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Proteins of the Extracellular Matrix Are Sensitizers of Photo-oxidative Stress in Human Skin Cells

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Sensitized production of reactive oxygen species after photo-excitation of endogenous chromophores is thought to contribute to skin photo-oxidative stress. Here we present experimental evidence in support of a potential role of extracellular matrix proteins as skin photosensitizers. Human and bovine type I collagen and elastin sensitized of hydrogen peroxide generation upon irradiation with solar simulated light or ultraviolet A. Induction of intracellular oxidative stress by extracellular matrix-protein sensitization was demonstrated by flow cytometric analysis of fibroblasts preloaded with the intracellular redox dye dihydrorhodamine 123 and exposed to pre-irradiated type I collagen. Pre-irradiated collagen and elastin induced pronounced inhibition of proliferation in cultured keratinocytes and fibroblasts, which was reversed by antioxidant or catalase treatment and reproduced by exposure to concentrations of H_2O_2 formed during extracellular matrix-protein irradiation. In fibroblasts, chromosomal DNA damage as a consequence of collagen-sensitized H_2O_2 formation was demonstrated using a single cell electrophoresis assay. The enzymatic cross-links pyridinoline and desmosine were examined as candidate sensitizer chromophores contained in collagen and elastin, respectively. Pyridinoline, but not desmosine, sensitized light-driven H_2O_2 production and inhibition of fibroblast proliferation. Our results support the hypothesis that extracellular matrix proteins play a functional role in skin photoaging and carcinogenesis by sensitization of photo-oxidative damage. ***J Invest Dermatol 121: 578–586, 2003***

A large body of experimental evidence supports the hypothesis that the ultraviolet (UV) component of solar radiation induces biologic effects such as skin photoaging and carcinogenesis by photo-oxidative mechanisms (Tyrrell, 1995; Scharffetter-Kochanek *et al*, 1997) that can be counteracted by effective skin antioxidant systems (Black, 1993; Thiele *et al*, 2001). Both UVA (320–400 nm) and UVB (290–320 nm) irradiation induce photo-oxidative stress in skin; however, in contrast to the formation of mutagenic pyrimidine base photoproducts as a consequence of direct absorption of UVB irradiation by skin cell DNA, UVA irradiation results in little photoexcitation of DNA directly and generation of reactive oxygen species (ROS) seems to be the key mechanism of UVA phototoxicity (reviewed in Gasparro, 2000). As most of the UV sunlight reaching the earth's surface (>95%) is in the deeply penetrating UVA region, solar irradiation-induced photo-oxidative stress effectively reaches into the human dermis (Sander *et al*, 2002). UV-driven ROS production has been detected in cultured human skin cells (Peus *et al*, 1998; Gniadecki *et al*, 2000; Peus and Pittelkow, 2001), skin homogenates (Nishi *et al*, 1991), and intact skin (Yasui and Sakurai, 2000).

Although ROS are widely accepted as crucial mediators of UV phototoxicity, the exact mechanism of their generation in irradiated skin is poorly understood and seems to be dependent on the presence of non-DNA chromophores acting as photosensitizers (Sander *et al*, 2002). Photosensitization occurs as a consequence of initial formation of excited states of chromophores and their subsequent interaction with substrate molecules (type I photoreaction) or molecular oxygen (type II photoreaction) by energy and/or electron transfer (Foote, 1991). Various chromophores contained in human skin have been proposed as endogenous UV sensitizers of photo-oxidative stress (Dalle Carbonare and Pathak, 1992; Scharffetter-Kochanek *et al*, 1997; Kipp and Young, 1999).

Upon UV photo-excitation human skin reveals a strong tissue auto-fluorescence indicative of the presence of various fluorescent UV chromophores, which in the dermis are mainly attributed to extracellular matrix (ECM) protein bound heterocyclic cross-links of enzymatic and nonenzymatic origin (Tian *et al*, 2001). Fiberoptic fluorescence excitation measurements on hairless mouse skin *in vivo* revealed (1) a dominant excitation maximum at 295 nm originating in the epidermis, attributable to tryptophan, and (2) two additional excitation maxima at 335 nm and 370 nm predominantly originating in the dermis, attributed to the presence of ECM protein fluorophores on collagen and elastin (Kollias *et al*, 1998). Recently, various investigators demonstrated that certain cross-link-chromophores called advanced glycation end products (AGE), which accumulate on long-lived dermal ECM proteins in human skin as a consequence of chemical damage by reactive carbonyl species (Dyer *et al*, 1993; Jeanmaire *et al*, 2001),

Manuscript received October 28, 2002; revised March 14, 2003; accepted for publication April 3, 2003

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Abbreviations: AGE, advanced glycation end products; BCA, bicinchoninic acid; DHR 123, dihydrorhodamine 123; ESI-MS, electrosprayionization-mass spectrometry; NBF, nitroblue diformazan; D-P, D-penicillamine; NBT, nitroblue-tetrazolium; SSL, solar simulated light.

may play an active part as mediators of skin phototoxicity acting as potent sensitizers of photo-oxidative stress (Masaki *et al*, 1999; Wondrak *et al*, 2002a, b). In our experiments using human fibroblasts, AGE-modified collagen strongly sensitized the anti-proliferative effects of irradiation with low doses of solar simulated light (SSL) and UVA, and light-driven formation of hydrogen peroxide (H_2O_2) was identified as the mechanism of AGE phototoxicity supporting the general hypothesis that ECM-bound AGE chromophores are endogenous sensitizers of UV phototoxicity in human skin. Unexpectedly, our preliminary observations revealed a significant formation of ROS upon SSL irradiation of ECM proteins (collagen and elastin) not modified with AGE, an effect not observed with other nonglycated proteins (Wondrak *et al*, 2002a). This observation lead us to test further the hypothesis that ECM proteins may play an active part as sensitizers of photo-oxidative damage in human skin. In this study, we examined the phototoxic effects of native nonglycated ECM proteins on skin cell proliferation and genomic integrity and describe a molecular mechanism of ECM protein photosensitization.

MATERIALS AND METHODS

Chemicals All chemicals were purchased from Sigma (St Louis, Missouri) and ECM proteins were of the highest purity commercially available: collagen, type I, from human placenta (Sigma, C7774); collagen, type I, from bovine achilles tendon (C9879); collagen, type I, from rat tail (C8897); collagen, type I, from calf skin (C3511); elastin, from bovine neck ligament (E1625). Desmosine, and acid-soluble human type I collagen from adult abdominal skin were obtained from Elastin Products Company, Inc. (Elastin Products Company, Inc., Owenville, MO). Purity of this human collagen preparation was at least 95% when examined by 7.5% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) analysis with residual low molecular weight fragments presumably from proteolytic degradation as specified by the manufacturer. Pyridinoline isolated from acid hydrolysates of bovine bone collagen (Robins, 1983) was kindly provided by Dr Simon Robins, The Rowett Research Institute, UK. The purity and identity of the preparation employed in the sensitization experiments was confirmed by UV spectroscopy, fluorescence spectroscopy, and electrospray mass spectrometry (ESI-MS; m/z 429, M^+) using a LCQ Classic quadrupole ion trap mass spectrometer from Thermo Finnigan (Thermo Finnigan, San Jose, CA).

