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Selective proteolysis by matrix metalloproteinases of photo-oxidised dermal extracellular matrix proteins

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ABSTRACT

Photodamage in chronically sun-exposed skin manifests clinically as deep wrinkles and histologically as extensive remodelling of the dermal extracellular matrix (ECM) and in particular, the elastic fibre system. We have shown previously that loss of fibrillin microfibrils, a key elastic fibre component, is a hallmark of early photodamage and that these ECM assemblies are susceptible *in vitro* to physiologically attainable doses of ultraviolet radiation (UVR). Here, we test the hypotheses that UVR-mediated photo-oxidation is the primary driver of fibrillin microfibril and fibronectin degradation and that prior UVR exposure will enhance the subsequent proteolytic activity of UVR-upregulated matrix metalloproteinases (MMPs).

We confirmed that UVB (280-315 nm) irradiation *in vitro* induced structural changes to both fibrillin microfibrils and fibronectin and these changes were largely reactive oxygen species (ROS)-driven, with increased ROS lifetime (D_2O) enhancing protein damage and depleted O_2 conditions abrogating it. Furthermore, we show that although exposure to UVR alone increased microfibril structural heterogeneity, exposure to purified MMPs (1, −3, −7 and − 9) alone had minimal effect on microfibril bead-to-bead periodicity; however, microfibril suspensions exposed to UVR and then MMPs were more structurally homogenous. In contrast, the susceptibly of fibronectin to proteases was unaffected by prior UVR exposure. These observations suggest that both direct photon absorption and indirect production of ROS are important mediators of ECM remodelling in photodamage. We also show that fibrillin microfibrils are relatively resistant to proteolysis by MMPs −1, −3, −7 and − 9 but that these MMPs may selectively remove damaged microfibril assemblies. These latter observations have implications for predicting the mechanisms of tissue remodelling and targeted repair.

1. Introduction

Chronic cutaneous exposure to solar radiation manifests clinically with the formation of wrinkles and histologically with extensive remodelling of the dermal extracellular matrix (ECM), in particular the microfibrillar components of the elastic fibre network, fibrillin-1 and fibulin-5 [1,2]. Fibrillin-rich microfibrils play important mechanical roles and contribute to tissue homeostasis *via* the regulation of cytokines [3–6]. To date, attention has focused on ultraviolet radiation (UVR)-mediated upregulation of ECM proteases as the main mediators of dermal remodelling in photodamaged skin [7]. In the direct hit model of photodamage, cellular photosensitisers absorb UVR, activating signalling pathways which promote the expression of ECM proteases [8–11]. The alternative bystander model proposes that UVR absorption by ECM-associated photosensitisers, located on post-translationally modified collagen or elastin, liberates extracellular ROS which in turn leads to ECM degradation *via* oxidation or protease induction [12,13]. As UVR up-regulates the expression of multiple ECM proteases (in particular matrix metalloproteinases [MMPs] -1, −3, −7, −8, −9 and − 12 [8,14–17]) and collectively these proteases can degrade most dermal ECM components, it is unclear how enzyme-mediated degradation alone accounts for the early and specific loss of fibrillin microfibrils in photodamaged human skin [1,18–20].

Proteins exposed to UVR are likely to be damaged *via* direct photon absorption resulting in an excited singlet state of their constituent UVR chromophores and/or photodynamic production of ROS (*e.g.* singlet

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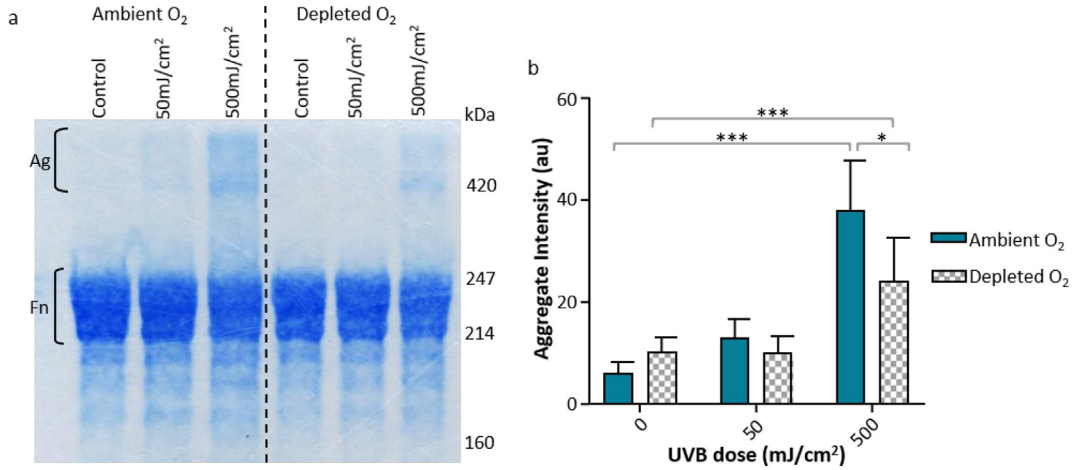

Fig. 1. Broadband UVB irradiation causes aggregation of fibronectin which is significantly abrogated in depleted O_2 conditions. Fibronectin (Fn) ran as an intense band with a molecular mass of 214–247 kDa and significantly aggregates (Ag) forming a band > 420 kDa following exposure to UVR ($P = .0005$; a&b). The mean pixel intensity of the Ag induced by 500 mJ/cm^2 of broadband UVB in ambient O_2 was significantly reduced at the 500 mJ/cm^2 dose in depleted O_2 conditions ($P = .0128$, $n = 3$).

