Enhanced Elastin and Fibrillin Gene Expression in Chronically Photodamaged Skin

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Cutaneous aging consists of chronologic aging as well as actinic damage, referred to as photoaging. Most of the morphologic changes associated with an aged appearance result from actinic damage to the skin. The morphologic changes in sun-damaged skin are associated with accumulation of material having the staining characteristics of elastin, known as solar elastosis, in the superficial dermis. Previous studies have demonstrated the presence of elastin within areas of solar elastosis; however, little is known about the mechanisms leading to elastin accumulation in photoaged skin. In addition, fibrillin, the fibrillar component of elastic fibers, has been found in small amounts in solar elastosis. In this study we demonstrate increased elastin mRNA levels in photoaged skin, as well as increased elastin and fibrillin mRNAs in skin explant-derived fibroblasts using Northern hybridizations, compared with controls from sun-protected sites of the same individual. Increased elastin mRNA levels result from transcriptional upregulation of the gene, as demonstrated by transient transfections with a human elastin promoter/chloramphenicol acetyltransferase construct. Elevated mRNA levels were also correlated with increased elastin and fibrillin deposition in paired biopsy specimens from photodamaged and non-sun-exposed skin, as demonstrated by immunohistochemical staining. Thus, approaches to counteract transcriptional activation of elastin gene expression may be useful in preventing the changes associated with cutaneous photoaging. *Key words: ultraviolet irradiation/cutaneous aging/elastic fibers/fibrillin/elastin. J Invest Dermatol 103:182–186, 1994*

Cutaneous aging consists of two components: chronologic aging, also known as innate or intrinsic aging, and actinic damage, referred to as photoaging. The clinical manifestations of chronologic aging include sagging and fine wrinkling of the skin, and reduced elasticity and recoil [1]. Superimposed on chronologic aging are the degenerative changes seen in sun-exposed skin resulting from actinic damage [2,3]. Most of the morphologic changes associated with an aged appearance result from photodamage [4,5]. Clinically, sun-damaged skin appears leathery and thickened with deep furrows.

When examined histopathologically, sun-exposed skin contains material that stains similarly to elastin, and the pathologic process is referred to therefore as solar elastosis [1–10]. Although this material stains strongly with elastic tissue stains [1–10], such as Verhoeff–van Gieson stain, its precise biochemical composition is unknown. Immunohistochemical analyses of sun-exposed skin have shown solar elastosis to be composed primarily of elastin [9,10], although the presence of several other matrix components, including microfibrillar antigen [9] or fibrillin [11], has been demonstrated. These conclusions have been supported by demonstration of elevated concentrations of desmosines, elastin-specific cross-link compounds in sun-exposed skin [12]. Elastin functions physiologically to provide resiliency to skin. Ultrastructural studies have demonstrated loss of normal elastic fiber architecture in photodamaged skin [1,4,8], apparently contributing to its loss of resilience and elasticity.

Although chronologic aging and photoaging result from different biologic mechanisms [8,12,13], it is clear that investigations of sun-induced changes found in skin may give insight into environmental factors that contribute to the aging process [7]. Consequently, in this study we have examined the expression of the elastin and fibrillin genes in photodamaged skin in comparison with sun-protected skin from the same individuals, thus negating the contribution of chronologic aging. Utilizing Northern analyses and transient transfections with a human elastin promoter/reporter gene, chloramphenicol acetyltransferase (CAT) construct, we demonstrate that transcriptional upregulation of elastin gene expression in photoaged skin is responsible for increased elastin expression. These changes are correlated with increased elastin and fibrillin deposition in photodamaged skin, compared with non-sun-exposed control sites from the same individual.