Cell culture The established cell line of human epidermal keratinocytes (HaCaT cells), a gift from Dr Norbert Fusenig (German Cancer Research Center, Heidelberg, Germany), and human dermal fibroblasts (CF-3 cells), a gift from Dr Robert Dell'Orco (Noble Center for Biomedical Research, Oklahoma City, Oklahoma) were routinely cultured in Dulbecco minimal Eagle's medium (DMEM) containing 10% fetal bovine serum and kept in a humidified atmosphere containing 5% CO_2 at 37°C. HaCaT cells were maintained in exponential growth by subculture twice weekly at a split ratio of 1:10. Cells used for experiments were of passage number 0 to 20 measured from thawing. CF3 cells, a diploid cell line, were maintained in exponential growth by subculture twice weekly at a split ratio of 1:4. Cells used for experiments had undergone between seven and 30 population doublings measured from thawing. During the 72 h cell growth inhibition experiment, control cells always underwent the same number of population doublings in each individual experiment.

Keratin extraction from cultured human skin keratinocytes Total keratin was extracted from HaCaT keratinocytes according to a standard procedure (Katagata *et al*, 1999). HaCaT cells (approximately 6×10^6) in exponential growth were washed twice with phosphate-buffered saline (PBS), collected by scraping with a rubber policeman and suspended in 3 mL ice-cold extraction buffer (10 mM Tris–HCl, pH 7.4, 10 mM ethylenediamine tetraacetic acid, 0.4 mM phenylmethylsulfonyl fluoride). After 15 s sonication using an ultrasonic disrupter (Vibracell from Sonics Materials, Inc., Danbury, Connecticut) and centrifugation at $8000 \times g$ at 4°C for 10 min, the resulting pellet was re-extracted by sonication and centrifugation four additional times. The final residue was suspended in distilled water and analyzed by reducing 12% SDS–PAGE revealing the presence of various keratin bands between 45 and 70 kDa.

Collagen extraction from cultured human skin fibroblasts A long-term culture of dermal human fibroblasts known to produce an organized matrix (termed "dermis-like structure") composed of type I collagen as a major constituent was performed according to a standard procedure (Hazeki *et al*, 1998). Human dermal fibroblasts (CF3) were seeded at 5×10^4 cells per dish on 35 mm dishes (Falcon, Franklin Lakes, New Jersey) and grown in DMEM containing 10% fetal bovine serum, supplemented with 0.2 mM ascorbate-2-phosphate, a potent stimulator of type I collagen production by fibroblasts. The culture medium was replaced with fresh medium twice a week. After 4 wk the dermis-like structure was washed twice with PBS, collected by scraping with a rubber policeman and suspended overnight at 4°C in 20 mM Na_2HPO_4 with 0.02% (w/v) sodium azide containing a protease inhibitor cocktail composed of 5 mM ethylenediamine tetraacetic acid, 1 mM *N*-ethylmaleimide, and 100 μM phenylmethylsulfonyl fluoride. The suspension was centrifuged at $16{,}000 \times g$ for 10 min and the pellet was resuspended overnight at 4°C in distilled water containing the protease inhibitor cocktail. After centrifugation at $16{,}000 \times g$ for 10 min the pellet was resuspended in distilled water. After pepsin treatment (in 0.5 M acetic acid, 24 h, 4°C), the solubilized material was analyzed by reducing 7.5% SDS–PAGE revealing the presence of collagen α1(I) and α2(I) chains and high molecular weight aggregates.

Preparation of glycated collagen Collagen, type I from bovine tendon (0.5 g) was stirred at 90°C in water (50 mL) in the presence or absence (control) of D-ribose (2 g). After 1 h reaction time, the solution was filtered and the soluble fraction was extensively dialyzed against water at 4°C (molecular cut-off: 12,000 Da). After lyophilization, glycation of the protein preparation was assessed by pentosidine determination as described below and quantified as 0.68 mmol pentosidine per mol lysine residues. No pentosidine was detected in the unglycated preparation.

Assessment of ECM protein glycation by pentosidine determination A 0.5 mg aliquot of the protein was hydrolyzed (6 M HCl, 110°C under nitrogen, overnight). The samples were dried using a speedvac (Savant, Holbrook, NY) and the residue redissolved in H_2O. An aliquot of the sample was injected on to a 4.6 mm analytical RP-C18-HPLC column (Vydac Inc., 218TP104, Hesperia, CA) using the following gradient (Varian pump, model 230, flow rate: 1 mL per min): 100% A (0.1% trifluoroacetic acid in H_2O) to 17% B (100% acetonitrile) over 35 min total run-time. Fluorescence was monitored using an Agilent (Agilent, Palo Alto, CA) 1100 series fluorescence detector (λex/λem 335/385 nm). Pentosidine was identified by comparison with a pentosidine standard obtained from Dr Vincent Monnier, Case Western Reserve University, Ohio (Sell and Monnier, 1989). The detection limit of our analytical system was 0.01 mmol pentosidine per mol lysine residues.

Irradiation with SSL and UVA A Kilowat (KW) large area light source solar simulator, model 91293, from Oriel Corporation (Stratford, Connecticut) was used, equipped with a 1000 W Xenon arc lamp power supply, model 68920, and a VIS-IR bandpass blocking filter plus either an atmospheric attenuation filter (output 290–400 nm plus residual 650–800 nm, for SSL) or UVB and UVC blocking filter (output 320–400 nm plus residual 650–800 nm, for UVA), respectively. The output was quantified using a dosimeter from International Light Inc. (Newburyport, MA), model IL1700, with an SED240 detector for UVB (range 265–310 nm, peak 285 nm), or a SED033 detector for UVA (range 315–390 nm, peak 365 nm), at a distance of 365 mm from the source, which was used for all experiments. At 365 mm from the source, SSL dose was 7.63 mJ per cm^2 per s UVA and 0.40 mJ per cm^2 per s UVB radiation. Using UVB/UVC blocking filter, the dose at 365 mm from the source was 5.39 mJ per cm^2 per s UVA radiation with a residual UVB dose of 3.16 μJ per cm^2 per s.

Assay for effects of ECM protein sensitization on skin cell proliferation Cells were seeded at 4×10^4 cells per dish on 35 mm dishes. After 24 h, human fibroblasts (CF3) were exposed to an ECM protein (bovine type I collagen or bovine elastin) filtrate (0.2 μm disposable Celtron 30/0 syringe filter, Schleier & Schuell, Germany), prepared after pre-irradiation of the protein suspended in Hank's balanced salt solution (HBSS) with SSL (300 s exposure, 120 mJ per cm^2 UVB and 2.30 mJ per cm^2 UVA). The final filtrate contained a residual amount of protein as determined by the BCA assay, which did not change with pre-irradiation of the sample (approximately 200 μg per mL collagen and 60 μg per mL elastin). HaCaT keratinocytes were treated the same way, but collagen received a higher pre-irradiation dose (600 s exposure, 4.58 J per cm^2 UVA, 240 mJ per cm^2 UVB). Following 30 min incubation, the exposure medium was removed and replaced with fresh DMEM. Cell number was determined 72 h later, and proliferation was compared with cells treated with filtrate from mock-irradiated collagen.

Detection of intracellular oxidative stress by flow cytometric analysis Intracellular levels of ROS were analyzed by flow cytometry using dihydrorhodamine 123 (DHR 123) as a specific fluorescent dye probe (Royall and Ischiropoulos, 1993; Peus *et al*, 1998). Fibroblasts were seeded at 2×10^5 cells per dish on 35 mm dishes and 48 h later cells were loaded with DHR 123 (50 μM) for 60 min and then washed three times with PBS. Cells were then exposed for 30 min to a preparation of collagen in HBSS (1 mL) obtained by filtration (0.2 μm) of a suspension of pre-irradiated (13.74 J per cm^2 UVA, 720 mJ per cm^2 UVB) bovine collagen type I (20 mg per mL) performed immediately after irradiation. Cell exposure to the filtrate of pre-irradiated collagen was performed in the absence or presence of catalase (400 u per mL). Cells were washed two times with PBS, harvested by trypsinization, suspended in ice-cold PBS/1% formaldehyde, and placed on ice in the dark. Intracellular accumulation of fluorescent rhodamine 123 was measured (10,000 cells each) using a FACScan flow cytometer (Becton-Dickinson, San Jose, California) with the excitation source at 488 nm and emission detected with a 580 nm long pass filter. Histograms were analyzed with the software program Cell Quest (Becton-Dickinson).