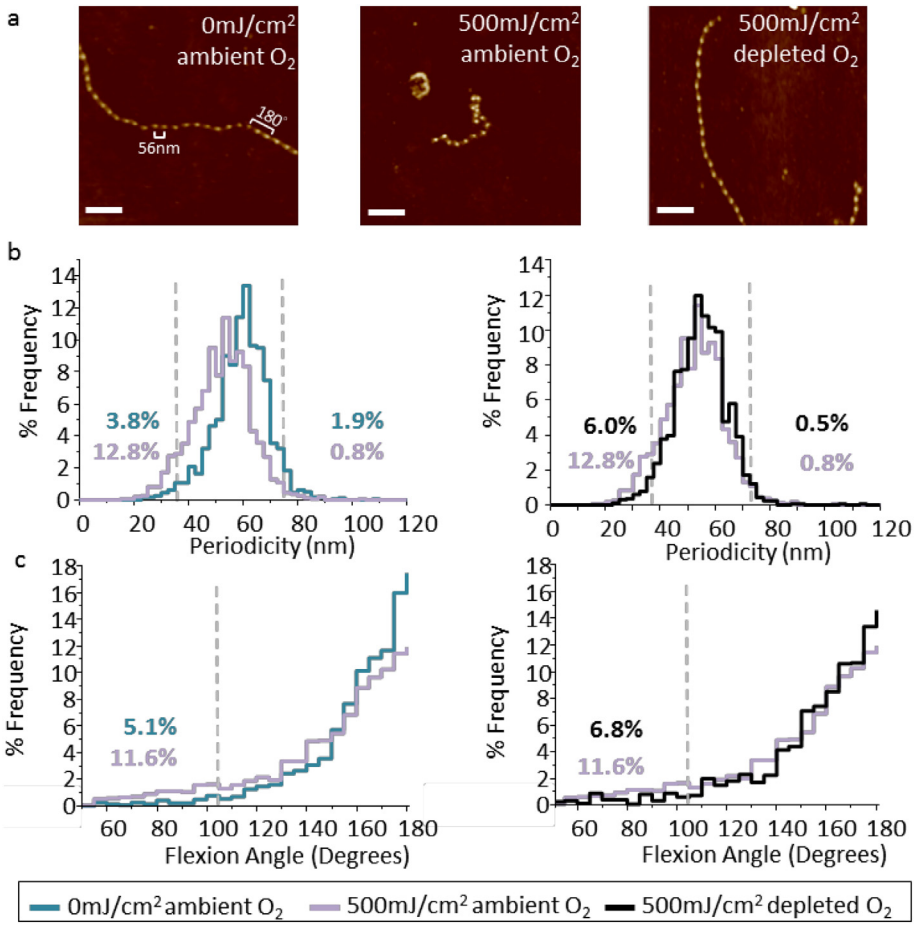

Fig. 2. Broadband UVB also causes structural alterations fibrillin microfibrils which are significantly abrogated in depleted O_2 conditions. Representative atomic force height images (scale = 200 nm) of unirradiated and 500 mJ/cm^2 ambient and depleted O_2 UVB-exposed microfibrils (a). Broadband UVB exposure significantly altered microfibril structure resulting in an increase in the outlier periodicity (Mean ± 2× STDEV [b]) and flexion angle measures (Mean – 2× STDEV [c]), these effects were significantly abrogated ($p < .0001$) in depleted O_2 conditions ($n = 3$ individuals and $n = 1500$ measures).

oxygen [$^1O_2$], superoxide and hydrogen peroxide [H_2O_2]). This latter pathway involves intersystem crossing of the excited singlet state to the longer-lived triplet state resulting in type I (electron transfer) or type II (energy transfer) reactions [21–23] with triplet state molecular oxygen (O_2). There is evidence from *in vivo* studies of UVR-induced ROS production [24] that this second pathway operates in human skin where there is a localised age-related accumulation of protein carbonyls (oxidation products) in the fibrillin microfibril-rich papillary dermis [25,26]. We have previously shown that physiologically relevant wavelengths and doses of UVR can selectively degrade ECM proteins which are rich in amino acid UVR-chromophores (Trp, Tyr, Cys and Cys = Cys) [27,28]. Crucially, these amino acid residues are also susceptible to oxidation [23]. Other groups have shown that exposure to relatively high doses of UVA radiation can increase the susceptibility of fibrillar collagen to proteases [29].

As ECM proteins in photoexposed skin may be subjected to degradation by multiple mechanisms (proteases, direct UVR and photodynamically-induced ROS) we have used biochemical and

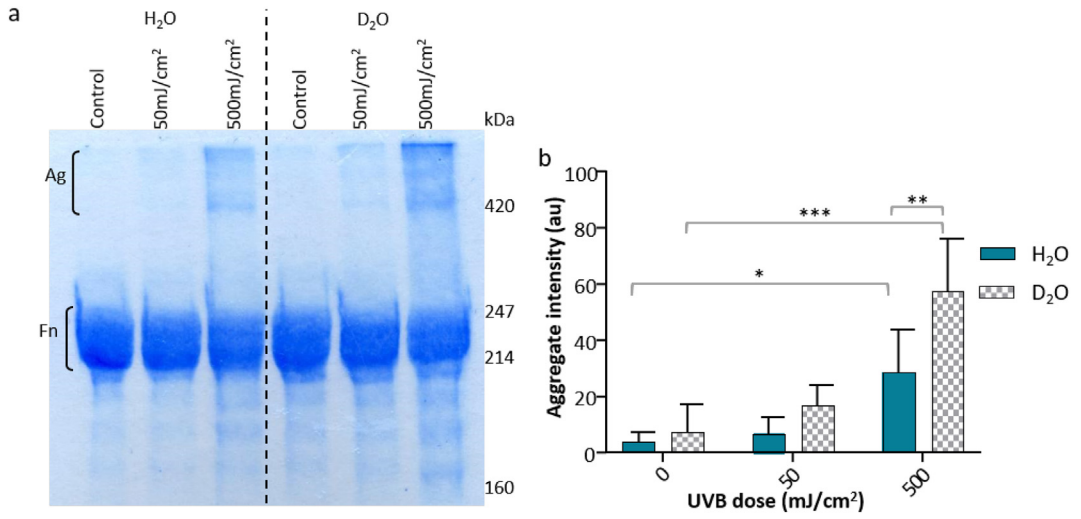

Fig. 3. UV irradiation in a D_2O environment significantly increased fibronectin aggregation. Fibronectin was reconstituted in PBS with either H_2O or D_2O. Unirradiated and irradiated fibronectin suspensions (at broadband UVB doses of 50 or 500 mJ/cm^2) were characterised by reducing SDS-PAGE (a). UVB exposure in an H_2O environment induces significant aggregation (Ag) of fibronectin (Fn) at 500 mJ/cm^2 (P = .0129). This UV-induced aggregation was enhanced in the D_2O PBS, shown by a significant increase of the Ag (P = .0074, n = 3) compare with irradiation in H_2O environments (b).

