Photosensitized Growth Inhibition of Cultured Human Skin Cells: Mechanism and Suppression of Oxidative Stress from Solar Irradiation of Glycated Proteins

Georg T. Wondrak,1 Michael J. Roberts,1 Myron K. Jacobson, and Elaine L. Jacobson
Department of Pharmacology and Toxicology, College of Pharmacy, Arizona Cancer Center, University of Arizona, Tucson, Arizona, U.S.A.

Chronic exposure to sunlight plays a role in skin aging and carcinogenesis. The molecular mechanisms of photodamage by ultraviolet A, the sunlight’s major ultraviolet constituent, are poorly understood. Here we provide evidence that advanced glycation end products on proteins are sensitizers of photo-oxidative stress in skin cells. Glycation is a process of protein damage by reducing sugars and other reactive carbonyl species leading to the formation of advanced glycation end products, which accumulate on long-lived proteins such as dermal elastin and collagen during skin aging. Growth inhibition as a result of advanced glycation end product photosensitization of ultraviolet A and solar-simulated light was demonstrated in human keratinocytes and fibroblasts. Using advanced glycation end product bovine serum albumin and advanced glycation end product collagen as model photosensitizers, ultraviolet A-induced formation of H$_2$O$_2$ was identified as the key mediator of skin cell growth inhibition as evidenced by complete protection by catalase treatment and equivalent growth inhibition of unirradiated cells treated with pre-irradiated advanced glycation end product protein. D-penicillamine protected against advanced glycation end product-photosensitized growth inhibition even when added following irradiation, suggesting the feasibility of therapeutic approaches for protection against skin ultraviolet A damage. Photosensitized growth inhibition increased with the degree of advanced glycation end product modification paralleled by the amount of H$_2$O$_2$ formed upon solar-simulated light irradiation of the protein. Photosensitization was not observed using bovine serum albumin modified with the major advanced glycation end product, N$^\alpha$-carboxymethyl-L-lysine, ruling out effects of cellular advanced glycation end product receptor (RAGE) stimulation. In contrast to bovine serum albumin, unglycated collagen showed photosensitization in CF3 fibroblasts and generation of H$_2$O$_2$ upon solar-simulated light irradiation. This study supports the hypothesis that advanced glycation end product-modified proteins are endogenous sensitizers of photo-oxidative cell damage in human skin by ultraviolet A-induced generation of reactive oxygen species contributing to photoaging and photocarcinogenesis.

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and the reaction volume was transferred into a dialysis bag and was prepared under the same conditions, except that glyoxylic acid was added glyoxylic acid (14.3 mg, 0.155 M) in a total volume of 1 ml per (CML)-modified bovine serum albumin (BSA).

Similarly, intracellular pheomelanin photosensitizes UVA-induced DNA damage in cultured human melanocytes (Wencel et al, 1998). In addition, UVA-induced intracellular accumulation of hydrogen peroxide in human HaCaT keratinocytes in the absence of an external sensitizer was linked to direct electron leakage from the mitochondrial respiratory chain (Gniadecki et al, 2000).

Besides photo-oxidative damage, increased cellular carbonyl stress mediated by glycation also can cause skin deterioration (Sell et al, 1992; Dyer et al, 1993; Mizutari et al, 1997; Chellan and Nagaraj, 1999). Glycation is a process of spontaneous protein damage by reducing sugars and other reactive carbonyl species (RCS) leading to the intracellular and extracellular accumulation of a complex mixture of modifications called advanced glycation end products (AGE) (Brownlee, 1995), many of which contain chromophores that absorb UVA. Accelerated tissue damage by glycation is observed under pathologic conditions such as diabetes mellitus contributing to the specific long-term complications of the disease, e.g., diabetic nephropathy, vasculopathy, and lens cataracts (Monnier et al, 1999). Moreover, significant accumulation of AGE occurs during normal human skin aging on long-lived proteins such as dermal elastin and collagen type I (Dyer et al, 1993; Verzijl et al, 2000). AGE are now well-accepted biomarkers of general tissue damage and rates of AGE accumulation in skin collagen are negative predictors of life span in mice (Sell et al, 2000). Glycation-induced cross-linking of collagen decreases its solubility and modifies its physical and metabolic properties interfering with proper extracellular matrix function (Rittie et al, 1999), e.g., during extracellular matrix remodeling in wound healing (Owen et al, 1998). Recently, ocular lens cataractogenesis has been linked to ROS production upon UVA absorption of AGE-modified lens protein, which functioned as an endogenous photosensitizer (Orwerth et al, 1995). Also, the endogenous chromophore, lipofuscin, was shown to have a pathologic role as a potential sensitizer of retinal photodamage and age-related maculopathy (Wassell et al, 1999). Moreover, dermal fibroblasts that were irradiated by UVA in the presence of AGE protein showed immediate cellular responses that peaked within a few hours (Masaki et al, 1997, 1999). Therefore, we initiated a study of the potential mechanisms by which photosensitization by AGE pigments may generate a source of photo-oxidative stress in human skin exposed to solar irradiation. In this study, we evaluated AGE-modified proteins as potential photosensitizers in two types of cultured human skin cells exposed to solar-simulated light (SSL) or UVA irradiation.

MATERIALS AND METHODS

Chemicals All chemicals were purchased from Sigma (St Louis, MO).

