 mRNA levels in severely photoaged forearm biopsies relative to photoprotected dermal sites. An immunohistochemical approach demonstrated that microfibrils at the dermal–epidermal junction were significantly reduced in moderate to severely photoaged forearm skin. Confocal microscopy revealed that the papillary dermal microfibrillar network was truncated and depleted in photoaged skin. These studies highlight that the fibrillin-rich microfibrillar network associated with the upper dermis undergoes extensive remodeling following solar irradiation. These changes may contribute to the clinical features of photoaging, such as wrinkle formation and loss of elasticity. ***Key words: elastic fibers/fibrillin microfibrils/photoaged skin. J Invest Dermatol 112:782–787, 1999***

Skin aging can be divided into intrinsic (or chronologic) and extrinsic due to environmental insults such as chronic sun exposure. Intrinsically aged skin has characteristic fine wrinkling and appears smooth (Montagna *et al*, 1989). At the histologic level, such skin shows general atrophy of the extracellular matrix (ECM) with reduced elastin and disintegration of elastic fibers (Braverman and Fornferko, 1982). Photoaged skin, in contrast, appears coarse, roughened, and deeply wrinkled with marked loss of elasticity and recoil (Smith *et al*, 1962; Warren *et al*, 1991). By routine light microscopy, photoaged skin contains abundant dystrophic elastotic material in the reticular dermis, which is immunopositive for tropoelastin and fibrillin (Mitchell, 1967; Chen *et al*, 1986; Mera *et al*, 1987; Werth *et al*, 1996). Recently, ultraviolet (UV) B irradiation has been shown directly to upregulate tropoelastin gene expression *in vivo* and *in vitro* (Uitto *et al*, 1997), and increased fibrillin expression and deposition have been reported within the reticular dermis of photoaged skin (Bernstein *et al*, 1994).

Elastic fibers are insoluble structural elements of connective tissues that have a central core of amorphous, hydrophobic cross-linked elastin surrounded by fibrillin-rich microfibrils (Mecham and Heusar, 1991). The process of elastic fiber formation is under tight developmental control with tropoelastin deposited upon a preformed microfibrillar template perinatally and during early development. In skin, the elastic fiber network forms a continuum from the dermal–epidermal junction (DEJ) to the deep dermis. It comprises thick elastin-rich fibers within the reticular dermis, a network of finer fibers with reduced elastin in the lower papillary dermis, and cascades of discrete microfibrillar bundles, without measurable elastin, in the upper papillary dermis terminating at the DEJ (Cotta-Periera *et al*, 1978; Dahlback *et al*, 1990). Fibrillin is a product of dermal fibroblasts, but keratinocytes also express fibrillin and assemble microfibrils (Haynes *et al*, 1997). Following keratinocyte autografting, a fine cascade of papillary dermal microfibrils is deposited in association with the DEJ (Raghunath *et al*, 1996). These studies demonstrate that loss of microfibrillar integrity in the upper dermis may contribute to the clinical appearance and reduced elasticity of photoaged skin. To examine this hypothesis, we have studied fibrillin expression and the distribution of fibrillin-rich microfibrils in sun-protected and sun-exposed skin of individuals with a clinical range of photoaging.

MATERIALS AND METHODS

Patients Sixteen healthy Caucasian volunteers (six male, 10 female; age range 24–80 y) were graded for overall severity of photoaging on the extensor forearm using an established 0–9 scale, where 0 is no clinically detectable photoaging and 9 is severe photoaging (Griffiths *et al*, 1992, 1993). Volunteers were separated into clinical groups: no clinical photoaging (grade 0; n = 3); minimal photoaging (grades 1–4; n = 3); and moderate to severe photoaging (grades 5–9; n = 10). Of the 10 individuals with moderate to severe photoaging, six had spent more than 2 y living and working abroad (India, Malaysia, and Australia). Eight of the 10 reported that they had previously been on sunbathing holidays (but not in the preceding 3 mo), although all admit to using sun-block and to wearing protective clothing. Seven patients had previously presented with skin tumors (six basal cell carcinomas; one squamous cell carcinoma) and one individual was a habitual user of a solarium. Of the minimally photoaged group, all reported to have been on sunbathing holidays, but similarly,

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these had not taken place within the previous 3 mo. None of the remaining individuals worked out of doors.