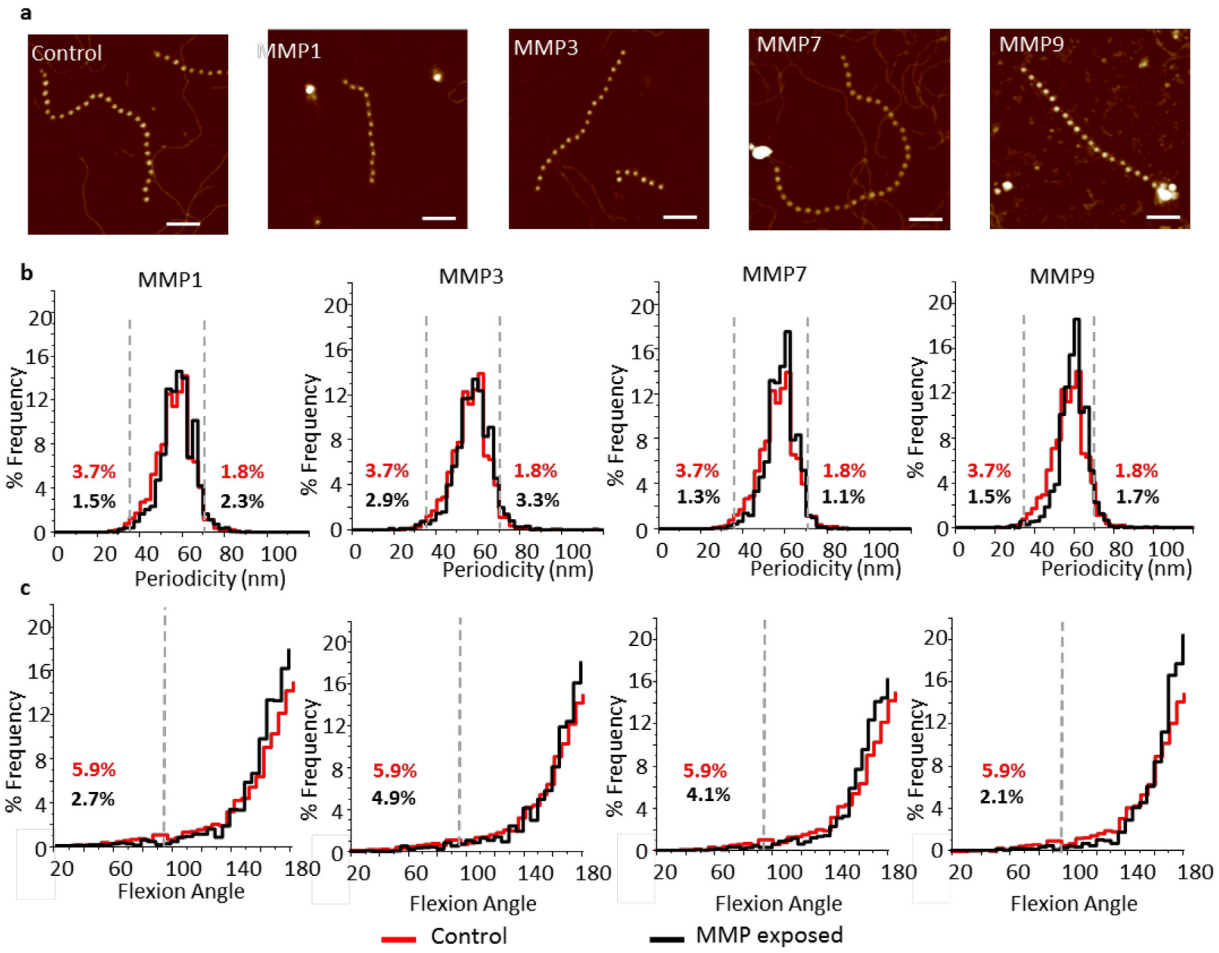

Fig. 4. Fibrillin microfibrils are relatively resistant to proteolysis by UVR-upregulated MMPs. Representative atomic force height images (scale = 200 nm) of control and MMP exposed microfibrils (a). When microfibrils are exposed to MMPs alone the population homogeneity become more tight, removing both periodicity and flexion angle measures that are more than 2SDs away from the mean of the control values (b&c), data from n = 3 individuals and n = 1500 measures.