MATERIALS AND METHODS

Subjects Sixteen male volunteers between 49 and 66 years of age were recruited from a Veterans Administration hospital. The study protocol was approved by the Jefferson Medical College Institutional Review Board for protection of human subjects. All individuals enrolled in the study had significant clinical evidence of chronic sun exposure. Specifically, the sun-exposed skin of the posterior neck was deeply furrowed and leathery, clinically diagnosed as cutis rhomboidalis nuchae. Sun-protected buttock skin from the same individuals was without clinical evidence of photodamage. Four-millimeter punch biopsies were taken from the neck and buttock skin

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of each volunteer. All samples were analyzed as pairs, i.e., one from sun-damaged neck skin and one from nonexposed buttock skin.

Cell Cultures Dermal fibroblasts were cultured by explanting tissue specimens and were utilized in the first passage. The cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (FBS), 2 mM glutamine, and antibiotics.

Northern Analyses Total RNA was isolated from paired frozen skin biopsy specimens from three volunteers (two from each site) or from fibroblast cultures derived from three additional volunteers, as previously described [14]. RNA was analyzed by Northern hybridization with ^{32}P-labeled 3.2-kilobase pair (kb) full-length human elastin (cHDE1) [15] and a 1.6-kb human fibrillin (MF-13) [16] cDNA probe. The [^{32}P]cDNA-mRNA hybrids were visualized by autoradiography, and the steady-state levels of mRNA were quantitated by scanning densitometry using a He-Ne laser scanner at 633 nm (LKB Produkter, Bromma, Sweden). Elastin and fibrillin mRNA levels were standardized to 7S rRNA levels in the same RNA samples. Due to the smaller amounts of mRNA isolated from frozen biopsy specimens, fibrillin mRNA levels were below the threshold for measurement on these samples, but could be measured in fibroblasts cultured from tissue explants.

Transient Transfections of Cultured Cells Pairs of fibroblast cultures derived from two volunteers were transfected in late logarithmic growth phase with 20 μg of plasmid DNA, pEP62/CAT, which contains 0.5 kb of 5′-flanking DNA region of the human elastin gene linked to the CAT reporter gene [17]. The transfections were performed with the calcium-phosphate/DNA coprecipitation method [18,19], followed by a 1-min (15%) glycerol shock [20]. After the glycerol shock, the cells were placed in medium supplemented with 10% FBS for 30 h. The cells were then harvested and lysed by three cycles of freeze-thawing in 100 μl of 0.25 M Tris-HC1, pH 7.8. The protein concentration of each extract was determined with a commercial assay kit (BioRad Laboratories, Richmond, CA), and identical aliquots (40 μg) were used for the CAT assay using [^{14}C]chloramphenicol as substrate [18,19].

Immunohistochemical Staining Paired punch biopsy specimens from eight volunteers were placed immediately into a modified Carnoy's fixative. Samples were dehydrated through a graded series of isopropanol over the next 2 h, impregnated with paraffin-amyl acetate, and embedded in Paraplast (Monoject Scientific, St. Louis, MO). Serial sections of 8-μm thickness were mounted onto glass Probe-on slides (Fisher Scientific Co., Philadelphia, PA) precoated with Cell-Tak cell and tissue adhesive (Collaborative Research Inc., Bedford, MD). Mounted sections were then baked to dryness. Before use, the sections were deparaffinized and rehydrated with a graded series of ethanol.

Antigenic sites crosslinked during formalin fixation were unmasked using Pepsin Reagent solution (Fisher Scientific), which was applied for 10 min at 37°C. After rinses in phosphate-buffered saline (PBS), sections were reacted with either an elastin or fibrillin antibody diluted 1:50 in 10% goat serum blocking solution (Zymed Laboratories, San Francisco, CA). The elastin antibody was a commercially available monoclonal antibody (Sigma Chemical Company, St. Louis, MO). The fibrillin polyclonal antibody was raised in rabbits against fibrillin, the gene of which is localized to chromosome 15 (fibrillin-1), and demonstrated no cross-reactivity with elastin or other fibrillins. This fibrillin demonstrates a high degree of expression in normal adult skin [21]. Following additional rinses in PBS, immunolabeling was visualized by means of the Histostain-SP kit (Zymed Laboratories), which employed a broad-spectrum biotinylated secondary antibody and a horseradish peroxidase-streptavidin conjugate. Negative control samples were incubated with pre-immune sera instead of primary antibody and subjected to identical processing.