Synthesis and characterization of N5-carboxymethyl-L-lysine (CML)-modified bovine serum albumin (BSA) Synthesis of CML-BSA was done according to a standard procedure (Kieda et al, 1996). Briefly, BSA (176 mg) and sodium cyanoborohydride (28.3 mg, 0.45 M) were dissolved in sodium phosphate buffer (0.2 M, pH 7.8). To this was added glyoxylic acid (14.3 mg, 0.155 M) in a total volume of 1 ml per reaction. The mixture was incubated for 24 h at 37°C. Control protein was prepared under the same conditions, except that glyoxylic acid was omitted. The reaction was terminated by acidification using 1 M HCl, and the reaction volume was transferred into a dialysis bag and extensively dialyzed (molecular cutoff: 12 kDa) against distilled water at 4°C for 48 h. After lyophilization, a 1 mg aliquot of the preparation was hydrolyzed (6 M HCl, 110°C, overnight) and, after OPA (o-phthalaldehyde) derivatization, amino acid analysis on a reversed phase system with fluorescence detection was performed according to a standard procedure (Lewisch and Levine, 1995). CML was identified by comparison with a synthetic standard and quantified as 330 mmol CML per mol lysine in CML-BSA.

Preparation and characterization of BSA modified with AGE (AGE-BSA) and AGE-modified RNAse A (AGE-RNAse A) AGE-BSA was prepared as described earlier (Takata, 1988). Briefly, 1.6 g of BSA and 3.0 g of D-glucose were dissolved in 10 ml of 0.5 M sodium phosphate buffer, pH 7.4, containing 0.05% NaN3. The solution was filter sterilized through a 0.45 μm filter and incubated in the dark for 90 d at 37°C. Following dialysis against water, the sample was lyophilized. Unless otherwise mentioned, this was the AGE-BSA preparation used for the photosensitization experiments. Additionally, analogous incubations were performed over various reaction times (between 1 d and 5 mo) to synthesize AGE-BSA preparations with increasing AGE modification. Molecular weight determination by matrix assisted laser desorption ionization-mass spectrometry (MALDI-MS) revealed a pattern of increasing molecular weight with glycation time [Mm/Ma] = 1.06/1.15 (reaction time 14 d); 1.07/1.15 (day 7); 1.08/1.15 (day 28); 1.09/1.15 (day 84); and 1.10/1.15 (day 150). Furthermore, the CML content of BSA and AGE-BSA, glycated for 84 d, was determined as an indicator of advanced glycation as described above and quantified as 60 nmol CML per mol lysine in AGE-BSA. No CML was detected in BSA. Progress of glycation was also followed by UV-VIS spectroscopy (Versamax microtiter plate reader, Molecular Devices, Sunnyvale, CA). AGE-modified RNAase A was prepared by incubation of RNAse (100 mg) with D-glucose (188 mg) in 625 μl of 0.5 M sodium phosphate buffer, pH 7.4, containing 0.05% NaN3. After reaction over 84 d at 37°C, the material was extensively dialyzed (molecular cutoff: 3 kDa), then lyophilized, and the protein preparation characterized by MALDI-MS and measurement of AGE fluorescence (Monnier, 1998) with a Spectramax Gemini XS (Molecular Devices, Sunnyvale, CA) (exc., em. 370, 440 nm; relative units per reaction time): 1.00, day 0; 1.25, day 1; 1.5, day 7; 7.5, day 14; 10.00, day 28; 13.50, day 74; 15.00, day 150. AGE-RNAse A (from bovine pancreas, type III A) was generated by incubation of RNAse (100 mg) with D-glucose (188 mg) in 625 μl of 0.5 M sodium phosphate buffer, pH 7.4, containing 0.05% NaN3. After reaction over 84 d at 37°C, the material was extensively dialyzed (molecular cutoff: 2 kDa), then lyophilized, and the protein preparation characterized by MALDI-MS and measurement of AGE fluorescence (exc., em. 370, 440 nm). The observed monomeric molecular weight of AGE RNAse A was 13458 Da, 9.3% Mw increase (∆m 1281 Da) and AGE fluorescence intensity (370/440 nm) of the glycated material was very similar to the one displayed by AGE-BSA of similar glycation time (data not shown).

Preparation and characterization of glyicated collagen Collagen type I from calf skin (0.5 g) was stirred at 90°C in water (50 ml) in the presence or absence (control) of D-glucose (2 g). After 1 h reaction time, the solution was filtered and the soluble fraction was extensively dialyzed against water at 4°C (molecular cutoff: 12 kDa). After lyophilization, glycation of the protein preparation was assessed by CML determination as described above and quantified as 47.4 nmol CML per mol lysine in AGE-collagen. No CML was detected in collagen.

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, OK) were routinely cultured in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal bovine serum and kept in a humidiﬁed atmosphere containing 5% CO2 at 37°C. Cells used for experiments were of passage numbers 0±20.

Irradiation with SSL and UV A A kW large area light source solar simulator, model 91293, from Oriel Corporation (Stratford, CT) was used, equipped with a 1000 W Xenon arc lamp power supply, model 68920, and a VIS-infrared bandpass blocking filter plus either an atmospheric attenuation filter (output 290–400 nm plus residual 650–800 nm, termed “solar-simulated light”) or UVB and UVC blocking.
filter (output 320–400 nm plus residual 650–800 nm, termed “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 cm² per s UVA and 0.40 mJ per cm² per s UVB radiation. Using UVB/ UVC blocking filter, the dose at 365 mm from the source was 5.386 mJ per cm² per s UVA radiation with a residual UVB dose of 3.16 mJ per cm² per s.