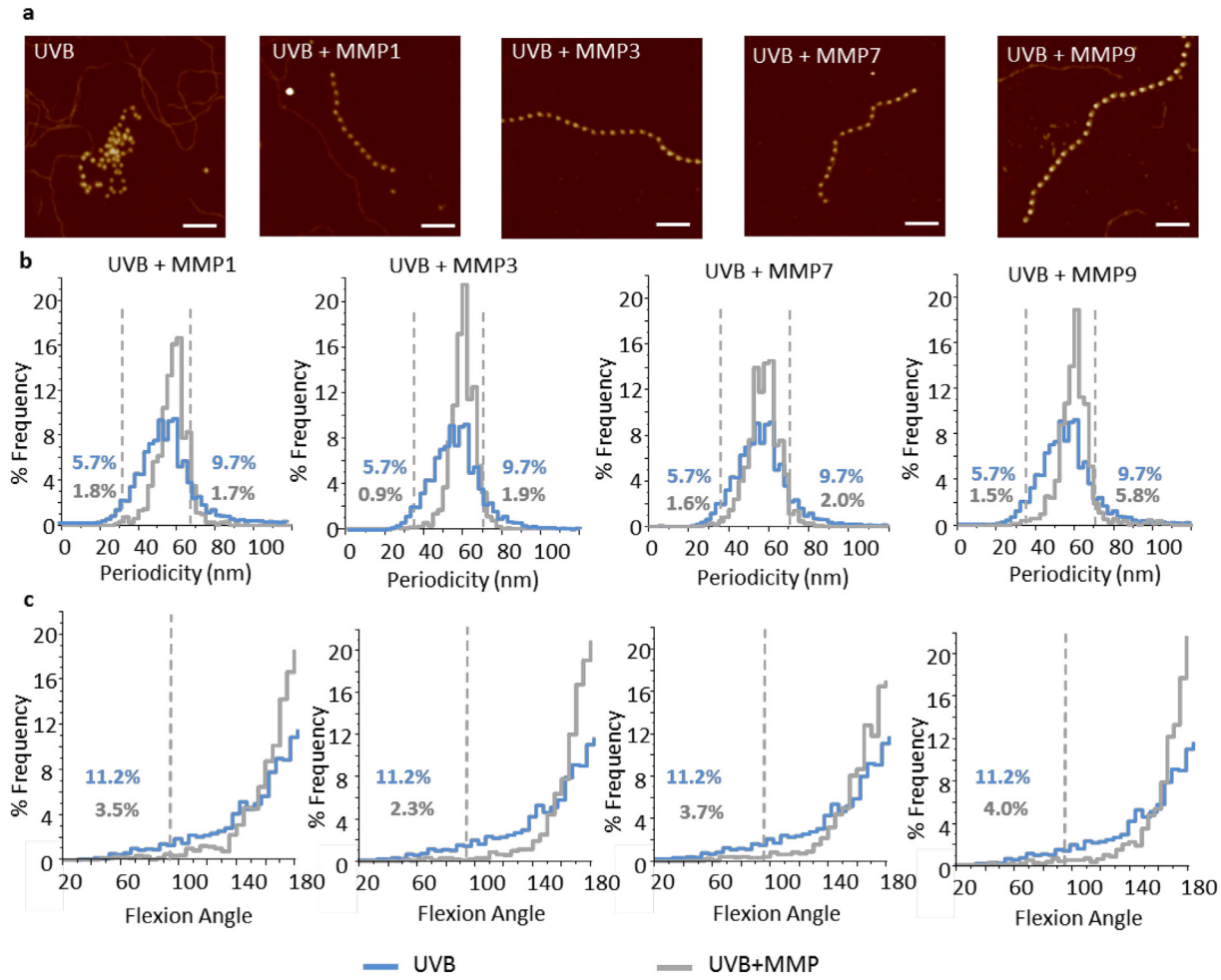

Fig. 5. MMPs appear to selectively degrade fibrillin microfibrils with abnormal periodicities and flexion angles. Representative atomic force height images (scale = 200 nm) of UVB irradiation and UVB + MMP exposed fibrillin microfibrils (a). As previously shown, UVB-irradiation caused significant changes to microfibril periodicity ($P < .0001$) as indicated by a 2.8 times increase in the outlier periodicities following irradiation (Control = 5.5%; UVB = 15.4% total). When this UVR-damaged population is then exposed to MMPs a shift in the population is seen, leaving only the intact population in place b). UVB induced microfibrils to become increasingly flexible, (Control = 5.9%; UVB = 11.2% outliers, $P < .0001$) and MMPs remove the microfibrils with increased flexibility following UV-damage (c) data from $n = 3$ individuals and $n = 1500$ measures.

ultrastructural imaging approaches to test the hypothesis that photo-oxidation is the primary driver of UVR-mediated degradation of fibrillin microfibril and fibronectin and that prior UVR exposure will enhance the subsequent proteolytic activity of UVR-upregulated MMPs. By testing these hypotheses we aimed to characterise the relative importance of different pathways in ECM degradation.

2. Materials and methods

All chemicals were of analytical grade and, unless otherwise stated, were purchased from Sigma Aldrich (Poole, UK). The ECM protein fibronectin (from bovine plasma) was also purchased Sigma, but fibrillin microfibrils were extracted from post-confluent human dermal fibroblasts.

2.1. Cell culture and microfibril isolation

Human dermal fibroblasts (HDFs; a gift from Dr. Stuart Cain, University of Manchester, originally from Cascade Biologics, Invitrogen; Paisley, UK) were maintained in 75cm^2 culture flasks (BD Falcon; Oxford, UK) with Dulbecco's Modified Eagle Medium + Glutamax (Invitrogen; Paisley, UK) supplemented with 10% fetal calf serum and 50 μg/mL streptomycin/penicillin, in a humid atmosphere at 37 °C and 5% CO_2. HDFs were cultured to six weeks post-confluency prior to isolation of fibrillin and collagen VI microfibrils by collagenase Type-IA digestion and size exclusion chromatography as previously described [30,31]. Briefly, cells were agitated at room temperature for 2 h with 0.5 mg/mL bacterial collagenase type IA in column buffer (0.4 M NaCl, 0.05 M Tris) supplemented with 10 mM $CaCl_2$ and protease inhibitors 2 mM phenylmethylsulfonyl fluride and 5 mM N-ethylmaleimide. The cell digest was centrifuged for 5 mins at 5000*g* and the supernatant chromatographed in column buffer through Sepharose CL-2B column on an AKTA prime plus (GE Healthcare; Little Chalfont, UK). All subsequent fibrillin microfibril experiments were performed using two pooled 1 mL fractions from the centre of the V_0 peak.