Sections were then examined by Nomarski differential interference contrast optics using a BH-2 microscope (Olympus, Lake Success, NY), and photographed with a Wratten 80B filter (Kodak, Rochester, NY). Sections were viewed by an observer blinded to their site of origin, and scored for degree of staining in the superficial to mid-dermis. Degree of staining in the superficial to mid-dermis was measured using a 7-point scale, similar to the method of Griffiths *et al* [22]. A score of 0 indicated no increased staining over baseline staining of fibers in a pattern similar to the deep dermis, and a score of 6 represented maximal confluent staining of the superficial dermis. Scores were compared using a paired Student t test analysis.

RESULTS

Northern Blot Analysis To examine the elastin gene expression at the mRNA level, total RNA was extracted directly from the biopsy specimens, and elastin mRNA levels were determined by Northern hybridizations. Photoaged neck skin demonstrated marked elevation of elastin mRNAs, compared with sun-protected buttock skin, after correction for 7S rRNA (Fig 1). Up to a 2.6-fold increase was seen in elastin mRNA expression measured from tissue samples taken from photoaged skin, compared with sun-protected skin from the same individual (Fig 1*b*). This increase in elastin mRNA expression persisted in the first passage of fibroblast cultures established from explants of sun-damaged skin, which revealed up to a 5.3-fold increase in elastin gene expression in photoaged skin (Fig 1*a*). Up to a 2.5-fold increase in fibrillin expression was measured in mRNA derived from fibroblast cultures (Fig 2).

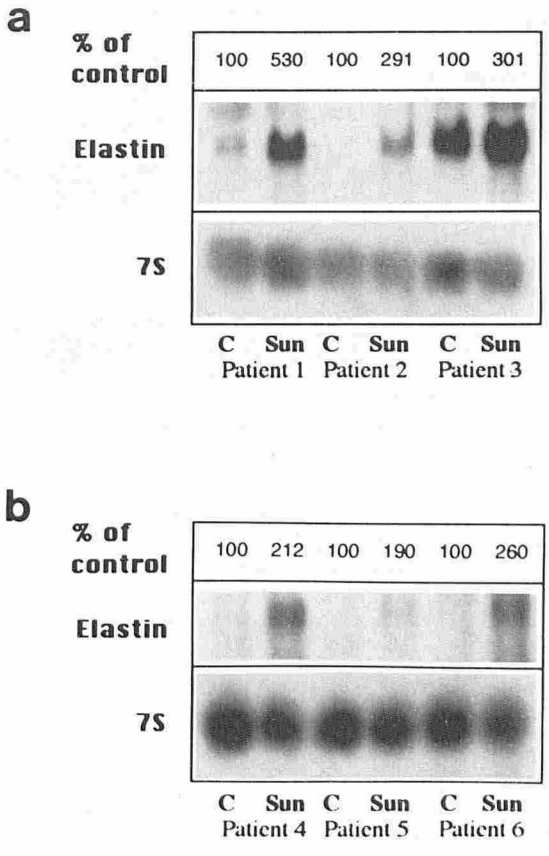

Figure 1. Elastin mRNA is increased in photoaged skin. *a*) Northern analysis, performed on pairs of first-passage fibroblast cultures established from three subjects, taken from photoaged neck skin and sun-protected buttock skin of each subject, reveals a mean increase in elastin mRNA of 420% in photoaged skin ($p < 0.01$; Student t test, *b*). Direct extraction of RNA from tissue revealed a mean increase of 220% in elastin mRNA content as compared with control non-sun-exposed buttock skin ($p < 0.01$; Student t test, *b*). All mRNA values are corrected for 7S rRNA content as shown.