Comet assay Fibroblasts were seeded at 1×10^5 per dish on 35 mm culture dishes (Sarstedt, Newton, NC) and left overnight to attach. Cells were then exposed for 30 min to a preparation of collagen in HBSS (1 mL) obtained by filtration (0.2 μm) of a suspension of pre-irradiated (13.74 J per cm^2 UVA, 720 mJ per cm^2 UVB) bovine collagen type I (20 mg per mL) performed immediately after irradiation. Cell exposure to the preparation of pre-irradiated collagen was performed in the absence or presence of catalase (400 u per mL). Cells exposed to a preparation of unirradiated collagen were used as a negative control. After exposure to collagen, cells were washed twice with HBSS and fresh DMEM was added. Two hours later, cells were trypsinized and analyzed by alkaline single cell gel electrophoresis (comet assay) according to a standard procedure (Singh *et al*, 1988), except that CometSlides (Trevigen, Gaithersburg, MD) were used. Comets were analyzed using a fluorescence based digital imaging system (OlymPix Astrocam CCD camera, Cambridge, UK; LSR OlymPix version 4.0 software). Tail moments were calculated using computerized image analysis (Euclid Comet Analysis, St. Louis, MO).

Quantification of sensitized superoxide formation Collagen- and pyridinoline-sensitized generation of superoxide anions was determined by nitroblue-tetrazolium (NBT) reduction and confirmed by scavenging of superoxide by superoxide dismutase (Wondrak *et al*, 2002b). A 200 μL reaction volume containing 0.8 μL of a Protoblot-NBT stock solution (50 mg per mL) from Promega (Madison, Wisconsin) and protein (10 mg per mL) or pyridinoline (500 μM) in HBSS was irradiated in the presence or absence of superoxide dismutase (3000 u) in triplicate on a 96 well microtiter plate. Replicate samples incubated in the dark were used as controls. Formation of nitroblue diformazan (NBF) as the photoreduction product was quantified measuring the absorbance at 560 nm on a Versamax microtiter plate reader (Molecular Devices, Sunnyvale, California) using a NBF standard curve.

Quantification of H_2O_2 formation H_2O_2 formed upon photosensitization by ECM proteins or ECM cross-links was quantified according to a standard procedure using the ferrous iron-xylenol orange assay (Jiang *et al*, 1990) with minor modifications as reported previously (Wondrak *et al*, 2002b). Samples were prepared as indicated above for the superoxide assay but without NBT, and, immediately after irradiation, deproteinized by spin-dialysis using a Microcon microconcentrator (Amicon, Beverly, Massachusetts; molecular cut-off: 3000 Da). The dialysates were then analyzed in triplicate on a 96 well microtiter plate. To ensure specificity of the assay, replicate samples were irradiated in the presence of catalase (400 u per mL) and the peroxide measurement suppressible by catalase was assigned to H_2O_2. Heat denatured catalase was employed as another control to exclude nonenzymatic effects of catalase treatment.

Immunodetection of collagen-bound carbonyl groups after UV irradiation Acid soluble type I collagen from calf skin in PBS (5 mg per mL) was irradiated with increasing doses of SSL in the absence or presence of catalase (400 u per mL). Derivatization of protein bound carbonyl groups with 2,4-dinitrophenylhydrazine (DNPH) and immunodetection were performed according to the procedure of Shacter *et al* (1994). Briefly, 5 μL of 12% SDS was added to a 5 μL aliquot of sample containing 5 μg of protein. After addition of 10 μL of 10 mM DNPH in 10% trifluoroacetic acid, the mixture was incubated for 20 min at room temperature. The reaction mixture was neutralized and prepared for SDS–PAGE by adding 7.5 μL of 2 M Tris base containing 30% glycerol (v/v). Protein samples (10 μL) were separated by SDS–PAGE (7.5%) and transferred to a nitrocellulose membrane using a semidry system. A second gel containing duplicate samples was stained with Coomassie Blue for control of equal loading. Additionally, equal transfer was monitored by reversible protein staining of the nitrocellulose membrane with Ponceau S. The anti-DNP antibody (V401) was supplied by DAKO Carpinteria, CA and used at a 1/4000 dilution. The secondary antibody was goat anti-rabbit conjugated with horseradish peroxidase (Jackson Immunoresearch Laboratories West Grove, PA) used at a 1/20,000 dilution. Immunoblots were visualized using ECL detection reagents supplied by Amersham (Piscataway, NJ) with exposure times between 5 s and 3 min.

Quantification of collagen-bound carbonyl groups after UV irradiation Protein carbonylation was quantified photometrically according to a standard procedure (Levine *et al*, 1990). Samples (333 μL) of acid soluble type I collagen from calf skin dissolved in PBS (3 mg per mL) were UV irradiated and analyzed in triplicate. After irradiation, 333 μL of 10 mM DNPH in 2 M HCl was added to each sample and left at room temperature for 30 min with repetitive vortexing. The protein was precipitated by addition of ice-cold trichloroacetic acid (10%, w/v, final concentration). The pellet collected by centrifugation (5 min, $14{,}000 \times g$) was extracted four times using ethanol/ethyl acetate (1:1, v/v), in order to remove unreacted DNPH. The pellet was re-suspended in 0.6 mL of a 6 M guanidine hydrochloride solution (in 20 mM phosphate buffer), adjusted to pH 2.3 with trifluoroacetic acid. Absorbance was measured at 360 nm using a Ultrospec III UV/Visible spectrophotometer (Pharmacia, Piscataway, NJ). Carbonyl concentration was calculated from the net absorbance using a molar absorbance of 22,000 per M per cm. To correct for differences in protein recovery after precipitation, protein concentrations of the final solutions were determined using a bicinchoninic acid protein assay kit (Pierce, Rockford, IL).

Spectroscopy Pyridinoline UV spectra were recorded using a Cary 100 Bio UV-Visible Spectrophometer from Varian, Inc. (Palo Alto, California). Pyridinoline fluorescence spectra were recorded using a Spectramax Gemini XS (Molecular Devices, Sunnyvale, California) 96 well-microtiter plate reader.

Statistical analysis The results are presented as mean (±SD) of at least three independent experiments. They were analyzed using the two-sided Student's t test ($^*p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$).