2.2. UVR irradiation

Protein suspensions were exposed to broadband UVB (280-315 nm) of 50, 100 or 500 mJ/cm^2. For this source (Philips TL-12: Philips, Eindhoven, The Netherlands) which emits UVC 0.4%, UVB 55.3% and UVA 44.3%, a dose of 50 mJ/cm^2 is equivalent to approximately one minimal erythemal dose (MED), the dose required to cause perceptible skin reddening, for lightly pigmented skin Fitzpatrick phototype I-II [25]. All irradiations were carried out in inert polyethylene lids (10 mm in diameter and 2 mm high) and exposure times were calculated from

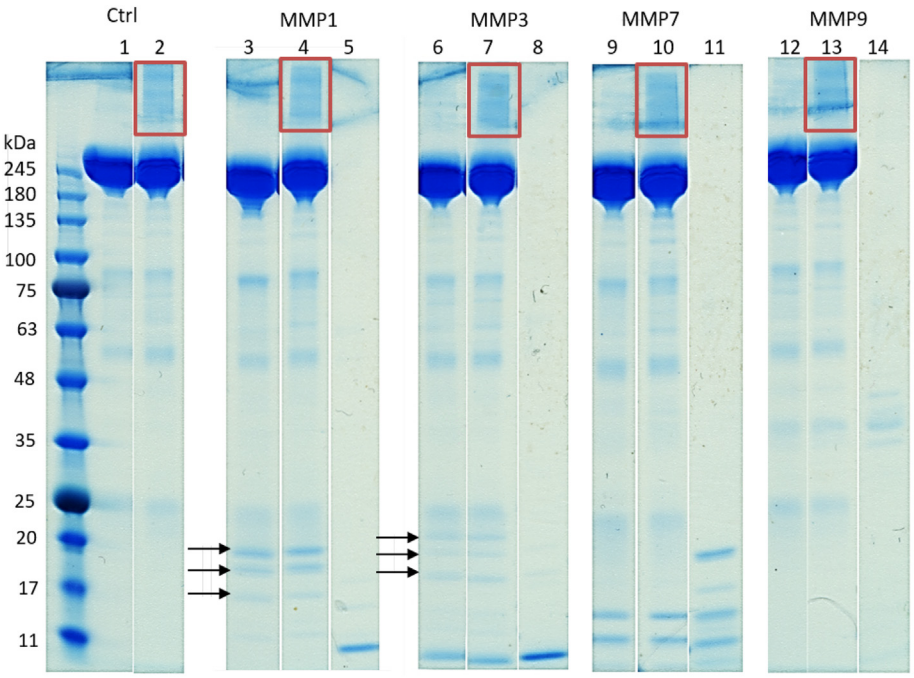

Fig. 6. MMPs to have little effect on the electrophoretic mobility of both non-UVB exposed and UVB exposed fibronectin. As previously shown, exposure to 100 mJ/cm^2 broadband UVB irradiation induced aggregation of fibronectin (lanes 1: Fn and 2: Fn + UVB). Exposure to MMPs −1 (lane 3) and − 3 (lane 6) produced three new low molecular weight bands (black arrows, 17–20 kDa), but MMPs-7 (lane 9) and − 9 (lane 12) had no apparent effect on the electrophoretic profile. Prior exposure to UVB did not appear to affect the protease susceptibility of fibronectin (lanes 4, 7, 10 and 13). The MMPs alone were also included so the banding produced by their addition could be clearly identified (lanes 5, 8, 11 and 14).

irradiances measured using a UVX radiometer with a UVX-31 detector (UVR products; Upland, CA, USA) as previously described [27].

2.3. Effects of dissolved [O_2] on the UVR-susceptibility of ECM glycoproteins

Dissolved O_2 was depleted by purging buffers for 10 min with O_2-free N_2 and the final [O_2] was determined by titration using the Winkler method [32,33]. Previously N_2 purging has been applied to H_2O, rather than the buffers used in these experiments. Given the difficulty of assessing the [O_2] in < 1 mL volumes by the Winkler method, we assayed the effects of 10 min N_2 purging on final [O_2] concentrations of 10 mL to 1 L volumes of column buffer and PBS. These data were then extrapolated to estimate the effects of N_2 purging on [O_2] in 600 μL microfibril suspensions. Subsequently, the [O_2] of microfibril-rich suspensions in high-salt column buffer, were depleted by direct N_2 purging for 10 mins directly prior to UVR irradiation.

Purging small volumes of suspended fibronectin induced substantial loss of protein by aggregation. Therefore, the [O_2] in fibronectin suspensions was depleted by dialysis against O_2-depleted PBS. Fibronectin suspensions reconstituted at 1 mg/mL, were diluted in PBS to a concentration of 250 μg/mL prior to dialysis for 8 h at 4 °C (dialysis membrane size 1; Medicell International Ltd.; London, UK) in a sealed 1 L bottle against 1 L of O_2-depleted PBS (purged with N_2 for 1 h and void between meniscus and lid filled with N_2). This dialysis step was repeated for a further 14 h against a fresh 1 L volume of N_2-purged PBS. Following [O_2] depletion, fibrillin microfibril and fibronectin suspensions were exposed to broadband UVB doses of 50 or 500 mJ/cm^2. UVR-induced changes in protein structure were assessed by AFM (fibrillin microfibrils) and fibronectin (SDS-PAGE) as described below and previously [27,34].

2.4. Modulating in the lifetime of UVR-induced ROS with D_2O

Fibronectin (250 μg/mL) suspended in PBS (reconstituted in either H_2O or D_2O) was exposed to doses of either 50 or 500 mJ/cm^2 broadband UVB radiation as described below. The effects of the solute on UVR-mediate damage were assessed by SDS-PAGE.

2.4.1. MMP exposure

MMPs −1, −3 -7 and − 9 (10 μg stocks; Enzo Life Sciences; Exeter, UK) were used to examine the potential cleavage of fibrillin microfibril and fibronectin, pre and post UV-irradiation. Fibrillin microfibrils were extracted from bisected 6 mm diameter punch biopsies excised from the photoprotected skin (upper inner arm) of three human volunteers (2 M, 1F; 30-50 yrs) in the same manner as described for the cell extracted protein. This study was approved by the North West Research Ethics Committee (Ref. 09/H1006/23) and adhered to Declaration of Helsinki guidelines for clinical research with volunteers giving written consent. The recombinant MMPs −1, −3, −7 and − 9 were added at a 10 μg/mL concentration to both proteins either alone, or post exposure to 50 (fibrillin microfibrils) or 100 (fibronectin) mJ/cm^2 UVB and incubated for 18 h at 37 °C. MMP activity was assessed using DQ™ gelatin zymography (ThermoFisher Scientific, UK). Briefly, 0.1 μg of each MMP was set in gelatin onto a glass slide with DQ gelatin and left to incubate at room temperature overnight. Each MMP showed positive fluorescence compared with a buffer control, confirming activity for our experiments (data not shown).