Four millimeter punch biopsies were taken from the extensor aspect of the forearm (photoaged) following 1% lidocaine local anesthesia, and from the photoprotected hip and upper inner arm (as an anatomic site control). Biopsies were snap frozen in liquid nitrogen and stored at −70°C until use. The study was approved by the Salford and Trafford local research ethics committee and all subjects gave written, informed consent.

***In situ* hybridization** Seven millimeters cryostat sections from biopsies taken from moderate to severely photoaged subjects were pretreated for *in situ* hybridization using standard protocols (Harrison and Pearson, 1990). Once pretreated (fixation in 4% paraformaldehyde, acetylation, serial dehydration and delipidation) sections were stored at −70°C until required. Fibrillin-1 anti-sense probe was 5′-ACA CAG GCC ATT TTT ACA CAC TCC TGG GAA-3′ and fibrillin-1 sense probe was 5′-TTC CCA GGA GTG TGT AAA AAT GGC CTG TGT-3′ (Pereira *et al*, 1993). Fibrillin-2 anti-sense probe was 5′-CAC AGT GAT ACC TAC TCC ACT ACT ACA AAG-3′ and fibrillin-2 sense probe was 5′-CTT TGT AGT AGT GGA GTA GGT ATC ACT GTG-3′ (Lee *et al*, 1991). Specificity of the chosen sequences (5′-untranslated sequences of both molecules) was checked using the MRC gene database and by northern blotting of total human skin RNA (Chomczynski and Sacchi, 1987; Farrell, 1993). Oligonucleotides (at a concentration of 2 pmol per ml) were 3′ tail-labeled with [^{35}S]αdATP using a commercially available kit (NEN/DuPont, Stevenage, U.K.). After purification, 10^6 cpm of labeled probe was applied to each section in hybridization buffer (4 × sodium citrate/chloride buffer; 1 × Denhardt's; 10 mM EDTA; 2 mg per ml denatured herring sperm; 1 mg per ml yeast tRNA; 1 mg per ml poly(A); 50% deionized formamide; 10% dextran sulfate; 20 mM phosphate buffer). Three sections per biopsy were hybridized for each anti-sense probe and one section for each control sense strand. Incubation was performed overnight and followed by stringency washes to remove unbound label. Incubation temperature (Ti) was 26°C and wash temperature (Tw) was 48°C. Following washing, sections were air-dried and message visualized by film autoradiography. Films were exposed for 21 d and developed (M35-M X-OMAT processor; Kodak, Hemel Hempstead, U.K.). Following film development, sections were subjected to autoradiographic emulsion (K5; Ilford, Mobberley, U.K.) to visualize cellular localization of mRNA. Sections were exposed for 6 wk and developed following standard protocols (Harrison and Pearson, 1990), stained with hematoxylin and eosin, and examined on a Leica Axioskop microscope. Quantitation of the number of cells positive for fibrillin-1 mRNA was done automatically using a Kontron KAT IBAS system with IBAS software (version 2.0) (Imaging Associates of Thame, Oxford, U.K.).

Quantitation of autoradiography Autoradiographic images were coded such that their anatomic origin was unknown and quantitated so as to produce mean optical densities for each section (Molecular Analyst, BioRad, Hemel Hempstead, U.K.).