RESULTS

ECM proteins as sensitizers of SSL-driven generation of H_2O_2 In a recent study on the photodynamic action of glycated proteins on cultured human skin cells by light-driven production of H_2O_2, we observed an unexpected sensitization activity of nonglycated, unmodified bovine collagen type I (Wondrak *et al*, 2002a). To examine the photodynamic potential of skin ECM proteins in more detail, SSL irradiation of collagen and elastin from various sources was performed and the sensitization of light-driven production of H_2O_2 was quantitatively analyzed (**Table I**). SSL irradiation (600 s exposure, 4.58 J per cm^2 UVA, 240 mJ per cm^2 UVB) of human type I collagen (10 mg per mL), taken from adult abdominal skin, placenta, or dermal fibroblasts cultured in the presence of 2-phospho-ascorbate to induce collagen synthesis (Hazeki *et al*, 1998), induced the production of micromolar concentrations of H_2O_2. In contrast, when the irradiation was performed in the presence of catalase (400 u per mL), no H_2O_2 was detectable (data not shown). Moreover, catalase suppression of H_2O_2 production was equally effective, when catalase was added after irradiation, but was not observed, when catalase was heat-deactivated before addition. Type I collagen taken from other sources such as bovine tendon, rat tail (**Table I**), and calf skin (data not shown) also sensitized the light-driven production of H_2O_2. Interestingly, the ECM protein elastin also showed photodynamic activity upon SSL irradiation, whereas keratin extracted from cultured human keratinocytes (HaCaT cells) did not. No H_2O_2 was detected from ECM proteins in the absence of light and irradiation of various other proteins such as bovine

Table I. Formation of Hydrogen Peroxide upon SSL-Irradiation of ECM Proteins*

ECM-protein, [source]	H2O2 [μM]
Collagen, type I, [human abdominal skin]	5.0 ± 0.9
Collagen, type I, [human placenta]	4.6 ± 1.1
Collagen, [human CF3-fibroblasts]	3.9 ± 1.5
Collagen, type I, [bovine achilles tendon]	3.7 ± 1.4
Collagen, type I, [rat tail]	2.0 ± 0.8
Elastin, [bovine neck ligament]	4.4 ± 0.9
Keratin, [human HaCaT-keratinocytes]	n.d.**

*SSL-Irradiation [4.58 J cm^{-2} UVA, 240 mJ cm^{-2} UVB] of suspended protein [10 mg/mL HBSS; 200 μl reaction volume] was performed and sensitized formation of hydrogen peroxide was determined as indicated under Materials and Methods.
**not detected, limit of detection: 0.5 μM.

serum albumin and ribonuclease A did not result in detectable amounts of H_2O_2 as reported previously (Wondrak *et al*, 2002b) (data not shown). Moreover, sensitization due to the presence of advanced glycation end products was excluded as a major source of light-driven H_2O_2 formation, as the ECM protein samples used in this study were nonglycated as evidenced by the lack of detectable amounts of the glycation marker pentosidine (<0.01 mmol per mol lysine residues) (Sell, 1989), previously shown to be a major AGE-type photosensitizer contained in glycated proteins (Ortwerth *et al*, 1997; Okano *et al*, 2001). Additional evidence against involvement of AGE sensitization came from the strong sensitization activity of collagen samples clearly not expected to have undergone long-term modification, such as from human placenta and cultured CF3 fibroblasts. In contrast, appreciable amounts of pentosidine were detected in collagen samples glycated *in vitro* (approximately 0.68 mmol per mol lysine residues), which strongly sensitized SSL-driven production of H_2O_2 as reported recently (Wondrak *et al*, 2002a).

Increasing doses of SSL or UVA caused a dose-dependent production of H_2O_2 upon irradiation of human skin and bovine tendon type I collagen (20 mg per mL) (**Fig 1**). Suspensions of bovine (**Fig 1*A***) and human (**Fig 1*B***) collagen were similarly potent sensitizers of SSL-driven H_2O_2 formation at doses used for proliferation assays with cultured skin cells in this study (up to 5 J per cm^2), but human collagen was less efficient as a sensitizer of UVA than bovine collagen, which was similarly effective with either light source in this dose range. At higher doses of irradiation human collagen was less efficient than bovine collagen in sensitizing the SSL-driven production of H_2O_2. The differential sensitization potency and wavelength dependency are consistent with the hypothesis that various protein-bound UVA and UVB chromophores and their relative proportions are responsible for the observed photodynamic characteristics of the collagen preparations. Extended SSL irradiation (up to 30 min exposure, 13.74 J per cm^2 UVA, 720 mJ per cm^2 UVB) of the collagen samples resulted in nonlinear increases in H_2O_2 production, probably indicating a partial chemical consumption or bleaching of the responsible ECM chromophores over the time of irradiation, which was more pronounced for human skin collagen.

We also measured chemical alterations to collagen molecules as a consequence of irradiation with doses of SSL sufficient to induce significant production of H_2O_2 (**Fig 2**). Acid soluble collagen type I samples from calf skin were exposed to increasing doses of SSL irradiation and protein integrity was examined by SDS–PAGE analysis (**Fig 2*A***) and carbonyl-immunostaining (**Fig 2*B***). Extensive protein fragmentation occurred as a consequence of prolonged SSL irradiation (30 min exposure, 13.74 J per cm^2 UVA, 720 mJ per cm^2 UVB), as discrete bands of collagen α and β chains were greatly reduced and formed a continuous protein smear following gel electrophoresis

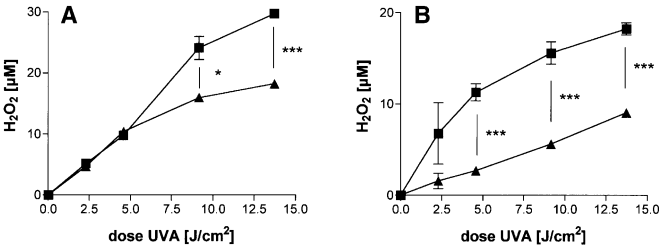

Figure 1. Type I collagen as a photosensitizer of H_2O_2 production. Production of H_2O_2 during SSL (squares) and UVA irradiation (triangles) of collagen, normalized for the total UVA dose applied to the sample. Protein suspensions (20 mg per mL in HBSS) were irradiated and H_2O_2 was measured immediately as described in *Materials and Methods*. Statistical analysis was performed for all doses, comparing UVA-induced with SSL-induced production of H_2O_2. (*A*) Dose–response of H_2O_2 production upon irradiation of bovine type I collagen from tendon. (*B*) Dose–response of H_2O_2 production upon irradiation of human type I collagen from skin.

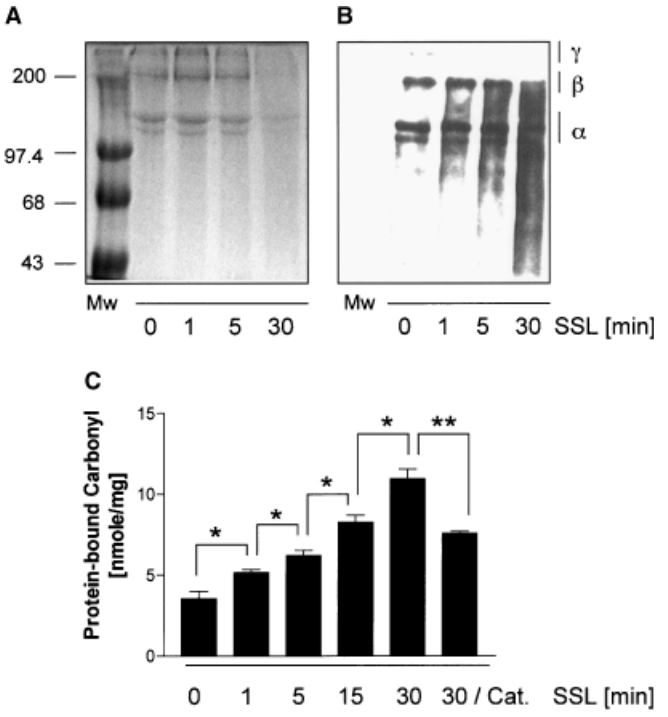

Figure 2. Collagen damage as a result of irradiation with SSL. Acid soluble type I collagen from calf skin was exposed to increasing doses of SSL irradiation (up to 30 min exposure, 13.74 J per cm^2 UVA, 720 mJ per cm^2 UVB) and protein integrity was examined by (*A*) 7.5%-SDS–PAGE analysis with Coomassie stain, and (*B*) carbonyl-immunostaining after derivatization with DNPH and western transfer. The migration positions of collagen α(I) monomer, β(I) dimer, and γ(I) trimer are indicated and molecular weight standards are given in kDa. (*C*) Quantification of collagen-bound carbonyl groups resulting from exposure to increasing doses of SSL irradiation. Photometric determination of protein-bound DNP-epitopes was performed as a direct measure of protein-bound carbonyl groups. Additionally, 30 min irradiation was performed in the presence of catalase (Cat, 400 u per mL).