2.5. Gel electrophoresis

Molecular changes to the fibronectin were assessed using SDS-PAGE. Gels were run using 20 * 20 cm glass plates with 6% running gel, 4% stacking gel or Novex™ 4–20% Tris-Glycine Mini Gels (ThermoFisher Scientific, UK). Samples were run under reducing conditions (1.43 M 2-mecaptoethanol) in Laemmli sample buffer for 5 h at 150 V (20 * 20 cm) or 50 mins at 225 V (Tris-Glycine) and stained with Instant Blue (Expedeon Ltd., Cambridge, UK). Dried gels were scanned using a high-resolution flatbed scanner at 800 dpi (Epson expression 1600). Intensity of the fibronectin aggregate bands were measured using ImageJ software [35], taking a mean intensity of the background which was subtracted from the mean intensity of the total aggregate area to produce the bar charts.

2.6. Atomic force microscopy

Microfibril solutions from each experimental group were directly adsorbed to glass coverslips prior to image capture with the multi-mode AFM (Nanoscope IIIa Multimode AFM; Veeco Instruments Inc., Santa

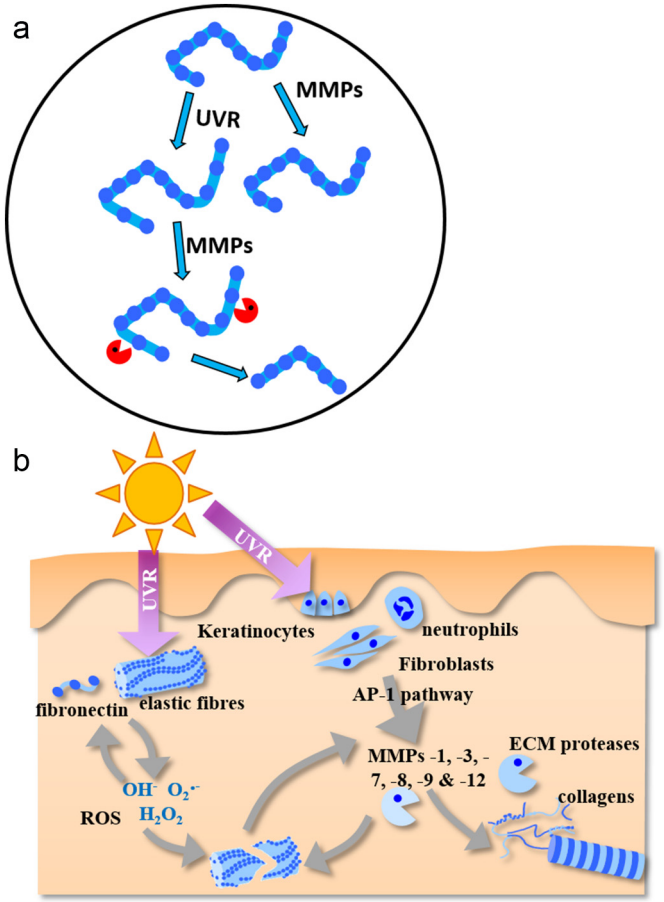

Fig. 7. ***Insights into mechanisms of photoageing.*** *(a) In vitro we hypothesise that MMPs are unable to degrade intact microfibril structure due to the structural integrity of the molecules. Once exposed to UVR we know that this structure is damaged resulting in more extended and flexible microfibrils, this could open up enzymatic cleavage sites within the microfibril, leaving only the intact population following MMP exposure. (b) Existing evidence in the literature shows that UVR appears to upregulate MMPs by inducing cell signalling pathways in keratinocytes, neutrophils and fibroblasts, via the activation of the AP-1 pathway [13]. Active MMPs can then go onto degrade numerous dermal matrix components including the collagens. Previous studies by our group have shown that the chromophore rich dermal matrix is directly susceptible to damage by UVR [27,28] and in this study we show that this is largely ROS mediated. Peptides from damaged microfibrils also upregulate MMP activity [51] which we show here might be part of a self-regulatory mechanism whereby, damaged matrix peptides induce MMP activity but only damaged matrix is susceptible to MMP cleavage, shown here for fibrillin microfibrils.*

Barbara, CA, USA). A series of 2 × 2 μm scans were captured at a scan rate of 1.97 Hz with Veeco OTESPA silicon cantilever with a pixel density of 3.9 nm using Nanoscope software [34]. For each set of experimental variables (all performed in triplicate) 500 bead repeats were measured to assess microfibril periodicity (bead-to-bead distance) and flexion angle (the angle between 3 bead repeats), using software routines written in Microsoft Visual Basic [3,34,36].

2.7. Statistical analysis

Significance was measured for the band intensities on the SDS-PAGE gels using ANOVA with a post-hoc Tukey test ($n = 3$ repeats). Any significant changes to the periodicity of fibrillin microfibrils were tested using Kolmogorov-Smirnov using a Bonferroni correction for multiple comparisons (a total of 1500 measurements (500 per experiment) were assessed).