**Assay for effects of potential photosensitzers on cell growth**

Cells were seeded at 4 × 10⁴ cells per dish on 35 mm dishes (Falcon, Franklin Lakes, NJ) and exposed to SSL (2289 mJ per cm² UVA, 120 mJ per cm² UVB) or UVB (2289 mJ per cm² UVA, 1.34 mJ per cm² residual UVB) in 1 ml Hank’s balanced salt solution (HBSS) containing protein with potential photosensitizer activity (10 mg per ml) or control protein at the same concentration and inhibitors of various concentrations (see figure legends). Following irradiation, the dishes were left for 30 min in the dark at room temperature (postirradiation incubation). The cells were washed twice with HBSS and fresh DMEM was added. Cell number was determined 72 h later. Photosensitization effects were measured by comparing the growth inhibitory effects of SSL alone, compared with that of SSL in the presence of the photosensitizer. Inhibitors of photosensitization were identified by their ability to reverse the effects of the photosensitizer on cell growth.

**Quantification of peroxide formation**

Photo-induced formation of peroxides was quantified according to a standard procedure (Jiang et al., 1990) with minor modifications. After irradiation of the test solution that contained the protein in 1 ml HBSS on 35 mm dishes in the absence of cells, a 300 μl aliquot was deproteinized by spin-dialysis using a Microcon microconcentrator (Amicon, Beverly, MA; molecular cutoff: 3 kDa). An aliquot (20 μl) of the dialyzed solution was added immediately to 380 μl 25 mM H₂SO₄. After addition of 400 μl containing 0.5 mM ferrous ammonium sulfamate, 200 μM xylol orange, and 200 mM sorbitol in 25 mM H₂SO₄ the mixture was incubated at room temperature for 30 min and the absorption was determined at 570 nm using a UV/Visible spectrophotometer (Ultraspex III, Pharmacia, Cambridge, UK). A standard curve was generated using 0, 10, 20, and 40 μM of H₂O₂ as standard. Samples were analyzed in triplicate. 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 H₂O₂. Moreover, heat denatured catalase was employed as another control to exclude nonenzymatic effects of catalase treatment.

**Mass spectrometry of glycated proteins**

Mass spectrometry was performed using a Bruker Reflex III MALDI-TOF-MS. Spectra were recorded in positive ion mode in linear configuration using sinapinic acid as the matrix.

**Statistical analysis**

The results are presented as mean ± SD of three independent experiments. They were analyzed using the two-sided Student’s t test (*p < 0.05, **p < 0.01, ***p < 0.001).

