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Malondialdehyde-Derived Epitopes In Human Skin Result From Acute Exposure To Solar UV And Occur In Nonmelanoma Skin Cancer Tissue

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Abstract

Cutaneous exposure to solar ultraviolet radiation (UVR) is a causative factor in photoaging and photocarcinogenesis. In human skin, oxidative stress is widely considered a key mechanism underlying the detrimental effects of acute and chronic UVR exposure. The lipid peroxidation product malondialdehyde (MDA) accumulates in tissue under conditions of increased oxidative stress, and the occurrence of MDA-derived protein epitopes, including dihydropyridine-lysine (DHP), has recently been substantiated in human skin. Here we demonstrate for the first time that acute exposure to sub-apoptogenic doses of solar simulated UV light (SSL) causes the formation of free MDA and protein-bound MDA-derived epitopes in cultured human HaCaT keratinocytes and healthy human skin. Immunohistochemical staining revealed that acute exposure to SSL is sufficient to cause an almost twenty-fold increase in general MDA- and specific DHP-epitope content in human skin. When compared to dose-matched solar simulated UVA, complete SSL was more efficient generating both free MDA and MDA-derived epitopes. Subsequent tissue microarray (TMA) analysis revealed the prevalence of MDA- and DHP-epitopes in nonmelanoma skin cancer (NMSC). In squamous cell carcinoma tissue, both MDA- and DHP-epitopes were increased more than three-fold as compared to adjacent normal tissue. Taken together, these data demonstrate the occurrence of MDA-derived epitopes in both solar UVR-exposed healthy human skin and NMSC TMA tissue; however, the potential utility of these epitopes as novel biomarkers of cutaneous photodamage and a functional role in the process of skin photocarcinogenesis remain to be explored.

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Keywords

Malondialdehyde; Lipid peroxidation; Photodamage; Photocarcinogenesis; Nonmelanoma skin cancer; DHP-lysine

1. Introduction

Cutaneous exposure to solar ultraviolet radiation (UVR) contributes to photoaging and photocarcinogenesis. Neoplasms of the skin represent the most common type of cancer in fair-skinned populations, and incidence rates of nonmelanoma skin cancer (NMSC), including both basal cell (BCC) and squamous cell carcinoma (SCC), are increasing worldwide [1–3]. Solar UV photons are established environmental carcinogens, and NMSC incidence correlates with high cumulative solar exposure [1, 4, 5]. Extensive research has been focused on determining the molecular processes linking solar UVR exposure to acute and chronic skin photodamage.

Beyond the formation of mutagenic DNA base photoproducts, UVR is thought to cause skin photodamage through induction of photooxidative stress [6]. UVR-driven generation of reactive oxygen species (ROS) may originate from endogenous non-DNA photosensitizers and other molecular sources including NAD(P)H oxidase and mitochondrial electron leakage [7–10]. Oxidative stress is now widely considered as a key mechanism contributing to the detrimental effects of acute and chronic UVR exposure, and both the UVB (290–320 nm) and UVA (320–400 nm) spectral regions have been shown to cause skin oxidative damage through structural and functional alterations of critical molecular targets [11–15].

Lipids are prominent molecular targets of photooxidative stress, and structural and functional alterations associated with skin photodamage have been linked mechanistically to lipid peroxidation observed upon exposure to isolated UVA, isolated UVB, and solar simulated UV light (SSL) combining the two spectral regions [16–18]. Initial membrane damage through free radical chain reactions leads to the formation of phospholipid peroxides and fatty acid-derivatives. For example, formation of 8-isoprostane, an arachidonic acid-derived prostaglandin F_{2α} isomer, has been demonstrated in human skin in response to UVB as well as SSL exposure [19, 20]. Further rearrangement and decomposition of initial lipid peroxides generate highly reactive carbonyl species (RCS) such as malondialdehyde (MDA), 4-hydroxynonenal, acrolein, and glyoxal [14]. Importantly, RCS accumulating under conditions of oxidative stress cause tissue damage through covalent adduction of bystander biomolecules including proteins and nucleic acids, an effect associated with formation of immunogenic damage-associated molecular patterns [21–23].

The reactive dialdehyde MDA is an established product of solar UVR-induced lipid peroxidation [24]. MDA formation has been documented in cultured skin cells and has also been observed in murine and human skin exposed to a wide range of spectral fractions and doses of solar UVR [24–26]. Consequently, the origin and role of MDA in photoaging, photocarcinogenesis, and inflammatory skin disorders has been the subject of investigation [27–29]. MDA-adduction of target proteins results in crosslinking, formation of fluorescent epitopes, and functional alterations [30, 31]. The MDA-lysine adducts N^ε-(2-propenal)lysine and dihydropyridine (DHP)-lysine ((S)-2-amino-6-(3,5-diformyl-4-methyl-4H-pyridin-1-yl)-hexanoic acid) have been detected in human tissue damaged by chronic oxidative stress [32]. However, the chemical identity of specific biological MDA-derived epitopes remains largely unresolved.