Immunohistochemistry To ensure the clinical assessment, elastin was visualized in 7 μm frozen sections (OFT cryostat, Bright Instruments, Cambridge, U.K.) using standard peroxidase histochemistry with the monoclonal antibody BA4 (Sigma, Poole, U.K.; Wrenn *et al*, 1986). For visualization of the fibrillin-rich microfibrils at the DEJ, triplicate sections were fixed for 10 min in chilled 100% acetone. Following washes in Tris-buffered saline (TBS; 100 mM Tris, 150 mM sodium chloride), sections were solubilized in 0.5% Triton X-100 for 15 min. Sections were blocked with 3% normal goat serum (Vector Laboratories, Peterborough, U.K.) plus 3% bovine serum albumin (BSA) for 1 h at room temperature before application of a well-characterized primary polyclonal anti-fibrillin-rich microfibril antibody (5507; Shuttleworth *et al*, 1992; Waggett *et al*, 1993; Kielty *et al*, 1994; Raghunath *et al*, 1996). Titration experiments ascertained an optimal working dilution of 1:200. Incubation was performed overnight at 4°C. Following rigorous washes in TBS to remove unbound antibody, sections were incubated with an FITC-conjugated goat antirabbit IgG secondary antiserum (45 min at room temperature). Sections were stained with 4,6-diamino-2-phenylindole (DAPI) to identify nuclei and mounted in aqueous mounting media containing *p*-phenylene diamine (an "antifade" agent). Negative controls were performed for each biopsy by omission of primary antibody.

Quantitation of immunostaining Immunostained sections were coded and randomized such that the single investigator (REBW) reading them was unaware of their anatomic site of origin. The staining intensity and both abundance and continuity of the microfibrillar arrays extending from the DEJ to the deeper dermis were assessed in four high power microscopic fields for each section and were graded using a 0–4 ordinal scale: 0, absent; 1, minimum; 2, moderate; 3, high; and 4, maximum. In all cases, both photoprotected and photodamaged skin from each individual was quantitated.

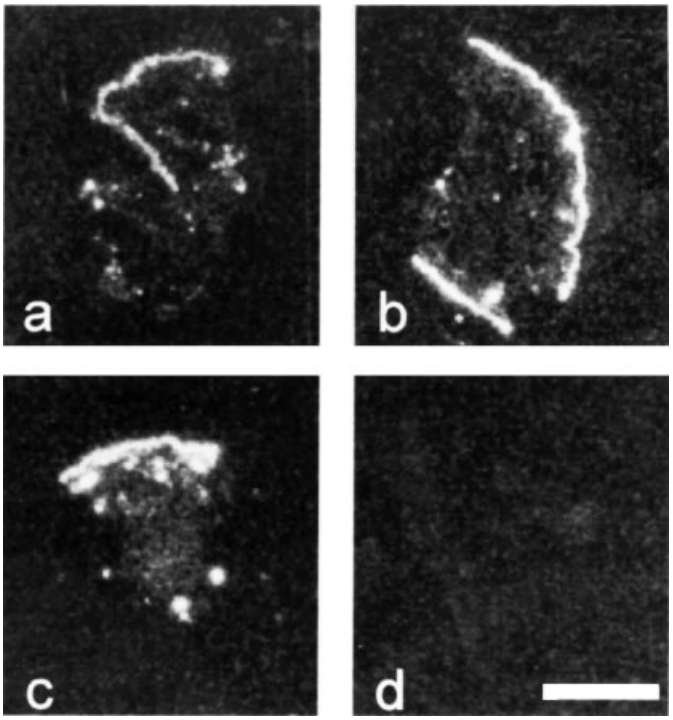

Figure 1. Fibrillin-1 mRNA in non-photoaged skin. Fibrillin-1 mRNA was detected in non-photoaged skin biopsies using [^{35}S]-labeled anti-sense and sense (control) oligonucleotide probes. Autoradiographs reveal abundant fibrillin-1 mRNA around the DEJ and in the dermis around hair follicles. Similar expression patterns were observed in photoaged skin. Fibrillin-1 mRNA localization to sun-protected hip (*a*), photoprotected upper inner arm (*b*), and non-photoaged forearm (*c*) from the same individual. Part (*d*) is control sense strand hybridization in photoprotected hip. *Scale bar*: ≈200 μm.

Confocal microscopy The same sections that were processed for immunofluorescence were also visualized by confocal microscopy (BioRad MRC 600 focal system). Fluorescence was visualized with an argon 25 mW laser (488 nm) to reveal the complete microfibrillar network running throughout the 7 μm specimen. Optical dissections (z series) were taken through the section every 0.7 μm (Kalman series, n = 8) with a mean pinhole diameter of 0.5 mm. Images were converted from BioRad plc format to Targa format and pseudo-colored using Confocal Assistant and Adobe PhotoShop, prior to printing on CL5000 film (Polaroid). These sections per specimen were merged to show extended focus views.