**RESULTS**

**AGE-modified protein as a photosensitizer of HaCaT keratinocyte growth inhibition**

To determine whether AGE function as photosensitzers in our human skin cell models, we assessed whether these compounds could affect cell growth. Human keratinocytes (HaCaT) were exposed to BSA or AGE-BSA (3 mo glycation time) in the absence or presence of a 300 s exposure to solar-simulated radiation (120 mJ per cm² UVB and 2289 mJ per cm² UVA) (Fig 1A). Following a 30 min postirradiation incubation, the exposure medium was removed and replaced with fresh DMEM. Cell number was determined 72 h later, and the growth of mock-treated cells was compared with those treated with BSA or AGE-BSA with or without SSL. BSA or AGE-BSA in the absence of SSL had no significant effect on the growth of HaCaT cells, but cells treated with SSL alone showed approximately 30% growth inhibition compared with mock-treated controls at the selected radiation dose. BSA protected slightly against the growth inhibitory effects of SSL, consistent with partial screening of deleterious UVB due to its strong UV absorbance below 300 nm (Masaki et al., 1999), although the effect did not reach statistical significance; however, treatment with AGE-BSA in the presence of SSL resulted in 80% growth inhibition compared with 30% for SSL alone, i.e., AGE-BSA treatment exhibited a photosensitization effect of greater than 50%. Interestingly, when fresh DMEM was added to cells immediately following exposure to SSL without a 30 min postirradiation incubation, no photosensitization effects were observed (Fig 1B). This observation, together with the finding that pronounced growth inhibition occurred upon addition of pre-irradiated AGE-BSA to unirradiated cells, suggested that a stable cytotoxic factor was produced upon SSL irradiation of AGE-BSA that required a postirradiation incubation to affect cell growth. Reproducibly, without the 30 min postirradiation incubation, AGE-BSA not only failed to photosensitize cells, but protected cells from the growth inhibition caused by SSL. Again, this is most likely due to screening effects caused by the pronounced UVA and UVB absorption by AGE-BSA (Masaki et al., 1999). To determine what portion of the SSL spectrum was responsible for the observed AGE photosensitization, we treated cells with a 425 s exposure to UVB/UVC-filtered UVA radiation in the presence or absence of AGE-BSA (Fig 1C) delivering the same UVA dose as upon SSL irradiation. Cells exposed to UVA alone (2289 mJ per cm² UVA, 1.34 mJ per cm² residual UVB) showed a significant growth inhibition of approximately 20%, whereas 60% growth inhibition was observed after irradiation in the presence of AGE-BSA. These results strongly suggested that the AGE photosensitization upon SSL irradiation in the presence of AGE-BSA was predominantly due to UVA. In an attempt to gain more insight into the generation of AGE photosensitzizers during the glycation of BSA, protein preparations of different degrees of glycation were tested for photosensitization activity towards HaCaT cells (Fig 1D). These preparations were obtained by incubating BSA with D-glucose for various lengths of time. Protein modification by D-glucose was evaluated by molecular weight increase using MALDI-MS, increased protein browning, and increased AGE-type fluorescence (as indicated in Materials and Methods). Incubation with AGE protein in the absence of irradiation had no significant effect on growth, although a trend towards growth inhibitory activity of heavily glycated protein (5 mo glycation time) was observed. Pronounced growth inhibition as a result of AGE sensitization clearly started with day 28 AGE-BSA with a marked increase over 5 mo of glycation time. Interestingly, day 14 AGE-BSA was the first to exhibit a visible brown pigmentation in solution as quantified by absorbance at 420 nm, characteristic of advanced stages of glycation. With glycation time, this pigmentation increased dramatically and paralleled increases in mass of the BSA that represented the accumulation of protein-bound AGE. These data suggest that early glycation products are not likely mediating the photosensitization effects observed, and that advanced stages of glycation are responsible for the formation of photosensitzing chromophores. To test further this hypothesis, we also examined the effects of 1-desoxy-1-morpholino-D-fructose in the presence and absence of SSL (Fig 1D). 1-desoxy-1-morpholino-D-fructose is a ketoamine of low molecular weight and an accepted structural analog of the protein-bound early glycation products called Amadori products (Lloyd and Marples, 1984). This compound showed no photosensitization on HaCaT cells even though it did have growth inhibitory effects at the dose studied. Many cells, including fibroblasts (Owen et al., 1998) and endothelial cells, express membrane receptors for AGE-products, the RAGE receptor in particular, which specifically recognizes the protein-bound AGE N’-carboxymethyl-L-lysin (CML) (Kislinger et al., 1999). CML is a nonfluorescent AGE and thus is not expected to display direct photosensitization effects, but could be activating this response by interaction with the RAGE receptor. To examine this, we incubated AGE-BSA with or without SSL. These preparations were mediated by CML stimulation of RAGE, we tested CML-BSA for cellular photosensitization properties over a wide range of CML-BSA concentrations from 0.1 to 10 μg per ml. As shown in
no photosensitization activity of CML-BSA was detected at 10 mg per ml or at the other concentrations tested (data not shown). Based on this finding together with the earlier observation that pre-irradiation of AGE-BSA confers growth inhibitory activity towards unirradiated cells, it was concluded that the AGE photosensitization of HaCaT cell growth inhibition presented here is likely not RAGE mediated.

**Mechanisms of AGE-photosensitized growth inhibition** To elucidate further the mechanism responsible for AGE photosensitization, a series of anti-oxidant inhibitors, including NaN₃ as a quencher of singlet oxygen and photoexcited states of organic molecules, mannitol as a hydroxyl radical scavenger, superoxide dismutase, and catalase were used. Additionally, deferoxamine mesylate, a ferric iron ion chelator previously shown to rescue fibroblasts from AGE photosensitization when used in millimolar concentrations (Masaki et al, 1999), and D-penicillamine, a potent copper ion chelator (Elgawish et al, 1996) and scavenger of RCS, previously shown to rescue skin cells from carbonyl stress (Wondrak et al, 2002), were tested. Both catalase and D-penicillamine fully blocked the photosensitization effects of AGE-BSA. Superoxide dismutase and deferoxamine mesylate did not protect against AGE photosensitization and showed growth inhibitory effects on HaCaT cells (Fig 2A). Whereas NaN₃ (500 μM and 50 mM) and mannitol (50 mM) exerted significant growth inhibition, no clear protection against AGE photosensitization was detected (data not shown). As catalase specifically degrades H₂O₂, the inhibitor experiments provided strong evidence that H₂O₂ is the toxic intermediate involved in growth inhibition caused by SSL irradiation of AGE-BSA. This finding also is consistent with the observation that a stable photo-induced toxic factor is formed as suggested by the AGE-BSA pre-irradiation experiment and the requirement of a postirradiation incubation for growth inhibition. Interestingly, catalase afforded complete protection of cells not only against photosensitization by AGE-BSA, but also against SSL irradiation in the absence of an externally added photosensitizer, consistent with reports from others that UVA-irradiated HaCaT cells produce H₂O₂ (Gniadecki et al, 2000). As opposed to the mechanistically well-defined protective action of catalase treatment, the protective effects of D-penicillamine could involve multiple mechanisms such as metal chelation or direct reaction with H₂O₂. The fact that the iron-chelator deferoxamine mesylate, which is taken up by cells only weakly, did not provide any protective effects demonstrates that extracellular iron ion-mediated Fenton chemistry was not the basis of toxicity in our experiments, but did not exclude intracellular metal-catalyzed production of hydroxyl radicals from hydrogen peroxide. Likewise, we cannot exclude the possibility that the lack of protection by mannitol is explained by inefficient cellular uptake (Byler et al, 1994). The lack of protection against AGE-photosensitization by NaN₃, together with the stability of the photo-generated toxic factor makes the involvement of singlet oxygen (Grether-Beck et al, 1996) as a mediator of AGE-photosensitized growth inhibition unlikely. As enhancement of an