Recently we have demonstrated the occurrence of MDA-derived epitopes of unknown origin in human skin, and have presented evidence in support of a specific role of DHP-epitopes as endogenous UVR-photosensitizers [33]. Other research has reported the occurrence of MDA-derived protein epitopes of undefined chemical structure in skin cancer tissue [34]. We therefore examined the possibility that acute exposure to solar UVR may cause the introduction of both general MDA- as well as specific DHP-epitopes in human skin. Here we demonstrate for the first time that (*I*) acute exposure to solar simulated UV light (SSL) causes the formation of free MDA and protein bound MDA- and DHP-epitopes in cultured human epidermal keratinocytes and healthy human skin, and (*II*) MDA- and DHP-epitopes occur abundantly in nonmelanoma skin cancer compared to adjacent normal controls as revealed by tissue microarray analysis.

2. Materials and Methods

2.1. Chemicals

Pentafluorophenylhydrazine (PFPH), deferoxamine mesylate, butylated hydroxytoluene (BHT), 1,1,3,3-tetraethoxypropane, 3-dimethylamino-2-methyl-2-propenal, sodium hydroxide (NaOH), potassium hydroxide (KOH), hydrochloric acid (HCl), perchloric acid (HClO₄), sulfuric acid (H₂SO₄), citric acid monohydrate, sodium phosphate dibasic, isooctane, acetone, isopropyl alcohol, (Sigma Chemical). All aqueous solutions were made with double distilled water. All other chemicals were of analytical grade.

2.2. Cell culture

The established cell line of spontaneously immortalized human epidermal keratinocytes (HaCaT), a gift from Dr. Norbert Fusenig (German Cancer Research Center, Heidelberg, Germany), was routinely cultured in low glucose Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal bovine serum (FBS) and kept in a humidified atmosphere containing 5% CO₂ at 37°C. Cell numbers were routinely determined after washing with phosphate buffered saline pH 7.4 without Mg²⁺/Cl²⁺ (PBS) and trypsin detachment using a Z1 Coulter counter (Beckman Coulter).

2.3. Human tissue acquisition

Healthy "normal" human skin was freshly obtained as remnant tissue from abdominoplasty procedures as per an Institutional Review Board approved protocol. Obtained skin specimens were remnants from designated standard care with no additional skin removed for research and tissue samples were assigned a unique specimen number and deidentified to protect patient confidentiality. Upon excision, the hypodermal fatty layer was removed from the dermal layer via scalpel and tissue samples were placed directly into transport medium (DMEM) on ice for the short duration of transport to the research laboratories. Immediately upon arrival, a scalpel was utilized to section the tissue specimens into treatment groups, which were placed into PBS. Specimens were irradiated or sham-irradiated as subsequently described; treatment times did not exceed 5 min. Immediately following treatment, specimens were placed into fresh medium (DMEM) and kept in a humidified atmosphere containing 5% CO₂ at 37°C for 30 min. Samples were then either sub-sectioned by scalpel and prepared for immunohistochemical analysis or partitioned into individual samples for analytical analysis utilizing a 4 mm dermal punch and stored at -80°C until analysis.

2.4. Immunocytochemical and immunohistochemical detection of MDA- and DHP-protein epitopes

HaCaT keratinocytes samples for immunocytochemistry (ICC) analysis were pelleted by centrifugation, fixed in 10% neutral buffered formalin for 24 hours, then transferred to 70%

ethanol for an additional 24 hours. Shandon Cytoblock Cell Block Preparation System (Thermo Scientific) was utilized per manufacturer instructions to render cellular samples suitable for routine processing and paraffin embedding. Five-micron sections were deparaffinised and rehydrated. Staining was performed using a streptavidin biotin peroxidase system with a phosphatase substrate and a hematoxylin counter stain. General MDA-adducted proteins were visualized using a validated commercial rabbit polyclonal antibody (ab6463 Abcam; dilution 1:500). Dihydropyridine (DHP)-lysine-adducted proteins were visualized using a validated murine primary monoclonal antibody (clone 1F83 MMD-030 JaICA, provided by Koji Uchida, Nagoya University, Japan; dilution 1:100) [35].

Normal human skin samples for immunohistochemistry (IHC) analysis were fixed in 10% neutral buffered formalin for 24 hours then transferred to 70% ethanol prior to routine processing and paraffin embedding. Five-micron tissue sections were deparaffinised and rehydrated. Sample were stained and visualized in the same manner as described for skin cells, with the exception that the general MDA polyclonal antibody was utilized at a dilution of 1:250.