Statistical analysis Data were tested nonparametrically to compare fibrillin immunostaining among the three anatomic regions from sun-protected and photoaged skin using Friedman's two-way ANOVA. Summary data are expressed as mean ± SEM. Data were analyzed with the SPSS package (SPSS, Chicago, IL). Autoradiographic data were analyzed using Student's t-test to identify differences between photoprotected and photoaged groups of each individual (with significance taken at the 5% level).

RESULTS

Confirmation of clinical status Localization of elastin in forearm skin sections by immunohistochemistry from non-photoaged and severely photoaged individuals confirmed the accumulation of abundant elastotic material in the upper papillary dermis following chronic sun exposure (data not shown).

Fibrillin expression following chronic sun exposure *In situ* hybridization of photoprotected and photoaged skin showed that fibrillin-1 mRNA was present in both dermal fibroblasts and epidermal keratinocytes (**Figs 1–3**). Film autoradiography high-

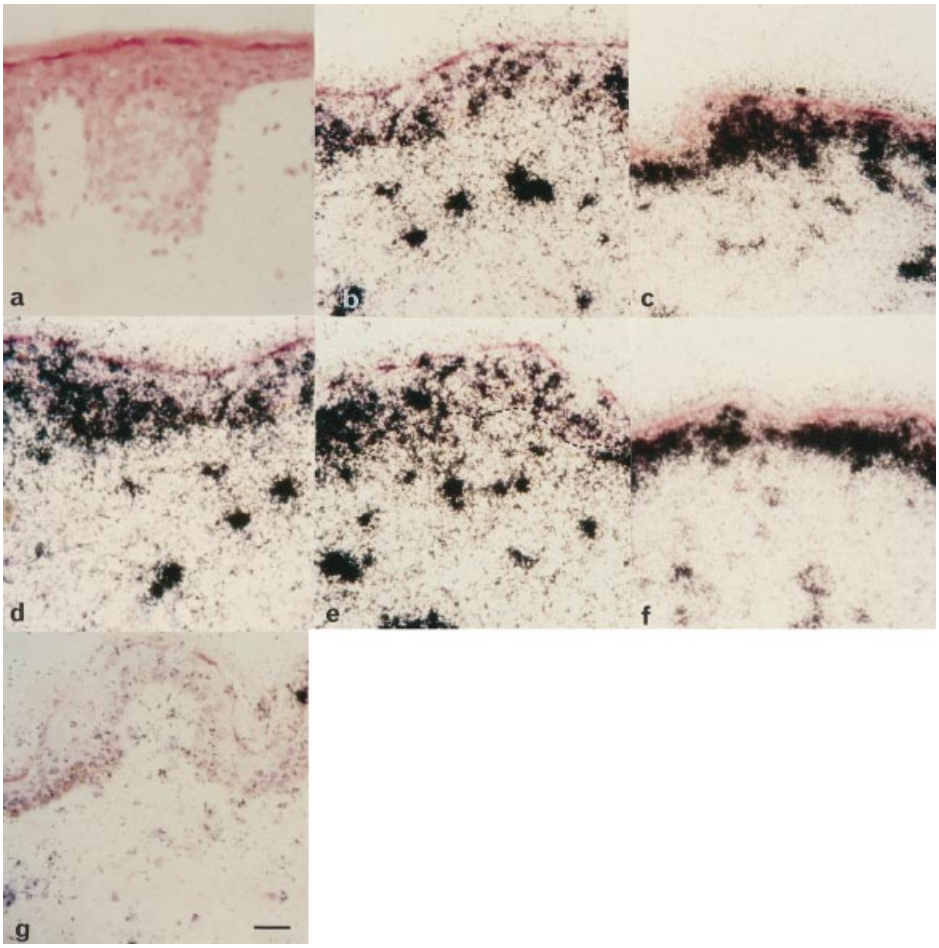

Figure 2. Cellular localization of fibrillin-1 mRNA. The cellular localization of fibrillin-1 mRNA in photoprotected skin and severely photoaged skin showed that keratinocytes were heavily labeled, and signals were also evident in dermal fibroblasts and around hair follicles. *Scale bar*: ≈25 μm. (*a*) Sense strand control; (*b*) photoprotected hip; (*c*) photoprotected upper inner arm; (*d*) non-photoaged forearm [parts (*a*)–(*d*) are from the same individual]; (*e*) photoprotected hip; (*f*) photoprotected upper inner arm; (*g*) severely photoaged forearm skin [parts (*e*)–(*g*) are from a second individual].