3. Results

3.1. Photooxidation is the primary mediator of UVR-induced damage of fibrillin microfibrils and fibronectin

Dissolved O_2 is required for the photodynamic generation of ROS [37]. Therefore, to characterise the mechanisms which drive UVR-mediated degradation of ECM proteins we adapted an existing technique where dissolved O_2 is replaced by N_2. This N_2 bubbling technique has previously been described for H_2O [38,39] but our proteins require suspension in salt buffers. Hence, we characterised the effects of removing dissolved O_2 in both physiological (PBS) and high salt (column buffer) solutions. The initial [O_2] was higher in PBS than in column buffer (mean initial [O_2] PBS = 9 mg/L and [O_2] column buffer = 3 mg/L) probably due to the high salt concentration of the column buffer [40]. Although it was not possible to directly measure [O_2] in the small volumes used for irradiation experiments, the logarithmic relationship between [O_2] and buffer volume suggests that following a 10 min purging with N_2, dissolved O_2 will be effectively removed from buffer volumes < 13 mL (Supplementary Fig. 1).

Fibronectin was visualised by SDS-PAGE as an intense band of molecular mass 214–247 kDa in both ambient and depleted O_2 conditions prior to irradiation with broadband UVB (290–315 nm; Fig. 1a). Irradiation of fibronectin in ambient O_2 induced UVB dose-dependent aggregation (~2 and 6 fold increases in optical density following 50 and 500 mJ/cm^2 UVB respectively [Fig. 1b]). However, when fibronectin was irradiated in depleted-O_2 conditions, the aggregation was significantly abrogated ($P = .0128$) at 500 mJ/cm^2 (reduced to 0 and 2 fold increases in optical density compared with the control, following 50 and 500 mJ/cm^2 UVB respectively [Fig. 1b]).

We also show that the bead-to-bead distance (periodicity) of isolated fibrillin microfibrils was significantly altered by exposure to broadband UVB in ambient O_2 conditions, detected as an increase in the outlier periodicities (mean ± 2*STDEV, 0 mJ/cm^2 = 5.7% total; 500 mJ/cm^2 = 13.6% total; $p < .0001$). UVR-induced changes to microfibril periodicity were significantly abrogated when exposed to UVB in O_2-depleted conditions (500 mJ/cm^2 = 6.5% total, 2.1 times less damage compared with the ambient O_2; $P < .0001$) (Fig. 2a&b). Furthermore, the flexibility (angle between three bead repeats) of non-exposed microfibrils (median flexion angle = 161°) was significantly increased, shown by a decrease in flexion angle (156°, $P < .001$), as were the outlier percentages (0 mJ/cm^2 = 5.1%; 500 mJ/cm^2 = 11.6%) after exposure to 500 mJ/cm^2 UVB in ambient-O_2 conditions. However, when microfibrils were exposed in depleted-O_2 conditions, the UVR-mediated increase in flexibility was also significantly abrogated (N_2 500 mJ/cm^2 = 160°; 6.8%; P < .001) (Fig. 2c).

Deuterium oxide (D_2O) increases the lifetime of both singlet oxygen [41] and superoxide [42]. As fibronectin is available commercially in a lyophilised state we reconstituted this glycoprotein in PBS made with either H_2O or D_2O. As in the previous experiment, fibronectin exposed to 500 mJ/cm^2 broadband UVB in H_2O PBS formed aggregates which were detectable by reducing SDS-PAGE (Fig. 3a&b). Compared to H_2O PBS however, exposure of fibronectin to UVR in D_2O PBS induced a greater than two-fold increase in the optical density of the resultant fibronectin aggregate band at both 50 and 500 mJ/cm^2 (Fig. 3a&b).

3.2. Fibrillin microfibrils are relatively resistant to proteolysis by MMPs

Here we show that exposure of fibrillin microfibrils to MMPs −1, −3, −7 or − 9 had little effect on the structurally homogenous populations. To compare any differences between the control; UVR exposed and; MMP exposed populations, we calculated the measures that were greater or < 2 SDs of the control mean (outliers). In the MMP exposed populations we see a small normalising effect of the periodicity (Control = 5.5%; MMP-1 = 3.8%; MMP-3 = 6.2%; MMP-7 = 2.4% &

MMP-9 = 3.2% [Fig. 4a&b]), with all but MMP-3 having a lower total percentage of outlier periodicities. The same pattern is also observed with microfibril flexibility with a small normalising of the outlier flexion angle measures (Control = 5.9%; MMP-1 = 2.7%; MMP-3 = 4.9%; MMP-7 = 4.1%; MMP-9 = 2.1% [Fig. 4c]).

3.3. MMPs appear to preferentially degrade damaged fibrillin microfibrils

Here we confirm that, exposure to broadband UVB significantly alters microfibril structure, as indicated by a higher percentage of outlier periodicities (control = 5.5% total; 50 mJ/cm^2 UVB = 15.4% total; $p < .0001$). Here we have shown that MMPs alone have very little effect on non-UVR exposed microfibril structure however, incubation with each of the four MMPs appears to remove UVB-damaged microfibrils, leaving a population with a periodicity frequency distribution which closely resemble those of the unirradiated population. This is clearly indicated by the significant reduction in outlier periodicities in each of the UVB + MMP samples (UVB = 15.4%; UVB + MMP-1 = 3.5%; UVB + MMP-3 = 2.8%; UVB + MMP-7 = 3.6% and; UVB + MMP-9 = 7.3% [Fig. 5a&b]). We also found this same pattern with the flexibility (flexion angle) of microfibrils, whereby all of the MMPs normalised the UVB irradiated microfibrils measured again by the outliers (control = 5.9%; UVB = 11.2%; UVB + MMP-1 = 3.5%; UVB + MMP-3 = 2.3%; UVB + MMP-7 = 3.7% and UVB + MMP-9 = 4.0% [Fig. 5c]).

3.4. UVR exposure has no discernible effect on the susceptibility of fibronectin to MMPs

As we have previously demonstrated that another key ECM component, fibronectin (Fn), is susceptible to physiological doses of UVR [27,28], we next determined whether broadband UVB exposure could modulate the protease susceptibility of Fn (Fig. 6). Exposure to MMPs −1, −3, −7 and − 9 had minimal effect on the electrophoretic profile of Fn, although MMPs −1 and − 3 did induce three additional low molecular weight bands (17-25 kDa).