2.5. Immunohistochemical detection of MDA- and DHP-protein epitopes in NMSC tissue microarray (TMA)

Two 5 × 10 commercial human skin cancer vs. normal TMAs (IMH-323 Imgenex, lot CX2) were utilized to assess the presence of general MDA-adducted proteins and specific DHP-lysine-adducted proteins in NMSC by IHC. Staining protocol, reagents, and dilutions were as described for normal human skin.

2.6. Image capture and quantification

Samples were acquired using a Leica DMR microscope (Wetzlar), and a 3CCD colour video camera (Sony). For each sample, three areas were chosen at random and imaged at 40X magnification under Kohler illumination upon a white balanced background. Images were quantified using ImagePro Plus (Media Cybernetics) software, written specifically for the analysis of immunohistologic specimens. All images and segmentation mask overlays were electronically saved and archived. Results from the three imaged areas were averaged for each sample to generate a positive area score ($\pm$ SD) as described by Einspahr et al. [36].

2.7. Gas chromatography-mass spectrometry quantification of MDA

Standard preparation—The MDA standard solution was prepared as described by Yeo et al. [37]. Briefly, a 10 mM solution of 1,1,3,3-tetraethoxypropane was prepared in 0.01 M HCl and allowed to hydrolyze at room temperature for 6 hr. The exact concentration of MDA was confirmed by sample dilution with 0.01 M HCl and spectrophotometric absorbance measurements at 245 nm, $\epsilon=13700 \text{ cm}^{-1} \text{ M}^{-1}$, with working serial dilutions prepared in PBS. The 2-methyl-propanedial (Me-MDA) internal standard solution was prepared as described by Claeson et al. [38]. Briefly, 500 mg 3-dimethylamino-2-methyl-2-propenal and 200 mg NaOH was dissolved in 700 mL of double distilled water. The mixture was heated to 70°C for 30 min. The sample was evaporated to dryness in a nitrogen atmosphere. The obtained crystals were repeatedly washed with a 1:1 mixture of acetone and ethanol. The purified crystals were again evaporated to dryness in a nitrogen atmosphere before weighing, separation into aliquots, and storage at -80°C . The exact concentration of Me-MDA solutions reconstituted in double distilled water was confirmed by spectrophotometric absorbance measurements at 274 nm, $\epsilon=29900 \text{ cm}^{-1} \text{ M}^{-1}$, with working dilutions prepared in PBS [39].

Analyte extraction from cells and tissue—For cultured human skin cells, following incubation for the indicated time after irradiation, cells were placed on ice and scraped into

1.5 mL sample tubes after addition of 1 mL buffered anti-oxidant solution containing the Me-MDA internal standard (1 mM deferoxamine mesylate, 10 μ M BHT in PBS with 250 pmole Me-MDA). Free MDA extraction was achieved utilizing a F60™ sonic dismembrator (Fisher Scientific) at 3 W for 10 seconds with 50 μ L sample aliquots removed for protein and DNA standardization. A 0.5 mL aliquot of sample was then neutralized to pH 5.5 by addition of 0.3 M citrate-phosphate buffer prior to derivatization. For human skin, samples were transferred from -80°C storage to liquid nitrogen. Samples were weighed prior to trituration by mortar and pestle in liquid nitrogen. Powdered sample was further homogenized in 1 mL 1 M HClO_4 containing 500 pmole of the Me-MDA internal standard. The supernatant was taken for analysis while the protein precipitate was processed for protein standardization. The supernatant was neutralized to pH 5.5 by addition of cold 2 M KOH/0.3 M citrate-phosphate and the precipitant salt was removed by centrifugation prior to derivatization.

Derivatization—The derivatization method was modified from Yeo et al. [37]. Briefly, for each neutralized sample, derivatization was achieved by the addition of 200 μ L 5 mg/mL PFPH. The derivatization reaction was allowed to proceed for 30 min at room temperature protected from light. The reaction was terminated by acidification utilizing 40 μ L of 9 N H_2SO_4 prior to derivative extraction. Extraction was achieved by addition of 150 μ L isooctane upon which the samples underwent 3 rounds of vortexing for 10 seconds each. Samples were centrifuged for 5 min to separate the two phases and approximately 100 μ L of the upper isooctane layer was then transferred to an autosampler vial for analysis.