Figure 1. AGE-photosensitized growth inhibition of HaCaT keratinocytes. (A) HaCaT cells were exposed to SSL irradiation (120 mj per cm² UVB and 2289 mj per cm² UVA) in the presence or absence of 10 mg per 1 ml BSA or AGE-BSA with a 30 min postirradiation incubation. After this time the cells were washed with HBSS. DMEM was added, and after 3 d, growth inhibition was assessed by cell counting. (B) As in (A), except for one sample, which was processed after irradiation without postincubation [AGE/SSL (± 30 min)] and another that included irradiation of AGE-BSA in the absence of cells and then exposing cells to the irradiated material for 30 min (Pre-irr AGE). (C) HaCaT cells were exposed to UVA irradiation (4847 mj per cm² UVA, 2.8 mj per cm² residual UVB) in the presence or absence of AGE-BSA with a 30 min postirradiation incubation and processing as in (A). (D) HaCaT cells were exposed to AGE-BSA prepared by various glycation times [up to 140 d (d₁−d₁₄₀)] with (hatched bars) or without (filled bars) 300 s of SSL irradiation followed by a 30 min postirradiation incubation and processing as in (A). Statistical significance is indicated in comparison with the respective (irradiated or nonirradiated) d₀ values as control. Moreover, 1-desoxy-1-morpholino-D-fructose (1 mM) and CML-BSA (10 mg per ml) were tested.
irradiation effect in the presence of deuterium oxide, acting by prolongation of singlet oxygen lifetime, is another probe for the involvement of singlet oxygen (Klotz et al, 2000), irradiation and 30 min postirradiation incubation were performed in 98% (vol/vol) deuterium oxide–phosphate-buffered saline vs normal phosphate-buffered saline (data not shown). An equal degree of AGE-sensitized growth inhibition was observed consistent with our conclusion that AGE-sensitized cell growth inhibition was not mediated by singlet oxygen. Next, we exposed HaCaT cells to SSL irradiation in the presence of AGE-BSA, but in the absence of catalase and D-penicillamine, and added these inhibitors only during the 30 min postirradiation incubation (Fig 2B). Using this procedure, catalase completely protected the cells from photosensitization, consistent with degradation of H2O2 before entering the cells. Remarkably, D-penicillamine also protected HaCaT cells from photosensitization, when present only during the postirradiation incubation, although it was less effective than catalase. The lower protection by D-penicillamine when added following SSL irradiation may point towards a contribution of thiol quenching of the photoexcited state of the sensitizer as a protective mechanism. As chemical pretreatment of AGE-BSA with D-penicillamine to scavenge protein-bound carbonyl groups or chelate copper ions did not diminish the photosensitization effect of AGE-BSA (data not shown), these results strongly suggest that D-penicillamine inhibits AGE photosensitization by direct scavenging of H2O2 and quenching of photoexcited states.

**AGE-photosensitized production of H2O2**

As production of various ROS during UVA irradiation of isolated AGE (Ortwerth et al, 1997) and AGE-BSA (Masaki et al, 1999) have been reported earlier, we looked for AGE-photosensitized production of H2O2 using a quantitative spectrophotometric assay for H2O2 as described in Materials and Methods. First, SSL irradiation of various glycated protein samples and nonglycated protein (BSA, RNase A) in the same concentrations as used for the cell experiments was performed in the absence or presence of inhibitory substances with subsequent determination of H2O2 (Fig 3A). BSA, whether irradiated or nonirradiated did not produce H2O2. Likewise, AGE-BSA (glycation time 84 d, as used in the cell growth inhibition assay) in the absence of SSL did not produce H2O2; however, if this sample was irradiated with an equal dose of SSL used in the cell growth inhibition assay approximately 35 μM H2O2 was produced. When the irradiation was performed in the presence of catalase (400 u per ml), no H2O2 was detectable. Sodium azide (50 mM) reduced the formation of H2O2 by 30%, possibly by direct quenching of the photoexcited state of the sensitizer with partial suppression of H2O2 formation, an effect that was not clearly identified in the cell protection experiment described above. As expected, mannitol (50 mM) did not interfere with the light-driven production of H2O2. Moreover, equal amounts of hydrogen peroxide were produced upon irradiation in 98% (vol/vol) deuterium oxide–phosphate–buffered saline vs normal phosphate-buffered saline (data not shown) disqualifying a role of singlet oxygen formation upstream of hydrogen peroxide generation. Neither CML-BSA (10 mg per ml) nor 1-deoxy-1-morpholinod-1-fructose (1 mM) produced H2O2 upon SSL irradiation, consistent with their lack of cellular photosensitization activity described above. To exclude the contribution of tryptophan and its oxidation products (e.g., N-formyl-kyurenine) as protein–bound UVA sensitizers (McCormick et al, 1976; Grossweiner, 1984), RNase A as a protein without tryptophan residues was glycated under similar conditions for comparative use with AGE-BSA in the photosensitization study (Ortwerth et al, 1995). Indeed, RNase A glycated for 3 mo (AGE-RNase) was a potent sensitizer of SSL-driven H2O2 production, whereas irradiation of unglycated RNase was ineffective as observed earlier with BSA demonstrating clearly that ROS production was a result of AGE sensitization. Next, increasing doses of SSL or UVA caused a concentration–dependent production of H2O2 upon irradiation of AGE-BSA (glycation time 28 d). These data have been normalized for the incident dose of UVA (Fig 3B). At doses used for cell irradiation in this study (up to 2.5 J per cm²), UVA was equally effective as SSL in driving production of H2O2, and at higher doses it was still approximately 70% as effective as SSL, consistent with earlier findings that isolated AGE chromophores responsible for photosensitization are excited by UVA wavelengths (Ortwerth et al, 1997). Remarkably, even extended SSL irradiation of the AGE-BSA sample resulted in linear increases in H2O2 production, giving no indication of chemical consumption or bleaching of the responsible AGE chromophores over the time of irradiation (up to 30 min). Finally, we compared the observed photosensitization potency of AGE-BSA preparations representing various degrees of glycation (Fig 3D) with light-driven production of H2O2 (Fig 3C). AGE-BSA samples glycated for various times were exposed to SSL irradiation under the same conditions as described in the cell growth assay, but in the absence of cells (Fig 3D). AGE-sensitized H2O2 production increased dramatically with glycation time supporting the hypothesis that H2O2 production correlated with the potency of AGE-photosensitization.