lighted that fibrillin-1 mRNA in skin from hip, upper inner arm and forearm was abundant around the DEJ and within the deeper dermis (**Fig 1**). In histologic sections of all skin sites, keratinocytes were heavily labeled, but signals were also evident in dermal fibroblasts and around hair follicles (**Fig 2**). Quantitation of label confirmed that the epidermal layer was more heavily labeled than the papillary dermis (**Fig 3**). Close examination showed that both hip and upper inner arm skin generally had higher levels of fibrillin-1 mRNA than forearm samples; there were no apparent differences between photoprotected areas. Fibrillin-1 mRNA expression was decreased in photoaged forearm compared with photoprotected hip and upper inner arm in eight of 10 moderate to severely photoaged individuals. This decrease was significant in two of these patients (**Fig 3**).

Low levels of fibrillin-2 mRNA were detected in dermis and epidermis of non-photoaged and minimally or severely photoaged skin biopsies (n = 10). Mean optical densities of 0.04 (hip), 0.035 (upper inner arm), and 0.045 (forearm) were recorded for severely photoaged skin (n = 6), and 0.015 (hip), 0.018 (upper inner arm), and 0.012 (forearm) were recorded for minimally photoaged skin (n = 3). There were no significant differences between photoprotected skin sites, or between minimally or severely photoaged forearm skin.

Microfibril changes in the upper dermis following photodamage Candelabra-like fibers extending perpendicularly from the DEJ into the papillary dermis were seen in all normal sections including photoprotected hip and upper arm and non-photoaged forearm (**Fig 4**). Severely photoaged extensor forearm sections also contained microfibrils at the DEJ, but in these sections the microfibrils were reduced in amount and appeared severely truncated (**Fig 4*f***) relative to the long arrays present in photo-

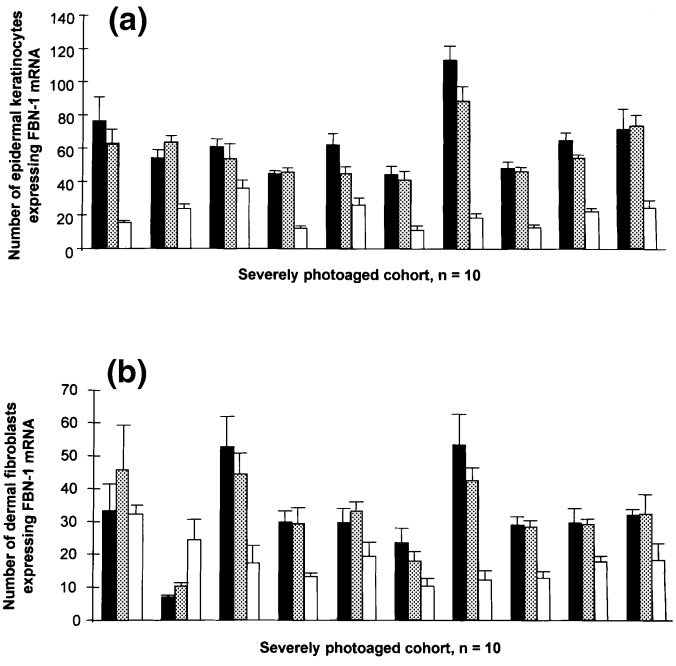

Figure 3. Keratinocytes and dermal fibroblasts show a reduction in the number of cells positive for fibrillin-1 mRNA. Histologic assessment of fibrillin-1 positive cells in individuals with moderate to severe photoaging (n = 10). (*a*) Epidermal keratinocytes; (*b*) dermal fibroblasts. Sites are photoprotected hip (*filled bars*), photoprotected upper inner arm (*shaded bars*), and photoaged forearm (*open bars*), respectively.