Gas chromatography and mass spectrometry (GC-MS) analysis—GC-MS analysis was performed on a Trace GC Ultra™ gas chromatograph coupled to a Trace DSQ™ mass spectrometer (Thermo Scientific). A DB-5 30 m $\times$ 0.25 mm MS column (J&W Scientific/Agilent Technologies) was utilized for compound separation using ultra-high purity helium carrier gas at a constant flow of 1.5 mL/min. Samples of 1 μ L were separated via temperature gradient: initial temperature of 50°C was held for 1 min before ramping at $20^{\circ}\text{C}/\text{min}$ to a final temperature of 300°C which was held for 5 min before equilibration to baseline. The Autosampler Plus (Thermo Scientific) tray was maintained at ambient temperature. Sample injection was performed in the PTV splitless mode with an injector temperature of 200°C . Mass spectrometer was operated with an electron energy of 170 eV and a source temperature of 200°C . Primary ionization peaks were determined to correspond to the principle molecular weight for each species, thus detection was conducted via selected ion monitoring of m/z 234 for PFPH-MDA and m/z 248 for PFPH-Me-MDA, retention times were 5.5 and 6.3 minutes, respectively. Data analysis was performed using Xcalibur™ software v 1.2 (Thermo Scientific).

2.8. Protein and DNA quantification assays

Protein determinations from cellular and skin tissue samples were conducted utilizing the Quick Start™ Bradford Protein Assay (Bio-Rad). The assay was conducted in standard microplate format utilizing bovine serum albumin as a reference standard according to manufacturer's instructions. Absorbance determinations were conducted on a Synergy 2™ multi-detection microplate reader (BioTek) at 590 nm.

Quantification of DNA from cellular and skin tissue samples were conducted utilizing the Quant-iT™ PicoGreen® dsDNA Kit (Invitrogen). The assay was conducted in 96-well plate format utilizing a lambda DNA standard as a provided kit component according to manufacturer's instructions. Fluorescence endpoint determination was conducted on a Spectra MAX Gemini™ microplate reader (Molecular Devices) at Ex/Em 480/520 nm.

2.9. Cell viability analysis

Cell viability was determined by annexin-V-FITC/propidium iodide (PI) dual staining of cells followed by flow cytometric analysis [40]. Cell staining was performed using an apoptosis detection kit according to manufacturer's specifications (APO-AF; Sigma-Aldrich). Flow cytometry analysis was performed on a FACScan analyzer (BD Biosciences) with results shown in a standard 4 quadrant display in which the lower left quadrant (AnnexinV⁻, PI⁻) represents viable cells, the lower right (AnnexinV⁺, PI⁻) represents early apoptosis, and the upper right quadrant (AnnexinV⁺, PI⁺) represents either late apoptotic or necrotic, non-viable cells.

2.10. Irradiation with solar simulated UV light (SSL) or UVA

Irradiation of cells and tissue samples with SSL or UVA was conducted utilizing a kilowatt large area light source solar simulator (model 91293, Oriel Corporation) equipped with a 1000 W Xenon arc lamp and power supply, model 68920, and a VIS-IR bandpass blocking filter combined with an atmospheric attenuation filter (output 290–400 nm plus residual 650–800 nm). The output was quantified using a dosimeter from International Light Inc., model IL1700, with an SED240 detector for UVB (range 265–315 nm, peak 285 nm), or a SED033 detector for UVA (range 315–400 nm, peak 365 nm), at a distance of 345 cm from the source, which was used for all experiments. Source irradiance ($\text{W} \cdot \text{m}^{-2} \cdot \text{nm}^{-1}$) was determined using a spectroradiometer, (OL754-PMT, Optronic Laboratories). At 345 cm from the source, the SSL dose was 550 mJ/cm²/min UVA + 30 mJ/cm²/min UVB radiation, and the UVA dose was 550 mJ/cm²/min UVA + 3.0 $\mu\text{J}/\text{cm}^2/\text{min}$ UVB radiation. Growth media was removed and replaced with an equal volume of PBS for both control and SSL irradiated HaCaT cells. For treatment, cells were irradiated for the indicated time while control cells were returned to the dark in a humidified atmosphere containing 5% CO₂ at 37°C. After treatment, the specified growth medium was returned to both treated and control cells that were then replaced in a humidified atmosphere containing 5% CO₂ at 37°C for the specified time.

2.11. Statistics

The results are presented as means ($\pm$ SD). In all bar graphs, comparative statistics were analyzed employing a one-way analysis of variance (ANOVA) with Tukey's multiple comparison post-test, (95% confidence). Differences were considered significant at $p < 0.05$. In all bar graphs, means denoted with different letters differ significantly ($p < 0.05$). Kruskal-Wallis ANOVA employing a Tukey's multiple comparison post-test, (95% confidence), was utilized for comparison between groups of the NMSC TMA. P values for NMSC TMAs originate from a two-tailed, unpaired students t test, (95% confidence). All statistical analyses were performed using Prism 5.0 software.