(**Fig 2*A***). As a sensitive measure of oxidative protein damage carbonyl-immunostaining after derivatization of the irradiated sample with the carbonyl reagent DNPH was performed (**Fig 2*B***). A dose-dependent introduction of protein-bound carbonyl groups was clearly discernible after SSL irradiation for

1 min (0.46 J per cm^2 UVA, 24 mJ per cm^2 UVB). Photometric quantification of protein carbonylation revealed increasing carbonyl epitopes up to approximately 11 nmol per mg protein (**Fig 2*C***), which was partially suppressed in the presence of catalase suggesting that H_2O_2 may be involved in photo-oxidative carbonylation of collagen during light-driven collagen oxidation. The high background of protein carbonylation observed in the unirradiated collagen sample (3.6 ± 0.7 nmol per mg protein as compared with 2.1 ± 0.1 nmol per mg detected in unirradiated bovine serum albumin) previously observed by others (Song *et al*, 2002) may be due to Schiff base-type reducible cross-links abundant in enzymatically modified collagen (Robins and Bailey, 1977). Based on these observations, it was concluded that SSL irradiation of skin ECM proteins *in vitro* induces photoreductive formation of H_2O_2 with concomitant protein oxidation and fragmentation.

Photodynamic action of the ECM proteins collagen and elastin on proliferation of cultured human skin cells To evaluate possible cellular consequences of the photodynamic action observed with irradiated ECM proteins, we tested whether ECM protein-sensitized formation of H_2O_2 could affect cell proliferation of cultured human skin cells. The similar sensitization characteristics with regard to formation of H_2O_2 by low doses of SSL shared between type I collagen from human skin and bovine tendon led us to use the latter, more easily available material for our cellular studies. To select for irradiation effects attributable to sensitized H_2O_2 formation and to suppress other potential photodynamic actions of ECM proteins such as direct energy transfer (singlet oxygen formation), which are not the subject of this study, the standard experiment was performed by exposing cells to a sterile filtrate (0.2 μm) of pre-irradiated ECM protein. First, human fibroblasts (CF3) were exposed to a collagen filtrate prepared after pre-irradiation of the protein suspended in HBSS (20 mg per mL) (300 s exposure to SSL, 120 mJ per cm^2 UVB and 2.30 J per cm^2 UVA) (**Fig 3*A***). Following 30 min incubation of cells with the filtrate, the exposure medium was removed and replaced with fresh DMEM. Cell number was determined 72 h later, and proliferation was compared with cells treated with filtrate from mock-irradiated collagen. Exposure of fibroblasts to the filtrate prepared from pre-irradiated collagen had a pronounced anti-proliferative effect (approximately 75% inhibition) (**Fig 3*A***) compared with the mock-irradiated control. If catalase (400 u per mL) was added to the collagen filtrate during the 30 min incubation, however, the anti-proliferative effect of the pre-irradiated collagen filtrate was fully reversed. The same protection was achieved by addition of 10 mM D-penicillamine (D-P), a potent thiol-antioxidant and carbonyl scavenger (Wondrak *et al*, 2002c). When cells were exposed for 30 min to a filtrate of mock-irradiated collagen, to which 5 μM H_2O_2, an amount equal to the concentration of H_2O_2 formed upon SSL irradiation of this preparation, was added, a similar effect on cell proliferation (approximately 70% inhibition) was observed strongly suggesting that H_2O_2 exerts the anti-proliferative action of the filtrate. As anticipated from the measurements of elastin-sensitized production of H_2O_2 (**Table I**), comparable anti-proliferative effects of pre-irradiated elastin preparations and their reversal by catalase and D-P were observed when this ECM protein was tested on the cultured fibroblasts (**Fig 3*B***). Cell proliferation after exposure to pre-irradiated elastin was suppressed by approximately 60% as compared with exposure to mock-irradiated elastin. The anti-proliferative effects of pre-irradiated collagen observed with human fibroblasts could also be observed, when cultured human keratinocytes (HaCaT cells) were exposed to a filtrate of pre-irradiated collagen (data not shown). These observations lead us to conclude that UV irradiation of ECM proteins exerts indirect phototoxic effects on skin cell proliferation by sensitized formation of H_2O_2.

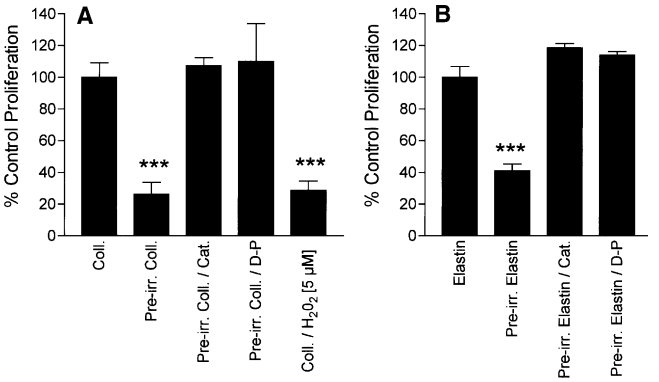

Figure 3. Anti-proliferative effects of ECM protein-sensitized formation of H_2O_2 on cultured human fibroblasts. Human fibroblasts (CF3) were exposed to a sterile filtrate (0.2 μm) of bovine type I collagen (*A*) or bovine elastin (*B*) prepared after pre-irradiation of the protein suspended in HBSS (20 mg per mL) with SSL (300 s exposure, 120 mJ per cm^2 UVB and 2.30 J per cm^2 UVA). Following 30 min incubation, the exposure medium was removed and replaced with fresh DMEM. Cell number was determined 72 h later by cell counting, and proliferation was compared with cells treated with filtrate from mock-irradiated ECM protein. Cell proliferation was equally assessed after cell exposure to filtrate from mock-irradiated collagen with externally added H_2O_2 (5 μM) and to filtrates of pre-irradiated ECM proteins in the presence of catalase (Cat, 400 u per mL) or D-P (10 mM).

Intracellular oxidative stress resulting from collagen phototoxicity Generation of intracellular oxidative stress resulting from ECM protein sensitization was examined exposing CF3 fibroblasts preloaded with the intracellular redox dye DHR 123 to bovine type I collagen, pre-irradiated with SSL (1800 s exposure, 720 mJ per cm^2 UVB and 13.73 J per cm^2 UVA) (**Fig 4**). Upon reaction with intracellular ROS, DHR 123 is irreversibly oxidized and converted to the fluorescent dye rhodamine 123, which allows quantitative analysis of intracellular redox stress by flow cytometric analysis (Peus *et al*, 1998). Exposure of cells to a filtrate of unirradiated collagen did not induce any significant change of the baseline fluorescence observed after preloading the cells with the redox dye. A nearly 3-fold increase of rhodamine fluorescence intensity was observed, however, after cells were exposed to a filtrate of pre-irradiated collagen containing approximately 30 μM H_2O_2 (**Fig 4*B***). If cell exposure to the preparation of pre-irradiated collagen was performed in the presence of catalase (400 u per mL), a significant reduction of rhodamine fluorescence intensity was observed increasing by only 30% over the control level. These findings provide evidence that H_2O_2 generated during SSL irradiation of collagen enters fibroblasts causing intracellular oxidative stress.