Transient Transfections of Cultured Cells To examine the mechanisms leading to elevated expression of the elastin mRNA steady-state levels in photoaged skin, first-passage fibroblast cultures were subjected to transient transfections with a human elastin promoter/CAT reporter gene construct. Paired assay of CAT activity demonstrated marked enhancement of elastin promoter activity in photoaged skin, compared with fibroblasts established from a sun-protected site (Fig 3). Elastin promoter activity was increased up to 5-fold, correlating with the up to 5.3-fold increase in elastin mRNA levels in cells from photoaged skin (Fig 1*a*).

Immunohistochemical Staining To examine elastin and fibrillin expression at the protein level, paired biopsy specimens from photoaged and control non-sun-exposed skin were stained with antibodies recognizing human elastin and fibrillin.

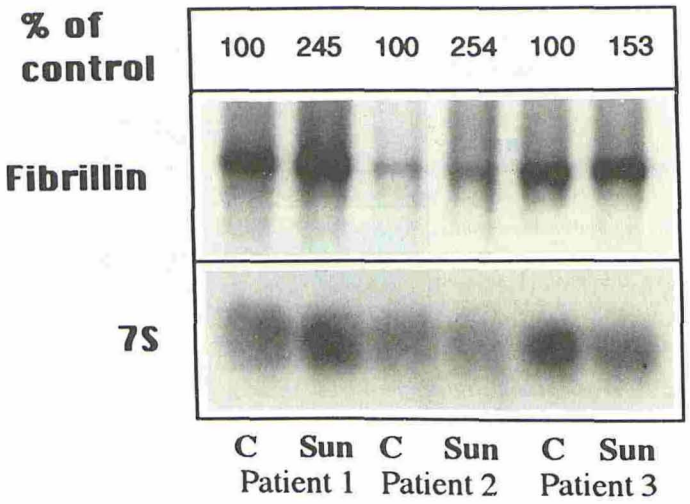

Figure 2. Increased fibrillin mRNA in photoaged skin. Northern analysis performed on pairs of first passage fibroblast cultures as shown in Fig 1*a*, reveals a mean increase in fibrillin mRNA of 217% in photoaged skin (sun) ($p < 0.05$; Student t test). All mRNA values are corrected for 7S rRNA content as shown.

Elastin: Elastin immunoreactivity in sun-protected skin was found in the papillary dermis just below the basement membrane and was in the pattern of small-diameter fibers mostly oriented perpendicular to the epidermis (Fig 4*a*). In deeper dermis, the fibers were thicker and more vertically oriented. Intense staining was also seen surrounding adnexal structures as well as in some of the larger vessels in the deep dermis and adipose tissue. The mean score for elastin staining in the papillary dermis of sun-protected skin was 0.62 ± 0.52 (mean $\pm$ SD, $n = 8$).

Photoaged skin demonstrated the similar small-diameter fibers just below the basement membrane within a zone lacking excessive staining (grenz zone), which was of variable thickness. Beneath this area of relatively sparse staining was a more homogeneously stained region of poorly formed, clumped, thick fibers (Fig 4*b*). Staining was clearly associated with the fragmented fibers present in areas of solar damage. This staining pattern occupied the superficial to mid-dermis, below which staining again resumed the pattern of well-defined, horizontally oriented fibers seen in sun-protected skin. The mean score for elastin staining in the superficial dermis was 5.0 ± 0.76 (mean $\pm$ SD). This represents an approximately eight-fold increase in elastin immunostaining in sun-damaged skin, compared with protected skin from the same individual ($p < 0.000001$). Deep dermal fibers both in sun-protected and exposed skin stained intensely. Sections treated with pre-immune serum demonstrated no staining.