3. Results

3.1. Acute SSL exposure increases intracellular levels of free MDA and drives the formation of both MDA- and DHP-protein epitopes in cultured human keratinocytes

The ability of solar UVR to induce MDA-derived protein epitopes in human skin cells was investigated by quantification of intracellular free MDA levels and epitope-specific immunocytological examination (Fig. 1).

A dose-dependent increase in intracellular levels of free MDA was observed in HaCaT keratinocytes after acute exposure to SSL (Fig. 1A), where identical response relationships were observed regardless of whether normalization was performed relative to cellular DNA or protein content. A significant elevation of cellular MDA levels was observed even in response to low doses of SSL (60 mJ/cm² UVB) that doubled MDA concentrations without

significant impairment of cellular viability as revealed by flow cytometric analysis of annexinV-PI stained cells (Fig. 1B). The subsequent analysis of MDA-derived protein epitopes was therefore performed at this dose.

Next, the SSL-induced occurrence of general MDA- and DHP-epitopes in human skin cells was examined by ICC analysis. HaCaT keratinocytes exhibited an increased intensity in epitope-specific staining as compared to sham-irradiated controls (Fig. 1C). This increase was observed for both general MDA- and specific DHP-epitopes displaying diffuse cytoplasmic staining. As a positive control, formation of MDA- and DHP-epitopes was also confirmed in response to exposure to free MDA generated by acid hydrolysis of 1,1,3,3-tetraethoxypropane (Fig. 1D), employed at concentrations (10 μ M, 24 h) that did not impair proliferative capacity of HaCaT keratinocytes (data not shown). Qualitative assessment of cellular staining patterns was confirmed by digital image quantification (Fig. 1C–D). Sham-irradiated control samples exhibited no quantifiable signal, while samples exposed to either SSL or free MDA exhibited a statistically significant increase in the levels of all investigated epitopes. Interestingly, an increase in staining intensity was apparent one hour post-irradiation and increased further at four hours post-irradiation, an observation consistent with the prolonged propagation of cellular peroxidation reactions after initiation by photooxidative stress [41].

3.2. Acute SSL exposure induces increased levels of free MDA and drives the formation of both MDA- and DHP-protein epitopes in human skin

Next, we examined if the results obtained in human keratinocytes would translate to intact human skin. First, formation of free MDA was examined in human skin after acute SSL exposure *ex vivo* (120 mJ/cm²) where MDA levels increased more than three-fold in exposed versus sham-irradiated skin (Fig. 2A). At higher doses (240 mJ/cm²) no significant further increase in MDA formation was detected regardless of whether normalization was performed relative to cellular DNA or protein content. This observation may be attributed to the potential sequestration of this reactive intermediate by tissue proteins resulting in the accumulation of MDA-derived epitopes.

The possible introduction of MDA- and DHP-epitopes in human skin in response to acute SSL exposure was examined by IHC analysis and qualitative and quantitative assessment of tissue staining patterns by digital image analysis (Fig. 2B). Indeed, a pronounced dose-dependent increase in staining intensity for both general MDA- and specific DHP-epitopes was observed as compared to sham-irradiated controls, with an almost twenty-fold elevation in epitope content in human skin exposed to the highest SSL dose. Immunoreactivity for SSL-induced MDA-derived epitopes was detected throughout epidermal and dermal layers displaying intra- and extracellular localization. Interestingly, for general MDA-epitopes, the penetration depth of dermal staining increased as a function of SSL dose. In contrast, immunostaining for DHP-epitopes was confined to the epidermis with only minor staining detectable throughout the dermis. Moreover, at every SSL dose examined staining intensity of general MDA-epitopes surpassed that of specific DHP-epitopes.

As biological photo-oxidative effects have been attributed to the UVA spectral region of solar light, the relative contribution of UVA to the observed MDA-related effects of acute SSL exposure on skin cells and tissue was explored. When comparing solar simulated UVA versus SSL delivered at doses normalized for UVA (spectral irradiance as a function of photon wavelength depicted in Fig. 3A), we observed that formation of free MDA by UVA exposure occurred with approximately 50% attenuated efficiency, a result obtained in both HaCaT keratinocytes (Fig. 3B) and human skin *ex vivo* (Fig. 3C). We also determined the UVA contribution to the SSL-induced formation of acute MDA- and DHP-epitopes in human skin by IHC analysis (Fig. 3D). Strikingly, even though UVA was sufficiently active

causing the formation of free MDA in HaCaT cells and skin (Fig. 3B–C), introduction of MDA-derived epitopes by UVA was strongly attenuated as compared to the effects of dose-matched SSL (Fig. 2B versus Fig. 3D). These data demonstrate that dose-matched solar UVA is less efficient than SSL in generating both free MDA and MDA-derived protein epitopes in human skin cells and tissue following acute exposure.