Genotoxic stress resulting from the photodynamic action of collagen Genotoxic effects of UV irradiation are partly mediated by ROS production (Kvam and Tyrrell, 1997). Therefore, we tested the effect of ECM protein sensitization on genomic integrity of skin fibroblasts using single cell gel electrophoresis assay, more commonly called the comet assay (Singh *et al*, 1988). The comet assay allows the sensitive detection and quantification of DNA damage by strand breaks, open repair sites, cross-links, and labile sites in single cells. Upon alkaline gel electrophoresis, damaged DNA is released from the nucleus forming a comet tail. Following fluorescent DNA staining, DNA damage is quantified calculating comet tail moments by computer image analysis. Untreated control cells as well as cells exposed to a preparation of unirradiated bovine type I collagen showed tight, compact nuclei with no comets and background

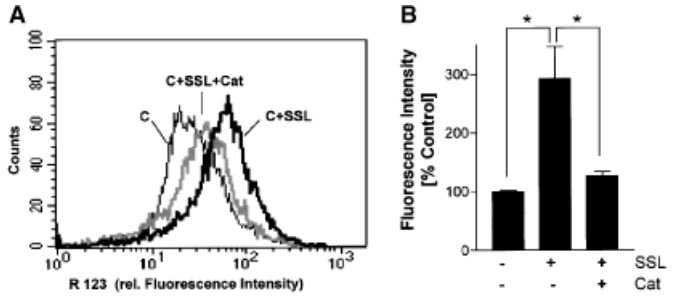

Figure 4. Intracellular oxidative stress resulting from collagen phototoxicity. CF3 fibroblasts preloaded with the intracellular redox dye DHR 123 were exposed to bovine type I collagen, pre-irradiated with SSL (1800 s exposure, 720 mJ per cm^2 UVB and 13.73 J per cm^2 UVA) and rhodamine 123 fluorescence indicative of intracellular redox stress was quantified by flow cytometric analysis. Exposure of cells to unirradiated collagen as control (C), to a filtrate of pre-irradiated collagen (C + SSL) and to a filtrate of pre-irradiated collagen in the presence of catalase (400 u per mL, C + SSL + catalase) was performed. Fluorescence intensity of 123 DHR-preloaded cells exposed to control collagen was not different from baseline fluorescence of untreated 123 DHR-preloaded cells (not shown). A representative experiment is shown in (*A*) and the relative fluorescence intensities (mean ± SD) calculated from three independent experiments are shown in (*B*).

tail moments (**Fig 5**) of 4 ± 4 (n = 13) and 4 ± 3 (n = 15), respectively, representative of DNA containing only a minimum number of inherent DNA strand breaks. In contrast, exposure to a preparation of SSL-irradiated collagen (600 s irradiation, 240 mJ per cm^2 UVB and 4.60 J per cm^2 UVA) for 30 min caused a pronounced increase in comet tail moment (20 ± 13; n = 11). Consistent with the protective effects of antioxidant treatment observed in the cell proliferation and oxidative stress assays described above, catalase treatment effectively reversed the genotoxic effects of cell exposure to preparations of pre-irradiated collagen reducing comet tail moments to control values (4 ± 2; n = 9). These data combined with our demonstration of ECM protein sensitized intracellular oxidative stress suggest that the photodynamic action of collagen may indirectly affect DNA integrity in intact skin fibroblasts by extracellular production of H_2O_2 followed by nuclear entry of ROS after membrane permeation.

Enzymatic ECM protein cross-link chromophores as candidate sensitizers of SSL-driven ROS formation In an attempt to identify candidate chromophores responsible for the unexpected photodynamic properties of elastin and collagen, the isolated pyridinium cross-links pyridinoline and desmosine, isolated from collagen and elastin, respectively, were tested for sensitization of light-driven production of ROS. With pyridinoline, a pronounced sensitization of SSL-driven production of superoxide, measured as superoxide dismutase-suppressible photoreduction of the redox dye NBT, was observed (**Fig 6*A***). Sensitized generation of superoxide anions occurred in proportion to UV dose and pyridinoline concentration. In contrast, desmosine did not display any sensitizing activity in this assay (data not shown). Next, we examined whether the ECM protein derived pyridinium cross-links would sensitize the production of H_2O_2 as the ROS responsible for the ECM protein phototoxicity observed in this study. Again, only pyridinoline sensitized the production of H_2O_2 in a UV dose-dependent manner (**Fig 6*B***) and desmosine was not photoactive (data not shown). Sensitization of H_2O_2 production by pyridinoline was a mixed UVA/UVB effect as irradiation with UVA achieved between approximately 30 (2.30 J per cm^2 UVA) and 60% (13.74 J per cm^2 UVA) formation of H_2O_2 as observed during irradiation with SSL normalized for the dose of UVA

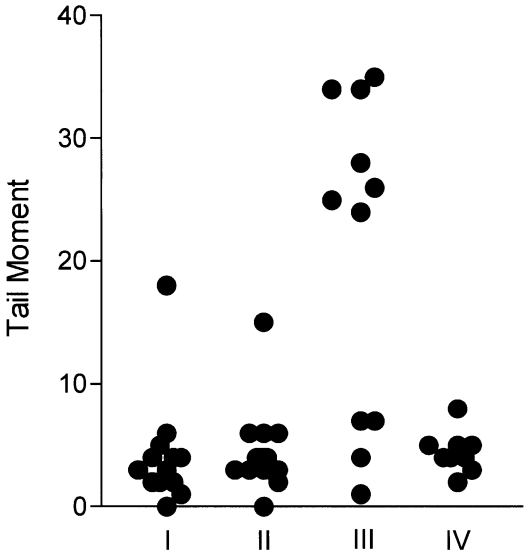

Figure 5. Skin cell DNA damage as a result of collagen phototoxicity. DNA damage resulting from 30 min exposure of CF3 fibroblasts to pre-irradiated collagen (600 s SSL irradiation, 240 mJ per cm^2 UVB and 4.60 J per cm^2 UVA) was analyzed using the Comet assay. Comet tail moments (each symbol represents an individual cell): untreated cells (I); cells exposed to a preparation of unirradiated bovine type I collagen (II), SSL-irradiated collagen (III), SSL-irradiated collagen in the presence of catalase (400 u per mL) (IV). A representative experiment out of at least three independent repeats is shown.