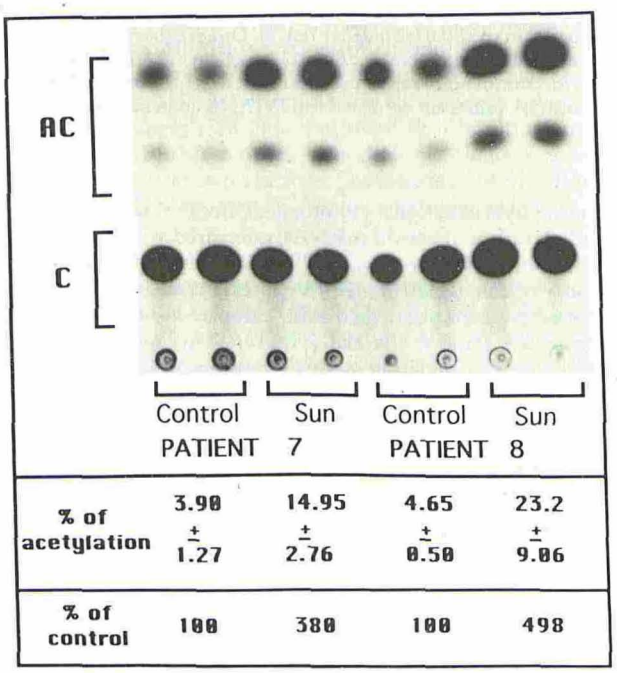

Figure 3. Elevated elastin promoter activity in fibroblasts from photoaged skin. Increased elastin promoter activity was demonstrated in the first-passage cell cultures subjected to paired transient transfections with a human elastin promoter/CAT construct taken from photoaged skin (sun), compared with cells from sun-protected sites in the same individuals (control). The mean increase in elastin promoter activity of 439% ($p < 0.01$; Student t test) agrees quite well with the 420% increase in elastin mRNA demonstrated in parallel cultures from photoaged skin of three different subjects shown in Fig 1*a*.

Fibrillin: Fibrillin immunostaining in sun-protected skin demonstrated a pattern similar to that of elastin. However, the fiber network in the zone just beneath the basement membrane was somewhat more extensive, finer, and closer to the basement membrane than that seen in sections stained for elastin (Fig 4*c*). The mean score for fibrillin staining in the papillary dermis of sun-protected skin was 0.75 ± 0.46 (mean $\pm$ SD). Fibrillin staining patterns in the deep dermis were virtually indistinguishable from those seen with elastin. Staining around adnexal structures and larger blood vessels was also similar (Fig 4*e*,*f*).

Photoaged skin also revealed an area of intense staining in the superficial to mid-dermis below a grenz zone of variable thickness. Staining of the superficial to mid-dermis was somewhat less intense than that seen with elastin stain but demonstrated an almost identical pattern (Fig 4*d*). Below the area of intense staining, the pattern again resembled the well-defined, horizontally oriented fibers seen in sun-protected skin. Staining was clearly associated with the fragmented fibers present in areas of solar damage. The mean score for fibrillin immunostaining in the superficial dermis of photoaged skin was 3.1 ± 0.64. This represents an approximately 4.2-fold increase in staining, compared with protected skin from the same individual ($p < 0.0001$). Immunostaining of deep dermal fibers was somewhat more intense than the elastotic material. Sections treated with pre-immune serum showed no staining.

DISCUSSION

Although several components of the extracellular matrix have been found to be altered in photodamaged skin, elastin is thought to be the most prominent. The histopathologic finding in photodamaged skin, when examined with routine hematoxylin-eosin staining, is an amorphous blue staining of the superficial to mid-dermis, referred to as solar elastosis [6,7,9,10]. This material stains intensely with elastic tissue stains, such as Verhoeff-van Gieson stain [1,2,5,8–10]. Immunohistochemical staining of sun-damaged skin has demonstrated the presence of elastin [9,10] as well as microfibrillar antigen [9]. Weak fibrillin staining has been shown within areas of solar elastosis [11]. Further evidence implicating elastin as a prominent component of photoaged skin is a fourfold increase in desmosine content in sun-exposed skin when compared with non-exposed skin in the same individual [12]. Desmosine is a lysine-derived cross-link compound present only in elastin among mammalian proteins and in relatively constant amounts, approximately 1.5 residues per 1000 amino acids in normal elastic fibers. Thus, its concentration in tissues reflects the amount of cross-linked elastin present [23].