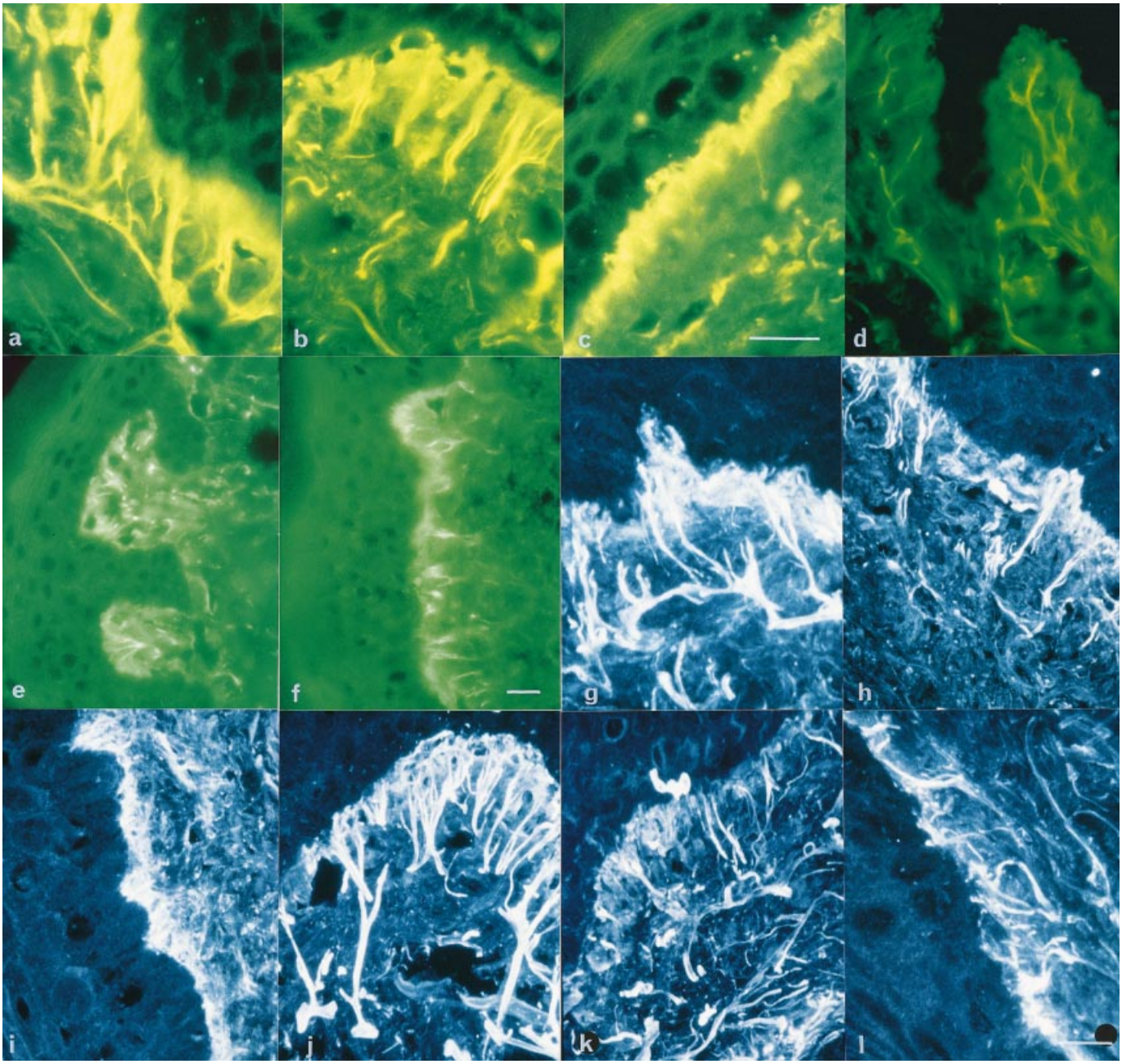

Figure 4. Fibrillin-rich microfibrils are reduced in photoaged forearm skin. Cascades of intertwining microfibrils from the DEJ into the papillary dermis are apparent in photoprotected skin at all biopsy sites. In contrast, in severely photoaged skin microfibril cascades are severely truncated, and immunopositive material is apparent only along the DEJ. The distribution and abundance of elastin in non-photoaged and photoaged forearm was also determined, both using immunohistochemistry and a histologic stain for elastin (orcein). In the non-photoaged skin, a network of elastin fibers are apparent within the deep dermis. In photoaged skin, abundant elastotic material is present throughout the dermis. Immunolocalizations were carried out on fresh frozen skin sections using a polyclonal anti-fibrillin-rich microfibril antibody (5507) or monoclonal anti-elastin antibody (BA4). Sections were visualized by fluorescence microscopy (*a–f*) and confocal microscopy (*g–l*). Fibrillin-rich microfibrils in skin from; photoprotected hip (*a, g*), photoprotected upper inner arm (*b, h*), and non-photoaged forearm (*c, i*) from the same individual. Immunolocalization of fibrillin-rich microfibrils in skin from photoprotected hip (*d, j*), upper inner arm (*e, k*), and severely photoaged forearm (*f, l*) of a second individual. *Scale bar*: ≈10 μm.

protected skin from the same patients (**Fig 4*d*, *e***) and in non-photoaged forearm skin (**Fig 4*c***). This analysis highlighted intraindividual differences between light-exposed and light-protected skin. Clinically, mild photoaged forearm samples showed similar marked disruption of the microfibrillar network (not shown). Semi-quantitative analysis confirmed that fibrillin-rich microfibril levels were significantly decreased in both severely and minimally photoaged forearm biopsies compared with sun-protected control sites ($p < 0.0001$ and $p < 0.05$, respectively).

Confocal microscopy of microfibrils subjacent to the DEJ confirmed and extended the light microscopy observations (**Fig 4*g–l***). Intact microfibrillar networks were observed in all sites from clinically non-photoaged skin and all photoprotected sites of patients with minimal or moderate-to-severe photoaging (**Fig 4*g–k***). In contrast, microfibrils were markedly disrupted and reduced in the papillary dermis of both minimally and severely photoaged skin (**Fig 4*l***).