3.3. Tissue microarray analysis reveals the abundance of MDA- and DHP-epitopes in nonmelanoma skin cancer

After demonstrating that acute exposure to SSL is sufficient to induce the formation of protein bound MDA- and DHP-epitopes in cultured human epidermal keratinocytes and healthy human skin, we employed TMA-based IHC analysis to examine the occurrence of these epitopes in NMSC, a group of malignancies with an established solar UVR-associated etiology.

Using a commercial NMSC tissue microarray that includes adjacent normal specimens, paired arrays were analyzed by immunostaining for general MDA-epitopes (Fig. 4A) and specific DHP-lysine epitopes (Fig. 4B). For general MDA-epitopes, across all examined microarray specimens (NMSC and adjacent normal), discernable immunoreactivity was observed. Adjacent normal tissue exhibited irregular staining patterns that varied widely in intensity. While the areas of highest intensity were primarily confined to the epidermal compartment, positive staining was also observed in the stratum corneum and to a lesser extent in the dermal layer, with increased intensity near the dermal-epidermal junction. Pooled image quantification for all samples in the adjacent normal subgroup yielded an average positive area score of 25.13 ± 12.63 , $n=9$ (Fig. 4A, inserts 1–3).

Basal cell carcinoma (BCC) tissue exhibited an irregular staining pattern, and intralesional samples displayed higher staining intensity than adjacent normal samples. Pooled image quantification for all samples in the BCC subgroup yielded an average positive area score of 40.88 ± 14.76 , $n=5$ (Fig. 4A; inserts 4–6). Comparative analysis between adjacent normal and BCC subgroups revealed that the differences in average positive area score, indicative of the total concentration of MDA-epitopes present, approached but did not reach the level of statistical significance ($p=0.0829$).

Strikingly, squamous cell carcinoma (SCC) tissue presented intense and consistent staining throughout lesional sections. Pooled image quantification for all samples in the SCC subgroup yielded an average positive area score of 77.81 ± 11.93 , $n=29$ (Fig. 4A; inserts 7–9). Comparative analysis between the SCC subgroup and both the adjacent normal and BCC subgroups indicated that MDA-epitopes were significantly elevated ($p=0.0011$ against BCC; $p<0.0001$ against normal adjacent).

For specific DHP-lysine epitopes, across all specimens the observed immunoreactivity was consistently less than that observed for general MDA-epitopes. However, staining intensity, while diminished, followed an identical pattern when compared across tissue subtypes. Adjacent normal tissue exhibited faint staining that varied between samples. As with *ex vivo* skin (Fig. 2B), areas of highest intensity were confined to the epidermal compartment, with little staining detectable in dermal layers. Pooled results of DHP-epitope image quantification for all samples in the adjacent normal subgroup yielded an average positive area score of 4.61 ± 4.16 , $n=9$ (Fig. 4B, inserts 1–3). Again, BCC tissue exhibited an irregular staining pattern with intra-lesional samples displaying higher staining intensities. In the BCC subgroup, pooled results of DHP-epitope image quantification for all samples yielded an average positive area score of 8.85 ± 5.55 , $n=5$ (Fig. 4B; inserts 4–6). Comparative analysis between adjacent normal and BCC subgroups revealed that the

difference in average positive area score, indicative of the total concentration of DHP-lysine epitopes present, did not reach the level of statistical significance ($p=0.1119$).

As observed for general MDA-epitopes, SCC tissue presented consistent DHP-lysine staining throughout lesional sections, and pooled image quantification for all samples in the SCC subgroup yielded an average positive area score of 22.03 ± 3.07 , $n=30$ (Fig. 4B; inserts 7–9). Comparative analysis between the SCC subgroup and both the adjacent normal and BCC subgroups indicated that DHP-epitopes were significantly elevated ($p=0.0004$ against BCC; $p<0.0001$ against normal adjacent).

4. Discussion

Protein damage is an important molecular consequence of skin exposure to solar UVR [14, 42, 43]. Even though posttranslational protein modifications represent a molecular hallmark of pathologies associated with redox dysregulation such as neurodegeneration, atherosclerosis, and diabetes, only limited research has examined the occurrence of protein epitopes derived from chemical adduction by RCS in human skin in response to solar UVR [29, 44, 45]. Remarkably, formation of MDA-derived protein adducts in skin exposed to solar UVR has not been investigated before, even though these epitopes are known to accumulate in tissue under conditions of pathologic oxidative stress [46–48]. In the current study, we demonstrate for the first time that acute exposure to SSL is sufficient to induce the formation of protein-bound MDA-derived epitopes in cultured human epidermal keratinocytes and healthy human skin. Moreover, we present TMA-based evidence indicating that these epitopes occur abundantly in NMSC.