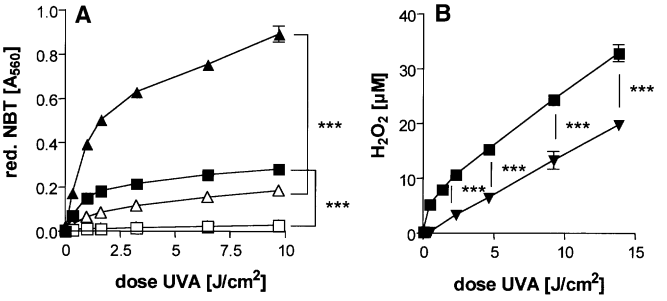

Figure 6. Pyridinoline as photosensitizer. (*A*) NBT photoreduction by SSL irradiation of pyridinoline (triangle, 500 µM; square, 100 µM) in the absence (closed symbols) or presence (open symbols) of superoxide dismutase. (15,000 u per mL). Statistical analysis was performed comparing photoreduction in the absence and presence of superoxide dismutase; $p < 0.001$ for all doses ≥ 0.32 J per cm^2. (*B*) Production of H_2O_2 during SSL (squares) and UVA irradiation (triangles) of pyridinoline (500 µM), normalized for the total UVA dose applied to the sample. Statistical analysis was performed comparing UVA-induced with SSL-induced production of H_2O_2; $p < 0.001$ for all doses ≥ 0.46 J per cm^2 UVA.

applied to the sample. The continuous sensitization activity observed with pyridinoline exposed to even high doses of SSL irradiation suggested a remarkable photostability of the material. We therefore examined the photostability of our pyridinoline preparation exposed to increasing doses of SSL irradiation using UV and fluorescence spectroscopy (**Fig 7**). Complete photodegradation of pyridinoline was observed within 300 s of exposure to SSL irradiation (120 mJ per cm^2 UVB and 2.3 J per cm^2 UVA) as shown by the pronounced fluorescence (excitation 305 nm) bleaching (**Fig 7*A***) and disappearance of the UV absorption peak (λmax 325 nm, **Fig 7*B***) suggesting that pyridinoline may sensitize its own photodegradation as commonly observed during photobleaching of other sensitizers

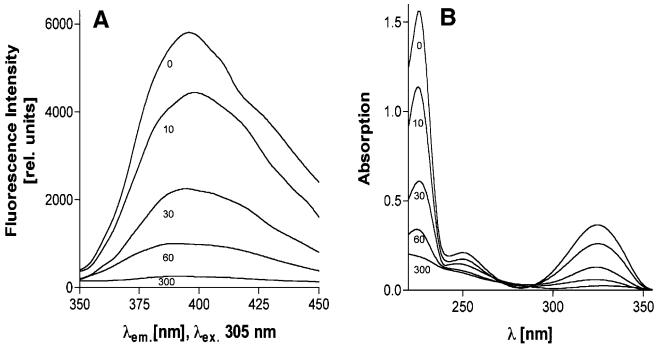

Figure 7. Photodegradation of pyridinoline. Disappearance of the pyridinoline chromophore (50 μM in PBS) upon SSL irradiation (up to 300 s exposure, 120 mJ per cm^2 UVB and 2.30 J per cm^2 UVA) was followed by fluorescence (*A*) and UV spectroscopy (*B*). Numbers indicate irradiation time in seconds.

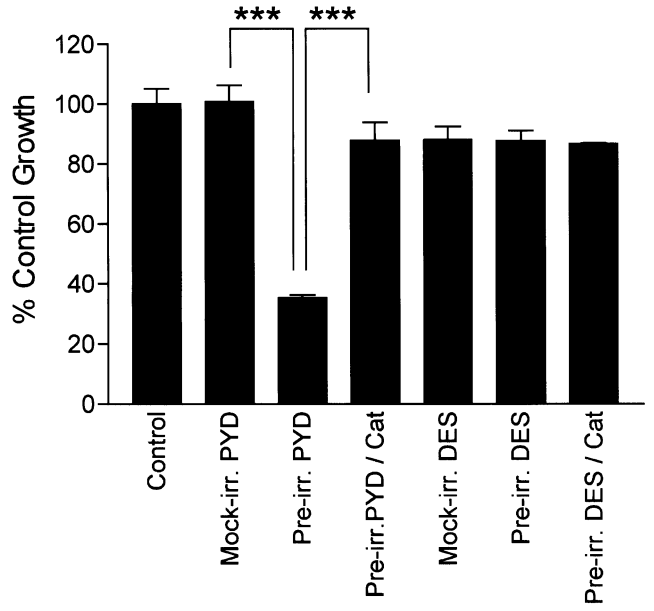

Figure 8. Anti-proliferative effects of pyridinoline-sensitized formation of H_2O_2 on cultured human fibroblasts. Unirradiated human fibroblasts (CF3) were exposed to pre-irradiated (300 s exposure to SSL, 120 mJ per cm^2 UVB and 2.30 J per cm^2 UVA) or mock-irradiated ECM protein cross-links desmosine (DES) or pyridinoline (PYD; 500 μM, each). Following 30 min incubation, the exposure medium was removed and replaced with fresh DMEM. Cell number was determined 72 h later by cell counting, and proliferation was compared with untreated cells. Moreover, cell exposure to pre-irradiated ECM protein cross-links was performed in the presence of catalase (Cat, 400 u per mL) or D-P (10 mM).

with formation of photolysis products that retain photosensitization activity (Fujimoto, 1985; Meddah *et al*, 2000). Next, the photodynamic action of ECM protein-derived pyridinium cross-links on proliferation of cultured human CF3 fibroblasts was examined (**Fig 8**). Neither pyridinoline nor desmosine (500 μM, each) displayed anti-proliferative effects, when cells were exposed to the nonirradiated cross-link preparations for 30 min; however, when pre-irradiated with SSL (300 s exposure, 120 mJ per cm^2 UVB and 2289 mJ per cm^2 UVA), pyridinoline, but not desmosine, suppressed cell proliferation by approximately 60%. Catalase treatment fully reversed the anti-proliferative action of pre-irradiated pyridinoline, consistent with the sensitized formation of H_2O_2 as the crucial mediator of the observed photodynamic effect. Further experiments examining the photodynamic activity of simple structural analogs and hydroxypyridine isomers of pyridinoline led to the identification of 3-hydroxypyridine as the minimum chromophore responsible for UV sensitization activity contained in pyridinoline but not present in desmosine (data not shown).

DISCUSSION

Photosensitization initiated by excitation of endogenous UV chromophores with subsequent formation of ROS has been suggested as a key mechanism of oxidative skin photodamage (Dalle Carbonare and Pathak, 1992; Scharffetter-Kochanek *et al*, 1997). Here we present experimental evidence in support of a functional role of ECM proteins as endogenous photosensitizers in human skin.

First, photodynamic activity of ECM proteins was demonstrated detecting formation of ROS in preparations of collagen and elastin irradiated with SSL and UVA. Human type I collagen, extracted from placenta, adult skin, and cultured human fibroblasts, and elastin of bovine origin strongly sensitized formation of H_2O_2. As recently observed with sensitized ROS formation by glycated proteins, formation of superoxide by single electron photo-reduction of oxygen might precede generation of H_2O_2 formed subsequently after spontaneous dismutation of the initially formed superoxide (Wondrak *et al*, 2002b). Consistent with this mechanism, the light-driven generation of superoxide anions, as assessed by superoxide dismutase-suppressible photoreduction of the redox dye NBT, was observed upon SSL irradiation of bovine collagen, but not bovine albumin (data not shown). In parallel with photo-reductive formation of ROS, oxidative protein damage as evidenced by extensive fragmentation and dose-dependent accumulation of protein-bound carbonyl groups was observed when collagen was irradiated with SSL.

We then examined the effects of ECM protein sensitization on skin cell function evaluating intracellular oxidative stress, proliferation, and genomic integrity in cultured human skin cells. Using 123 DHR cell loading as an intracellular fluorescent redox probe (Peus *et al*, 1998), clear experimental evidence was obtained indicating that ECM protein sensitized formation of ROS induced intracellular oxidative stress in CF3 fibroblasts. Furthermore, pronounced inhibition of skin cell proliferation and extensive DNA damage as evidenced by the comet assay were observed as strong indicators of cellular stress resulting from ECM protein phototoxicity. The similar anti-proliferative effect observed when H_2O_2 alone was added to cells in concentrations formed during irradiation of collagen and the effective suppression of all cellular consequences of ECM protein sensitization by catalase confirmed the involvement of H_2O_2 as the crucial phototoxic mediator.