DISCUSSION

The fibrillin-containing microfibrillar network of the papillary dermis, close to the DEJ is greatly re-organized and reduced following chronic sun exposure. Previous studies have examined

elastin and fibrillin distribution in the deep dermis of photoaged skin and showed that both of these matrix components are increased following chronic sun exposure (Bernstein *et al*, 1994; Schwarz *et al*, 1995). Little attention has been paid to the fibrillin-containing microfibrils (devoid of elastin), however, which are found in close proximity to the DEJ. In photoprotected skin (hip and upper inner arm) we observe a complex, intertwining system of small fibers running from the DEJ into the papillary dermis. This distribution is in agreement with that previously described in normal skin (Cotta-Periera *et al*, 1978; Dahlback *et al*, 1990). The distribution of fibrillin-positive fibers, however, in severely photoaged skin shows that, although immunohistochemically identifiable material is present along the DEJ, discrete microfibrils are markedly reduced in number. Examination of minimally photoaged skin reveals a similar marked loss of fibrillin-positive structures at the DEJ. Such data implies that this molecule is one of the first constituents of the dermal extracellular matrix to be affected or damaged by solar irradiation. Mutations in the genes encoding fibrillin-1 (FBN-1) and fibrillin-2 (FBN-2) have been shown to be causative in a number of inherited diseases such as Marfan syndrome (FBN-1; Lee *et al*, 1991) and congenital contractural arachnodactyly (FBN-2; Lee *et al*, 1991). Of interest in these diseases is the presentation of striae distensae in the skin (Pinkus *et al*, 1966; Aoyama *et al*, 1995). Recent work in this laboratory has identified reductions in the elastic fiber network in striae distensae, where both fibrillin microfibrils and elastin in the papillary dermis are affected equally (Watson *et al*, 1998). It, therefore, seems unlikely that the observed reduction in fibrillin-rich microfibrils in photoaged skin is due to any spatial confinement brought about by increased abnormal elastin production and deposition in the papillary dermis.