As catalase completely suppressed AGE-photosensitized cell growth inhibition and various inhibitors of other ROS had very limited effectiveness, we compared the effects of exposure to AGE-BSA (glycation time 84 d) in combination with SSL irradiation on HaCaT cells with the effects of direct exposure to 40 μM H2O2 in
the absence of irradiation (Fig 4), an amount slightly above the concentration of H$_2$O$_2$ formed upon SSL irradiation of this AGE-BSA sample. H$_2$O$_2$ (40 μM) in the absence of SSL induced a growth inhibition similar to that of irradiated AGE-BSA. Full growth inhibition of HaCaT cells resulted when treatment with H$_2$O$_2$ (40 mM) was combined with SSL irradiation. We conclude that H$_2$O$_2$ formation is likely responsible for most of the AGE-photosensitized cell growth inhibition in HaCaT cells.

AGE-BSA as a photosensitizer of human fibroblast growth inhibition To elucidate further the effects of AGE photosensitization on various human skin cells, human CF3 fibroblasts were tested next, employing the same growth inhibition assay as described above for HaCaT keratinocytes (Fig 1A), with the exception that a different AGE-BSA preparation (only 28 d glycation time) with a somewhat attenuated photosensitization potency was chosen due to the expected higher oxidative vulnerability of fibroblasts as compared with keratinocytes (Leccia et al., 1998). In the absence of SSL irradiation, treatment with BSA, AGE-BSA, or CML-BSA had no effect on the growth of CF3 fibroblasts (Fig 5A). CF3 fibroblasts were more sensitive to SSL irradiation alone, showing over 60% growth inhibition compared with 30% for keratinocytes. In agreement with our results on HaCaT cells and an earlier report on AGE-photosensitized cell toxicity to fibroblasts (Masaki et al., 1999), AGE-BSA in the presence of SSL resulted in 100% growth inhibition, even though AGE-BSA of attenuated photosensitization activity (1 mo glycation time) was used. As human skin fibroblasts are known to express the RAGE receptor (Owen et al., 1998), we tested RAGE involvement in AGE-sensitized growth inhibition of CF3 fibroblasts using CML-BSA as a known ligand for the RAGE receptor (Kislinger et al., 1999). CML-BSA had no suppressive

Figure 4. Hydrogen peroxide treatment is responsible for the AGE-photosensitized growth inhibition of HaCaT cells. A cell growth inhibition experiment was performed as described in Fig 1(A). Additionally, cells were treated with hydrogen peroxide (40 μM) with or without SSL irradiation, followed by a 30 min postirradiation incubation.

Figure 3. AGE-photosensitized production of hydrogen peroxide. (A) Production of hydrogen peroxide during SSL irradiation of BSA preparations. Protein preparations (10 mg per ml in HBSS) were SSL irradiated under the same conditions as given in the legend of Fig 1(A). Hydrogen peroxide was measured immediately as described in Materials and Methods. AGE: glycated protein. Non-irradiated BSA was used as control for statistical analysis. (B) Dose-response of hydrogen peroxide production during irradiation of AGE-BSA (10 mg per ml), glycated for 28 d, with SSL (■) or UVA (▲), normalized for the total UVA dose applied to the sample. Statistical analysis was performed for all doses, comparing UVA-induced with SSL-induced production of H$_2$O$_2$. *p < 0.039 for all doses > 2.3 J per cm$^2$. (C) SSL-driven hydrogen peroxide production as a function of AGE content as based on glycation time of the AGE-BSA sample. Protein samples were treated as described in Fig 1(A), except that no cells were present, and hydrogen peroxide levels were determined immediately after irradiation. Irradiated BSA (d0 sample) was used as controls for statistical analysis.
effect on the growth of CF3 fibroblasts in the absence of SSL irradiation, and likewise did not function as a photosensitizer; however, because other AGE receptors are known to exist (Sano et al., 1999), the possibility that some other epitope in AGE-BSA stimulates another type of AGE receptor cannot be ruled out.