Despite the numerous studies implicating elastin as the major component of solar elastosis, little is known about the mechanism and regulation of elastin production within photoaged skin. In this study, we found that fibrillin and elastin are greatly increased in photodamaged skin, compared with control skin from the same

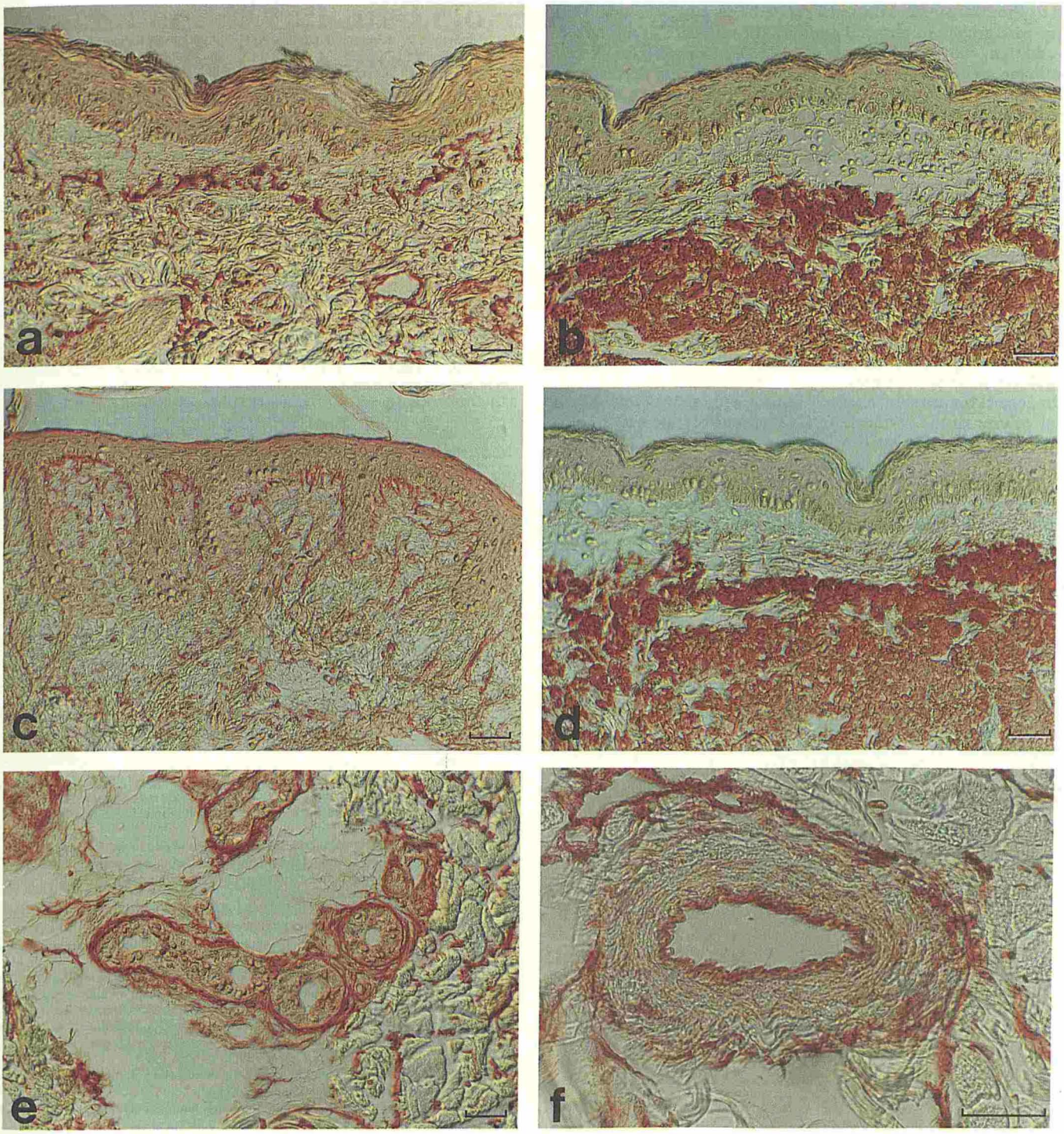

Figure 4. Increased elastin and fibrillin in photoaged skin is demonstrated by immunohistochemical staining. *a)* Staining of sun-protected skin for elastin reveals discrete fibers below the epidermis and sparsely throughout the dermis. *b)* Photoaged skin stained for elastin demonstrates a dense accumulation of large, clumped fibers in the superficial dermis. *c)* Staining for fibrillin in sun-protected skin reveals a network of fine fibers with increased density just below the dermal-epidermal junction. *d)* Fibrillin immunostaining of photoaged skin shows a pattern almost identical to that of elastin with large, clumped fibers in the superficial dermis. *e)* Fibrillin immunostaining reveals dense bands surrounding adnexal structures, elastin immunostaining showed essentially identical staining *(f)*. The internal and external elastic lamina of larger blood vessels located at the dermal-subcutaneous junction was evident on immunostaining for fibrillin. Elastin immunostaining revealed an identical pattern. *Bar,* 50 μm.

individual. These changes were accompanied by increased elastin and fibrillin mRNA levels in fibroblasts derived from tissue culture explants and increased elastin mRNA, as measured by direct extraction of RNA from biopsy samples. Fibroblasts derived from tissue culture explants, in the first passage, demonstrated a greater increase in elastin mRNA versus controls than did direct extraction of mRNA from biopsies. This difference may be due to differential rates of proliferation or survival between the superficially located cells sub-

ject to photodamage and those deeper in the dermis, although use of early-passage cells might well diminish the influence of this factor. In addition, the small amounts of mRNA extracted from biopsy specimens, as compared with cultured fibroblasts, may decrease the sensitivity of the results from the former. However, both methods demonstrated increased elastin mRNA in photoaged, compared with non-sun-exposed, skin. These data suggest that matrix expression *in vivo* may be reflected in tissue culture, at least in early-passage cells.