Fibrillin-1 expression by keratinocytes and dermal fibroblasts in photoprotected and photoaged skin suggests that both cell types contribute actively to maintaining the integrity of the microfibrillar network that extends from the DEJ into the papillary dermis. The presence of fibrillin-1 mRNA even in photoprotected adult skin further suggests that remodeling of the microfibrillar network occurs throughout adult life. Investigation of the distribution of fibrillin-1 mRNA-positive cells revealed extensive labeling within the epidermis. This labeling was not confined to basal keratinocytes, but extended into the granular cell layer. Such a distribution provides evidence that epidermal keratinocytes have potential to make a major contribution to the production of microfibrillar arrays in normal skin, similar to that observed with type VII collagen and anchoring fibrils in the same region. Examination of fibrillin-1 gene expression in skin derived from sites of severe photoaging indicates that long-term exposure to solar irradiation has major effects at the mRNA level. Decreases in the number of keratinocytes and dermal fibroblasts expressing this mRNA were observed in 80% of the severely affected extensor forearm biopsies examined. This decrease mirrors those observed in other major matrix components of the papillary dermis in photoaging; collagen VII-containing anchoring fibrils of the DEJ (Craven *et al*, 1997) and procollagens I and III (Talwar *et al*, 1995). It can be argued that this reduction is a general one, a result of the pathobiology of photoaging itself. It would appear, however, that not all matrix proteins within the papillary dermis are affected by solar irradiation. Studies examining the distribution of the microfibrillar collagen type VI and its mRNA expression in photoaged skin have identified little alteration with sun exposure (Boorsma *et al*, 1996). The distribution and role of fibrillin-2 in normal human skin has not been examined in detail. It is, however, known to be localized to predominantly elastic tissues such as cartilage and bronchal tissue (Boileau *et al*, 1997). Ashcroft *et al* (1997) examined the distribution and abundance of fibrillin-1, fibrillin-2, and elastin in wound healing. These studies reveal fibrillin-1 to be the major component of the microfibrillar arrays in skin repair following punch biopsies, with the distribution of fibrillin-2 mainly confined to areas within the dermis proximal to blood vessels. This is very much in accord with data presented here on expression of fibrillin-2 mRNA in photoaged skin. Fibrillin-2 is expressed in much lower amounts than fibrillin-1 and there appears to be little difference in the number of cells expressing it, nor in the level of expression in photoprotected *versus* photoaged skin.

Examination of severely photoaged skin provides data for understanding the end point of the pathologic process due to prolonged exposure to UV irradiation. Conclusions cannot be drawn from these data as to the effect of acute UV irradiation on the expression of either fibrillin-1 or fibrillin-2. It is well known that UV has the ability to modulate quickly the expression of elastin via transcriptional promoters (Schwarz *et al*, 1995, 1998; Bernstein *et al*, 1996; Uitto *et al*, 1997). It is, therefore, vital to examine further the effect of acute UV irradiation on the turnover of both these molecules *in vivo*.

Microfibril depletion observed as a result of prolonged, chronic UV exposure could reflect inflammatory cell proteinase activities such as neutrophil elastase, which we have previously shown capable of rapidly degrading intact microfibrils (Kielty *et al*, 1994). Fibrillin degradation is also possible by matrix metalloproteinases (Ashworth *et al*, 1999). Fisher *et al* (1997) have further shown that matrix metalloproteinase expression is enhanced in photoaged skin. It is, therefore, likely that the end process observed in photoaging is due (at least in part) to the effects of these enzymes. It remains to be determined if the microfibril-immunopositive material at the DEJ of photoaged skin is newly deposited fibrillin-rich microfibrils elaborated as a repair attempt similar to that previously shown to follow keratinocyte autografting of burns (Raghunath *et al*, 1996). Additional data need to be acquired to address this situation.

In conclusion, these studies suggest that the fibrillin-containing microfibrillar elements of the DEJ are susceptible to damage caused by solar irradiation, likely to be due to the activity of matrix degrading enzymes, and that such alterations may contribute to some of the clinical characteristics of chronically sun-exposed skin.

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