Glycated collagen as a photosensitizer of human fibroblast growth inhibition To add further experimental evidence towards a possible role of AGE-proteins as photosensitizers in skin we tested the photosensitization effects of glycated type I collagen on the growth of human CF3 skin fibroblasts (Fig 5B); however, treatment with heat-solubilized collagen significantly inhibited normal growth of CF3 fibroblasts most probably as a result of some growth inhibitory extracellular matrix–cell interaction. This effect was equally apparent, when we used collagen solubilized by a short collagenase digestion (data not shown). Equally, AGE collagen in the absence of SSL irradiation displayed a growth inhibitory effect. Unexpectedly, both unglycated collagen and AGE-collagen showed a photosensitization effect in CF3 fibroblasts upon SSL irradiation (Fig 5B); however, AGE collagen sensitized 100% cell growth inhibition and was clearly more pronounced than collagen-sensitized growth inhibition. The unexpected photosensitization effect of nonglycated collagen suggested that it may generate H₂O₂ upon SSL irradiation and this was confirmed in vitro. Heat-solubilized and untreated collagen in suspension produced significant amounts of H₂O₂ upon SSL irradiation (Fig 5C). AGE collagen displayed massive photosensitized H₂O₂ production, and low amounts of H₂O₂ were detected even in the absence of irradiation. In the CF3 fibroblast model system, catalase protected the cells only slightly from the effects of SSL irradiation alone (Fig 5B), contrary to the full protection of HaCaT cells observed above. Interestingly, the degree of protection provided by catalase treatment in fibroblasts was the same for SSL-irradiated AGE-collagen as for SSL irradiation in the absence of sensitizer. As HaCaT keratinocytes were fully rescued by catalase treatment from the growth inhibitory effects of SSL irradiation (Fig 2) and fibroblasts were not, this result suggests that keratinocytes growth inhibition is mediated entirely by externally produced H₂O₂; whereas in fibroblasts, production of intracellular ROS not accessible to external catalase and/or involvement of 1O₂ are likely to occur.

DISCUSSION

The UVA component of solar radiation induces biologic effects such as photoaging and carcinogenesis of cutaneous cells by photosensitized reactions involving endogenous chromophores (Scharffetter-Kochanek et al, 1997). Accumulation of AGE-modified protein as a result of protein glycation by RCS is thought to contribute to skin deterioration and aging (Monnier et al, 1992; Brownlee, 1995). Recently, AGE-photosensitized production of ROS has been shown to cause immediate cytotoxicity in cultured human fibroblasts as assessed by loss of neutral red uptake and increased lipid peroxidation (Masaki et al, 1997, 1999). Here we demonstrate AGE-photosensitized cell growth inhibition of two cultured human skin cell lines, HaCaT keratinocytes and CF3 fibroblasts, irradiated with SSL or UVA. Consistent with earlier reports on AGE-photosensitization (Masaki et al, 1997, 1999), H₂O₂ was identified as the primary mediator of AGE-photosensitized cell growth inhibition as evidenced by the complete protective effects of catalase treatment and cell growth inhibition of...
Singlet oxygen formation may occur as a result of direct energy transfer from the excited AGE sensitizer to molecular oxygen (photosensitization type II, equation II A) (Ortwerth et al., 1997). In addition, the formation of superoxide radical anion as a precursor of \textit{H}_2\textit{O}_2 is likely to occur, either by a variant of photosensitization type II with production of a sensitizer radical cation (equation II B) or by intermediate reduction of the sensitizer by a substrate (R) to the semireduced state followed by single electron reduction of oxygen (photosensitization type I, equation I) (Foote, 1991; Scharffetter-Kochanek et al., 1997). The predominance of \textit{H}_2\textit{O}_2 formation as observed in our cellular assay suggests that superoxide formed by either type I or II photosensitization undergoes spontaneous dismutation with the production of \textit{H}_2\textit{O}_2 that finally decays upon Fenton-type metal catalysis into highly reactive hydroxyl radicals (Scharffetter-Kochanek et al., 1997). Consistently, superoxide dismutase was not protective in our cellular assay. The formation of an AGE protein–bound free radical cation (equation II B) as a result of photoreduction of oxygen to the superoxide anion radical is hypothetical at this point, but consistent with earlier ESR studies \textit{in vitro} (Masaki et al., 1999; Wondrak et al., 2000).

Hypothetically, AGE-photosensitized production of ROS not only exhibits direct cellular toxicity, but likely induces the further generation of RCS from lipid peroxidation and sugar autoxidation, thereby establishing a vicious cycle between RCS and ROS formation driven by solar light. Thus, the significantly higher CML levels of human skin elastin observed in sun-exposed than in unexposed areas (Mizutani et al., 1997) may be due to increased UV-induced oxidative stress leading to formation of the RCS glyoxal, a direct precursor of CML, from lipid peroxidation and/or glycation (Baynes and Thorpe, 2000). Additionally, cellular toxicity as a result of direct RCS action and/or chronic AGE-mediated stimulation of RAGE with subsequent NF-{\kappa}B activation (Lander et al., 1997), may be of relevance in skin, although not observed during the short exposure times examined in this study. Thus, the well-established age- and disease-dependent accumulation of AGE-modified proteins in human skin may have a fundamental role in the generation of chronic photo-oxidative stress.