First, the ability of solar UVR to generate reactive free MDA was confirmed in both cultured human keratinocytes and normal human skin, a finding consistent with prior publications that reported MDA-generation in the context of photooxidative lipid damage [24, 49]. In cultured human skin cells, SSL exposure caused a dose-dependent increase in intracellular levels of free MDA that was observable at sublethal doses (60 mJ/cm^2 UVB; Fig. 1A & B). Next, we demonstrated that this dose of solar UVR is sufficient to induce the formation of MDA-derived protein epitopes (both general MDA- and specific DHP-epitopes; Fig. 1C). Importantly, solar UVR-driven formation of free and protein-bound MDA (both general MDA- and specific DHP-epitopes) was also observed in normal human skin irradiated *ex vivo*. Consistent with DHP-epitopes representing only a fraction of total MDA-derived protein-adducts, staining intensity as displayed by a monoclonal antibody (1F83) directed specifically against DHP-lysine epitopes was consistently weaker than that achieved by a polyclonal antibody that recognizes general MDA-epitopes [35]. Using TMA-based analysis we demonstrate that both general MDA- and DHP-epitopes are abundant in NMSC (Fig. 4), a finding consistent with earlier research suggesting the occurrence of MDA-derived epitopes of unknown structure in cancerous human skin [34].

In the context of RCS formation and subsequent protein adduction in human skin exposed to solar UVR, it should be mentioned that accumulation of the glyoxal-derived epitope N^ε-(carboxymethyl)lysine (CML) has been demonstrated in actinic elastosis characteristic of photoaged human skin [50]. Interestingly, immunochemical analysis demonstrated that CML levels of sun-exposed human skin were significantly higher than those of sun-unexposed control areas originating from the same donor [51]. Moreover, solar UV-driven formation of CML-epitopes has been demonstrated in skin cells and human skin *ex vivo*, findings consistent with our observation of UVR-induced MDA-epitopes [52–54].

Our data that demonstrate the occurrence of MDA-derived epitopes in both solar UVR-exposed healthy human skin and NMSC TMA tissue suggest the potential utility of MDA-

derived protein epitopes as biomarkers of tissue damage, resulting either from acute solar insult or chronic photocarcinogenic exposure. However, a larger and more detailed follow up study employing tissue samples with defined solar exposure history and donor-matched controls will be needed to stringently validate a biomarker function for these skin epitopes. Of note, detection of 8-isoprostane has recently emerged as a potential biomarker of acute solar exposure in human skin [19], and the MDA-derived DNA adduct M1dG has been proposed as a biomarker of oxidative stress [55]. However, both of these markers are relatively short lived, and there are no markers currently available that would integrate the cumulative risk of chronic insult from repetitive UVR exposure, a function potentially served by MDA-epitopes that may accumulate in human skin.

The solar etiology of NMSC initiation and progression is firmly established, and MDA has been recognized as a chemical carcinogen [56, 57]. Therefore, based on our finding that acute UV exposure is sufficient to generate free MDA as well as MDA- and DHP-epitopes in healthy skin, it is tempting to speculate that MDA-derived epitopes may play a causative role in skin photocarcinogenesis, a hypothesis to be explored by future experimentation. Indeed, autoantibodies against MDA-modified Lys residues have been detected under conditions of inflammation, and photooxidation products of endogenous skin lipids have been shown to mediate the SSL-induced inflammatory response in human keratinocytes [58, 59]. Additionally, the activity of DHP-epitopes as potent photosensitizers and potential enhancers of skin photooxidative stress has recently been substantiated [33]. Further evidence in support of a functional role of lipid peroxidation- and glycation-derived protein adducts in skin carcinogenesis originates from the fact that these epitopes have recently been shown to act as potent RAGE (receptor for advanced glycation endproducts) agonists causing inflammatory dysregulation involved in both tumor invasion and metastasis [60–64]. It remains to be seen if UVR-driven formation and occurrence of MDA-derived epitopes observed in both healthy human skin and NMSC play a mechanistic role in skin photocarcinogenesis.