4. Conclusions

Current proposed mechanisms of skin photodamage focus on the upregulation of MMPs by UVR and subsequent ECM protein degradation [13, 43, 44]. In previous studies we have shown that ECM molecules are differentially susceptible to UVR-induced damage, with those high in UVR-chromophore content (including fibrillin microfibrils and fibronectin) being more susceptible to damage in an MMP-free system [27,28]. We also know that fibrillin microfibrils and fibulin-5 are particularly susceptible to *in vivo* photo-exposure and are early and specific biomarkers of mildly photoaged skin [1,2]. However, the UVR-mediated upregulation of low-substrate specificity MMPs cannot readily explain this specific loss of elastic fibre-associated components. Here we show that not only can fibrillin and fibronectin be degraded by UVR in an enzyme-free system, but that ROS are key mediators of this ECM protein damage. Significantly, we also show that MMPs appear primarily to be mediators of UVR-damaged protein removal, rather than the instigators of initial protein damage.

In this study we demonstrate that the photodynamic production of ROS plays a key role in mediating the degradation of UVB-exposed proteins *in vitro*. Purging solutions with N_2 is a recognised method for reducing the concentration of dissolved O_2 (9, 30, 31, 37) whilst conversely, the lifetime of both singlet oxygen and superoxide is extended in a D_2O environment [41,42]. Using these complimentary approaches we demonstrate that the UVR-mediated degradation of key ECM components is partially dependent on the photodynamic production of ROS. In future studies it would be informative to characterise the relative contributions of UVR and ROS in induced modifications in these proteins. Here, exposure to broadband UVB reduced the periodicity and increased the flexibility of fibrillin microfibrils, similar ultrastructural consequences can be induced by the chelation of bound calcium from the calcium-binding epidermal growth factor (cbEGF)-like domains which constitute the major motif in fibrillin-1 [45]. The presence of calcium stabilises these cystine-rich cbEGF motifs inducing a rod-like conformation [36]. Our data show an increase in microfibril flexibility, suggesting that UVR/ROS can mediate conformational changes in fibrillin cbEGF-like domains, resulting in the loss of Ca^{2+} and hence loss of the rod-like conformation.

Whilst profound histological changes of chronically UVR-exposed skin have been well documented, the causative mechanisms behind such changes remain poorly defined. In our proposed multi-hit model of photodamage, we suggested that UVB irradiation of skin may selectively damage ECM components either directly or *via* the photodynamic production of ROS [46]. The data presented in this study confirm that irradiation with UVB can damage ECM proteins *via* the photodynamic production of ROS. However, the induction of UVB-induced damage even following extended N_2 bubbling to remove O_2 also suggests that these proteins may undergo structural changes as a consequence of direct UVR absorption [47].

We have previously characterised the susceptibility of fibrillin microfibrils to MMP degradation [48]. A subsequent study showed that MMPs −3 and − 9 are capable of degrading fibrillin peptides, in addition to recombinant fragments being susceptible to MMPs −2, −3, −9, −12, −13 and − 14 [48,49].Whilst our 1999 study concluded that MMPs degrade fibrillin microfibrils, our new data suggests that the interaction of MMPs with fibrillin microfibrils is more complex and nuanced. Using AFM, we demonstrate that intact fibrillin microfibril structure appears relatively resistant to degradation by ubiquitous tissue MMPs (−1, −3, −7, and − 9) and that it is the UVR-damaged microfibril population that is targeted by these enzymes. Crucially, data in Ashworth et al. also suggests that MMP-13 (in addition to the MMPs analysed in the current study) can normalise periodicity, in this case of a chronically photo-exposed zonular microfibril population [48]. Collectively, these observations suggest that the packing of fibrillin monomers into microfibrils may mask MMP cleavage sites, which then may be exposed by the actions of UVR/ROS, but that the native microfibril is relatively resistant to MMP-mediated cleavage. Similar observations have been made for collagen I, whereby the structure is only susceptible to degradation by bacterial collagenase following UVR irradiation [29]. In contrast, fibronectin appears to be differentially susceptible to MMP action, with MMPs −1 and − 3 inducing fragmentation, but prior UVB-exposure having no detectable effect. These observations suggest that whilst the interactions between protease- and UVR-mediated damage are complex, differential degradation of ECM components could be an important part of targeted repair following, for example, the application of topical retinoids which induce fibrillin deposition in photo damaged skin [50].

It has been established that fibrillin fragments which contain an RGD site upregulate MMP-1 and MMP-3 expression [51]. A study by the same group also show that mutations in fibrillin peptides associated with Marfan's Syndrome also lead to an increase in peptide susceptibility to MMP degradation [52]. Additional evidence on fibronectin has identified that fragments of this glycoprotein can also induce MMP activity [53,54]. Therefore, if protein fragmentation can increase expression of MMPs and damaged proteins are more susceptible to degradation by MMPs, a self-regulatory mechanism between proteins and their degrading enzymes may be at work *in vivo*, aiding the maintenance of tissue homeostasis within the ECM (Fig. 7a).

These finding offer insights into potential mechanisms of photodamage; it has been previously established that MMPs are upregulated following exposure to UVR and we have shown previously that key ECM molecules that are high in UVR chromophores are susceptible to damage in enzyme-free environments. Here we show that the photodynamic production of ROS is a key mechanism for non-enzymatic protein damage and that for fibrillin, at least, MMP proteolysis only

occurs when the microfibrils are already damaged (Fig. 7b). Fibrillin microfibrils are readily susceptible to oxidation and that (at least some) MMPs preferentially degrade UVR damaged proteins, these insights could also offer models of tissue repair mechanisms throughout body sites which are susceptible to oxidative stress.

Declarations of interest

The authors declare they have no conflicts of interest with the contents of this article.

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Author contribution statement

SAH performed all of the experimental work, analysis and prepared the manuscript. MJS was responsible for the initial concept. MJS, NKG, REBW and CEMG secured the programme grant for this work and were involved in finalising the manuscript.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cellsig.2018.11.024.

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