Although effective anti-oxidant systems, such as catalase, glutathione peroxidase, and anti-oxidant compounds, exist in skin (Black, 1993), differential sensitivity of skin cell types towards photosensitized oxidative stress may be expected and genotypic differences in anti-oxidant response may vary among individuals with different skin types. The possibility of AGE-photosensitized production of significant amounts of \textit{H}_2\textit{O}_2 in skin has important implications for skin carcinogenesis. Specifically, interference of AGE-mediated photoproduction of \textit{H}_2\textit{O}_2 with \textit{H}_2\textit{O}_2-related mitogenic redox signaling (Schmidt et al., 1996) seems likely. Various human tumor cells produce substantial amounts of \textit{H}_2\textit{O}_2

### Figure 6. A model of AGE photosensitization in human skin.

Extracellular accumulation of AGE-modified proteins as a result of RCS-mediated glycation induces solar light-driven ROS production targeting cellular membranes, proteins, and DNA. Involvement of AGE receptors on human skin cells and the indicated chemistry of light-driven ROS production are hypothetical. Flashes indicate ROS- and RCS-mediated cellular damage. AGE*: photoexcited AGE; PS: photosensitization.

Similar magnitude in AGE-BSA photosensitization experiments as with \textit{H}_2\textit{O}_2 alone when used at concentrations produced by SSL-irradiated AGE-BSA. D-penicillamine protected against the photosensitization, most likely by direct interaction with \textit{H}_2\textit{O}_2 or additional inhibitory effects on Fenton-reaction induced hydroxyl radical production by chelation of transition metal ions. Upon irradiation of AGE-BSA, \textit{H}_2\textit{O}_2 formation correlated with increasing AGE content of the glycated protein, as reflected by increasing molecular weight, browning, and formation of AGE-type fluorescence as a function of glycation reaction time. The UVA portion of the SSL spectrum accounted for most of the SSL-induced \textit{H}_2\textit{O}_2 production \textit{in vitro}. Keratinocytes were clearly more resistant to AGE-photosensitized growth inhibition as compared with fibroblasts. This finding is consistent with the well-known differences in anti-oxidant defense capacities of these cell lines (Yohn et al., 1991; Leccia et al., 1998). Interestingly, the cytotoxic effects of SSL- and UVA-irradiated AGE protein described above are very similar to the cytotoxicity of the UVA-irradiated endogenous photosensitizer riboflavin (Sato et al., 1995). As observed with AGE/photosensitization, the long-lasting oxygen-dependent cytotoxicity in human fibroblasts of irradiated riboflavin also was abolished by catalase treatment. Further, the cytotoxicity of UVA-irradiated riboflavin correlated with the concentration of \textit{H}_2\textit{O}_2 formed in the irradiated solution. In contrast to SSL-irradiated BSA, SSL irradiation of nonglycated skin collagen \textit{in vitro} was another source of significant \textit{H}_2\textit{O}_2 production in our experiments, suggesting photosensitization activity of collagen-associated chromophores (Kollias et al., 1998). AGE modification of collagen, however, clearly enhanced its potency as a UV sensitizer of cell growth inhibition and ROS production \textit{in vitro}, supporting our hypothesis that glycated components of the extracellular matrix may act as sensitizers in skin. The relative contribution of native collagen and elastin cross-links, as UVA-irradiation of elastin, although lacking any tryptophan residues (Petruska and Sandberg, 1968), induced ROS formation and cell growth inhibition and the isolated collagen cross-link pyridinoline was an equally effective sensitizer (data not shown). Moreover, as our experiments used type I collagen from calf skin, the question of whether comparable sensitizing effects occur with human skin collagen requires further studies.

Our results qualify AGE-modified protein as a candidate chromophore for the mediation of UVA-induced skin damage (Fig. 6). Extracellular accumulation of AGE-modified proteins as a result of RCS-mediated glycation of long-lived extracellular matrix components induces solar light driven ROS production targeting cellular membranes, proteins, and DNA. The chemistry of AGE-photosensitized ROS production is not clear and may involve both type I and type II photo reactions (formulas I and II).
spontaneously, a process that has been interpreted as an autocrine maintenance of proliferative signaling in melanoma cells (reviewed in Brar et al, 2001). Moreover, mitogenic signaling in fibroblasts through both Ras and Rac is mediated by superoxide anion production with spontaneous $H_2O_2$ formation. Interestingly, scavengers of ROS, especially catalase, are potent inhibitors of melanoma cell proliferation, further demonstrating that endogenous redox stress seems to be responsible for constitutive NF-kB activation in malignant melanoma cells (Brar et al, 2001).

Owing to the involvement of UVA photosensitization in skin pathology and photoaging (Scharffetter-Kochanek et al, 1997), the pharmacologic suppression of AGE-photosensitized skin cell growth inhibition, as shown in this study for catalase and D-penicillamine, may be of therapeutic relevance. In this respect, the recent report on the prevention of SSL-induced photodamage in the hairless mouse dorsal skin by topical application of the iron chelator kojic acid should be mentioned (Mitani et al, 2001). Currently, our experimental model is being evaluated for the screening of novel therapeutic agents for the suppression of skin photodamage as a result of photosensitization. Future studies are needed on intact human skin with elevated AGE-content to test the hypothesis raised in this study.

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