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Abbreviations

UVR	ultraviolet radiation
SSL	solar simulated UV-light
UVB	ultraviolet B (280–315 nm)
UVA	ultraviolet A (315–400 nm)
MDA	malondialdehyde
DHP	dihydropyridine-lysine ((S)-2-amino-6-(3,5-diformyl-4-methyl-4H-pyridin-1-yl)-hexanoic acid)
NMSC	nonmelanoma skin cancer
ROS	reactive oxygen species
RCS	reactive carbonyl species

PFPH	pentafluorophenylhydrazine
BHT	butylated hydroxytoluene
NaOH	sodium hydroxide
KOH	potassium hydroxide
HCl	hydrochloric acid
HClO₄	perchloric acid
H₂SO₄	sulfuric acid
HaCaT	immortalized human epidermal keratinocytes
DMEM	Dulbecco's Modified Eagle Medium
FBS	fetal bovine serum
PBS	phosphate buffered saline
ICC	immunocytochemistry
IHC	immunohistochemistry
TMA	tissue microarray
Me-MDA	2-methyl-propanedial
GC-MS	gas chromatography and mass spectrometry
PI	propidium iodide
ANOVA	one-way analysis of variance
BCC	basal cell carcinoma
SCC	squamous cell carcinoma
CML	N ^ε -(carboxymethyl)lysine
RAGE	receptor for advanced glycation endproducts

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- Exposure to acute solar simulated UV drives formation of free MDA in human skin
- UV exposure generates cutaneous MDA-derived protein epitopes including DHP-lysine
- MDA- and DHP-epitopes are prevalent in nonmelanoma skin cancer tissue microarrays

Figure 1.

Exposure to sub-apoptogenic levels of SSL results in a dose-dependent increases in measured levels of free MDA and drive the formation of MDA- and DHP-protein epitopes in human skin cells. (A) Intracellular concentrations of free MDA quantified from human (HaCaT) keratinocytes 30 min post irradiation with SSL as quantified by GC-MS. Average of 3 independent experiments; n=9 (mean ±SD). (B) Viability was determined 24 h after exposure to increasing doses of SSL using flow cytometric analysis of annexin V (AV-FITC)-propidium iodide (PI) stained cells (numbers indicate % viable of total gated cells located in lower left quadrant; n=3, (mean ±SD)). (C) Representative images of MDA- (1–3) and DHP- (4–6) epitopes in HaCaT cells visualized by ICC. Sham-irradiated control (1, 4)

and SSL-irradiated cells (60 mJ/cm² UVB) harvested at indicated time post treatment (2, 3, 5, 6), and the corresponding image quantification, representing an average of 3 independent samples per group (mean $\pm$ SD). (D) Representative images of MDA- (1, 2) and DHP- (3, 4) epitopes in HaCaT cells visualized by ICC. Untreated control (1, 3) and cells harvested 24 hours after exposure to 10 μ M MDA (2, 4), and the corresponding image quantification, representing average of 3 independent samples per group (mean $\pm$ SD). With (n.d.) indicating no staining detected and bars labelled with different letters differing significantly, $p < 0.05$.

Figure 2.

Exposure to physiologically relevant levels of SSL results in a dose-dependent increase in detectable levels of free MDA and both MDA- and DHP-protein adducts in human skin. (A) Concentrations of free MDA quantified from healthy human skin approximately 30 min post irradiation with SSL (120 or 240 mJ/cm² UVB). Average of 3 samples from an individual donor (mean $\pm$ SD). (B) Representative images of MDA- and DHP-adducts visualized by IHC in human skin after indicated dose of SSL exposure, and the corresponding image quantification, representing average of 3 independent samples per group (mean $\pm$ SD). Bars labelled with different letters differ significantly, $p < 0.05$.

Figure 3.

Levels of free MDA are lower in both human skin cells and in human skin when exposed to SSL-matched doses of UVA alone. Exposure to UVA alone is less efficient than SSL at generating MDA- and DHP-protein adducts in human skin. (A) Spectral power distribution (irradiance) of SSL (UVB + UVA, solid line) and UVA (dashed line) provided by the solar simulator equipped with appropriate cut-off filters. (B) Free MDA in cultured human skin cells after exposure to SSL or SSL-matched doses of UVA alone. (C) Free MDA in human skin after exposure to SSL or SSL-matched doses of UVA alone. (D) Representative images of MDA- and DHP-adducts visualized by IHC in human skin after exposure to indicated dose of UVA alone, and corresponding image quantification, representing average of 3 independent samples per group (mean ±SD). Bars labelled with different letters differ significantly, p<0.05.

Figure 4.

A commercially available human NMSC tissue microarray was interrogated to determine the presence of MDA- and DHP-adducts in SCC, BCC, or adjacent normal skin. (A) Representative images of MDA-adducts visualized by IHC separated by diagnosis subgroup (adjacent normal 1–3, BCC 4–6, SCC 7–9). Results of image quantification for diagnosis subgroups (adjacent normal $n=9$, BCC $n=5$, SCC $n=29$), (mean $\pm$ SD). (B) Representative images of DHP-adducts visualized by IHC separated by diagnosis subgroup (adjacent normal 1–3, BCC 4–6, SCC 7–9). Results of image quantification for diagnosis subgroups (adjacent normal $n=9$, BCC $n=5$, SCC $n=30$), (mean $\pm$ SD).