Earlier findings have demonstrated that intracellular generation of ROS occurs in response to direct irradiation of cultured skin cells with UVA (Gniadecki *et al*, 2000) and UVB (Peus and Pittelkow, 2001), primarily ascribed to intracellular sensitization by pigments such as porphyrins or light-driven electron leakage from the mitochondrial respiratory chain. In contrast, our experiments point towards a functional role of ECM proteins as extracellular effectors of skin cell phototoxicity by sensitized formation of ROS. H_2O_2, the crucial mediator of ECM protein dependent photodynamic action identified in this study, may be sufficiently stable to migrate in dermal skin and then exert cellular damage distant from its source of generation. By avoiding co-irradiation of cells and ECM protein and exposing unirradiated cells to pre-irradiated protein preparations instead, the experimental design of our cellular studies selected against cellular effects of short lived excited species such as singlet oxygen or protein-bound free radicals as other potentially important mediators of skin photodamage (Grether-Beck *et al*, 1996; Scharffetter-

Kochanek *et al*, 1997; Krutmann, 2000) currently being examined in a separate study.

As a first structural model on how a particular chromophore contained exclusively in ECM proteins might explain their unusual activity as sensitizers, the enzymatic collagen cross-link pyridinoline (Fujimoto *et al*, 1978; Robins, 1983) contained in appreciable amounts in our preparation of bovine collagen (approximately 250 mmol per mol collagen, Simon Robins, personal communication), was examined for photodynamic activity. Isolated pyridinoline clearly acts as a UVA-sensitizer of superoxide and H_2O_2 formation with subsequent anti-proliferative action on fibroblasts, but no obvious quantitative correlation exists between sensitization potency of our various collagen preparations and their pyridinoline content suggesting the presence of additional chromophores involved in sensitization. Collagen extracted from normal human dermis or cultured human fibroblasts, highly active in our sensitization assays, is generally low in pyridinoline (approximately 16 mmol per mol collagen (Pasquali *et al*, 1997; Gineyts *et al*, 2000)), and elevated levels of pyridinoline occur only during conditions of skin remodeling following wounding, such as hypertrophic scar tissue and keloids (approximately 100 mmol per mol collagen (Moriguchi and Fujimoto, 1979; Uzawa *et al*, 1998) and in scleroderma (Brinckmann *et al*, 2001). More experimental evidence is therefore needed to define a possible role in skin photosensitization for this particular model UVA-sensitizer. In contrast to the pronounced photodynamic activity of pyridinoline, the elastin-derived cross-link chromophore desmosine was inactive in our sensitization assays. As involvement of tryptophan- (McCormick *et al*, 1976) or AGE-mediated sensitization in elastin phototoxicity can be excluded based on the fact that these alternative sensitizers are not contained in our elastin preparation (Petruska and Sandberg, 1968) other major elastin-derived cross-links, such as the recently identified potent fluorophore cyclopentenosine (Akagawa *et al*, 1999), may be involved in the observed photodynamic activity of elastin.

Recent work has demonstrated that skin proteins are important targets of chemical photo-oxidative damage *in vitro* (Dalle Carbonare and Pathak, 1992; Au and Madison, 2000; Miles *et al*, 2000) and during human photoaging *in vivo* (Sander *et al*, 2002). Moreover, UV-dependent induction of matrix metalloproteinases (MMP) leads to the increased enzymatic degradation of dermal collagen as a major hallmark of skin photodamage (Petersen *et al*, 1992; Herrmann *et al*, 1993; Fisher *et al*, 1996) and MMP-generated collagen fragments were shown to be important mediators of photosuppression of fibroblast collagen synthesis (Varani *et al*, 2001). On the basis of our experimental observations described above, we envision a novel causative role of dermal ECM proteins in skin photodamage as summarized in **Fig 9**: Photo-excitation of dermal ECM protein chromophores by solar light (mainly by the deeply penetrating UVA-portion) in the presence of oxygen sensitizes the formation of ROS, with concomitant oxidative damage to the sensitizing protein itself as revealed by collagen carbonylation and fragmentation observed in this study. Subsequently, reactive species of sufficient stability, such as H_2O_2 detected in our experiments, enter target skin cells causing subsequent oxidative cell damage presumably by Fenton-type chemistry after transition metal ion-catalyzed peroxide decomposition. The resultant intracellular oxidative stress is expected to severely impair skin cell structure and function as demonstrated in our study by the suppression of skin cell proliferation and accumulation of chromosomal DNA damage. The pronounced deterioration of skin cell genomic integrity resulting from ECM protein phototoxicity, as revealed in this study using the Comet assay, points towards a novel mechanism of DNA photodamage in skin that does not require the direct UV irradiation of cells. Thus it might be hypothesized that ECM protein phototoxicity enhances the genotoxicity of a given dose of UV irradiation, if cells are irradiated in the presence of ECM proteins. Moreover, this may be a relevant mechanism for the induction of genotoxic effects in cells situated in deeper layers of skin receiving little UV exposure due to prior absorption by the epidermis and upper dermis. Future studies on human skin are needed to substantiate the hypothesis that repetitive ECM protein sensitization of light-driven oxidative insults on genomic integrity contributes to photoaging and carcinogenesis of human skin.

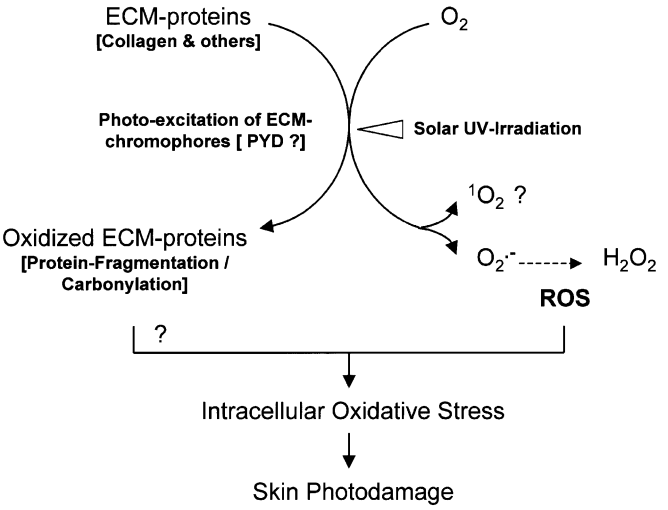

Figure 9. A proposed role of dermal ECM proteins as sensitizers of skin photodamage. Photoexcitation of dermal ECM protein chromophores by solar light in the presence of oxygen sensitizes the formation of ROS. Oxidative damage to the sensitizing protein (protein carbonylation and fragmentation) occurs as a direct result of photoreductive activity and by ROS attack. ROS cause subsequent oxidative skin cell damage with deterioration of skin cell proliferation and genomic integrity. The involvement of pyridinoline (PYD) as a candidate sensitizer is hypothetical at this point and ECM protein sensitized formation of singlet oxygen has not been evaluated in this study.

This research was supported by grants from the National Institutes of Health (CA43894, NS38496) and Niadyne, Inc. M.K.J. and E.L.J. are principals in Niadyne Inc., whose sponsored research is managed in accordance with University of Arizona conflict-of-interest policies. We thank Dr Simon Robins, The Rowett Research Institute, UK, for providing a gift of pyridinoline. Technical assistance of our student Desiree Tilbury is gratefully acknowledged. Mass spectral analyses were performed at the SWEHSC/AZCC Proteomics Core Facility, College of Pharmacy, University of Arizona. The Core Facility is directed by Dr George Tsaprailis and supported by NIH grants NIEHS ES-06694 and NCI CA-23074. Flow cytometric analysis was performed at the AZCC flow cytometry laboratory.

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