Transient transfections of fibroblast cultures derived from sun-damaged skin using an elastin promoter/CAT construct revealed an approximately fourfold increase in elastin promoter activity, compared with cultures grown from protected skin in the same individual. Quantitatively, this fourfold increase accounts for the fourfold elevation in elastin mRNA in fibroblast cultures, and correlates with the previously described fourfold increase in desmosine content of photodamaged skin [24]. Elastin mRNA levels as measured directly from extraction of mRNAs from biopsy specimens were somewhat less elevated, up to 2.6 times.

In normal human skin, elastin comprises only about 2–4% of the total volume of the dermis [25,26]. The fourfold increase in promoter activity, elastin mRNA, and desmosine content [23] of sun-exposed skin does not account for the massive accumulation of the elastotic material seen histopathologically in the superficial and mid-dermis. This suggests that most of the material staining as elastin in photoaged skin is not composed of normal cross-linked elastic fibers with the physiologic properties of elastin [13]. This discrepancy could have several explanations. First, it is possible that elastin degradation does not keep pace with increased production of elastin, resulting in massive accumulation of partially degraded elastic fibers. Secondly, degradation of desmosine may account for the inability of desmosine determinations to reflect the dramatic increase in elastin accumulation. Finally, although elastin may be the major component of solar elastosis, other matrix components have been shown to be present in actinically damaged skin as well [9].

Upregulation of elastin and fibrillin mRNAs is accompanied by a coordinate increase in elastin and fibrillin in the superficial dermis of photoaged skin. This excess material may be responsible for some of the clinical manifestations of aging skin. Further studies to identify the specific factors responsible for increased elastin promoter activity and elevated fibrillin mRNA will allow more precise insight into the pathogenesis of photoaging. By understanding the mechanisms of altered matrix deposition in photoaged skin, therapeutic strategies may then be developed to counteract the detrimental effects of sunlight on skin. Approaches to normalize elastic fiber synthesis, such as downregulation by cytokines and growth factors, or by anti-sense RNA technologies, could potentially prevent or reverse some of the degenerative changes associated with cutaneous aging.

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