Responses of Black and White Skin to Solar-Simulating Radiation: Differences in DNA Photodamage, Infiltrating Neutrophils, Proteolytic Enzymes Induced, Keratinocyte Activation, and IL-10 Expression

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Black skin is more resistant to the deleterious effects of ultraviolet radiation than white skin. A higher melanin content and a different melanosomal dispersion pattern in the epidermis are thought to be responsible for this. Our purpose was to compare skin responses in black and white skin following exposure to solar-simulating radiation (SSR) to further investigate the photoprotective properties of black skin. Six volunteers of skin phototype I–III (white) were exposed to (doses measured directly with a Waldmann UV detector device) 12,000–18,000 mJ per cm^2 (2 MED) of SSR and compared with six volunteers of skin phototype VI (black) exposed to 18,000 mJ per cm^2 (<1 MED) of SSR. The presence and distribution of skin pigment, DNA photodamage, infiltrating neutrophils, photoaging associated proteolytic enzymes, keratinocyte activation, and the source of interleukin 10 (IL-10) in skin biopsies taken before and after exposure were studied. In all white skinned subjects, 12,000–18,000 mJ per cm^2 of SSR induced DNA damage in epidermal and dermal cells, an influx of neutrophils, active proteolytic enzymes, and diffuse keratinocyte activation. Additionally, in three of the white skinned volunteers IL-10 positive neutrophils were found to infiltrate the epidermis. Except for DNA damage in the supra basal epidermis, none of these changes was found in black skinned subjects. Increased skin pigmentation appears to be primarily responsible for the observed differences in skin responses. Our data could provide an explanation as to why black skin is less susceptible to sunburn, photoaging, and skin carcinogenesis.

Keywords: DNA damage/interleukin-10/keratinocyte activation/neutrophils/photoaging
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Black skin is better protected against the damaging effects of ultraviolet radiation (UV) than white skin. These effects include sunburn (acute effect), photoaging, and skin carcinogenesis (chronic effects) (Taylor, 2002). The mechanisms underlying the photoprotective properties of black skin are still poorly understood. A higher melanin content, however, and a different melanosomal dispersion pattern in the epidermis ensuring a greater barrier to UV, are thought to be responsible (Kaidbey *et al*, 1979; Taylor, 2002; Ito and Wakamatsu, 2003).

Documented effects of UV on the skin, which have, or may be compared between persons of different skin color are as follows: DNA photodamage, infiltration of inflammatory cells, induction of photoaging-associated proteolytic enzymes, keratinocyte proliferation, and immune suppression.

Abbreviations: AP, alkaline phosphatase; IL-10, interleukin 10; K, cytokeratin; MED, minimal erythemal dose; MMP, matrix metalloproteinase; PBS, phosphate-buffered saline; SPT, skin phototype; SSR, solar-simulating radiation; UV, ultraviolet radiation; X mJ per cm^2-CIE eff, calculated wavelength weighted UV doses (X) using the CIE erythema action spectrum; X mJ per cm^2-Waldmann, UV dose (X) computed using direct readings from a Waldmann UV detector device

A previous investigation has studied the protective effect of skin pigment with respect to UV-induced DNA damage (Sheehan *et al*, 2002). Persons with skin phototypes (SPT) IV and II were exposed to repeated suberythemogenic doses of solar-simulating radiation (SSR) and the outcome was compared. It appeared that the levels of DNA damage induced were dependent on the physical (in Joules per cm^2 rather than the erythema effective dose (in MED or minimal erythemal dose) of SSR. Although the two groups differed in terms of the mean minimal erythemal dose (IV $>$ II), the amount of epidermal DNA damage induced was similar in both groups after exposure to equal physical doses of SSR. A more recent study has described a difference in the distribution of UV-induced DNA damage between subjects with SPT I/II and SPT V/VI (Fisher *et al*, 2002). In the former, DNA photoproducts were found in both the epidermis and the upper dermis. In the latter, however, DNA photoproducts were restricted to post-mitotic cells in the upper epidermis.

Inflammatory cells infiltrate the skin following UV exposure, and neutrophilic granulocytes are known to be present in sunburned skin (McGregor and Hawk, 1999). Neutrophils are potent producers of a wide array of proteolytic enzymes, including neutrophil elastase and

matrix metalloproteinase 9 (MMP-9) (Ford Bainton, 1999). These products are potentially capable of inflicting serious extracellular matrix damage (Doring, 1994; Opdenakker *et al*, 2001; Shapiro, 2002). MMP-1, produced by keratinocytes and fibroblasts, is the matrix metalloproteinase most often associated with extracellular matrix damage in the pathogenesis of photoaging of the skin (Fisher *et al*, 1996, 1997).

UV radiation can activate keratinocytes and induce (hyper) proliferation *in vivo* as demonstrated by increased epidermal thickness following UV exposure (de Winter *et al*, 2001). This may be regarded as an adaptive process since a thicker epidermis serves as a more effective physical barrier. Activated, hyperproliferative keratinocytes express a different set of cytokeratins (K6, K16, and K17) as compared with basal (K5, K14) and differentiating keratinocytes (K1, K2, and K10) (Freedberg *et al*, 2001). Immunohistochemical staining of these proteins thus enables the possibility of distinguishing between different activation/proliferation states of keratinocytes.

Selgrade *et al* (2001) have previously investigated UV-induced immunosuppression in subjects with different SPT. They demonstrated that the degree of immunosuppression induced with a solar simulator was dependent on the erythemal reactivity. Subjects with a weak or no erythemal response (mainly SPT IV–VI) showed a flat dose–response curve, i.e., no erythema and no immunosuppression.

The cytokine associated with UV-induced immunosuppression is interleukin 10 (IL-10) (Asadullah *et al*, 1999). IL-10-producing inflammatory cells infiltrate the epidermis following exposure to erythemogenic doses of UV (Kang *et al*, 1994). IL-10-producing cells may be involved in skin carcinogenesis (Kim *et al*, 1995; Asadullah *et al*, 1999; Alamartine *et al*, 2003) and thus play a role in the difference found between black and white skinned persons in (UV associated) skin cancer incidence.

The purpose of this study was to further compare skin responses in black and white skin after UV exposure. A solar simulator was used as a UV source because it best approaches the emission spectrum of natural sunlight. The following parameters were investigated: SSR-induced DNA photodamage, infiltrating neutrophils, the presence and enzymatic activity of (selected) proteolytic enzymes, keratinocyte activation markers, and the presence and source of IL-10.

Results

DNA photodamage Irradiating black and white skin with the same physical dose of SSR-induced thymine dimers throughout the supra-basal epidermis in both skin types, (Fig 1). Numbers of positive staining cells in these layers of the epidermis did not show great inter-individual variance and also seemed independent of SPT. In contrast, thymine dimers in basal keratinocytes were SPT dependent. Although they could scarcely be detected in basal keratinocytes of black skinned persons, they were abundantly present in basal keratinocytes of white skinned persons (particularly in the supra-papillary region). Furthermore, thymine dimer positive cells were effectively absent in the dermis of irradiated black skin, although they were present in large numbers in the dermis of irradiated white skin. These thymine dimers (superficial- to mid-dermal regions) were also induced when exposing white skinned individuals to suberythemogenic doses of SSR (0.5 MED, data not shown). To exclude the possibility that dermal thymine dimer positive cells had in fact migrated from the epidermis, additional biopsies were taken immediately following 2 MED of SSR exposure. Localization of thymine dimer positive cells at this time point proved to be similar to that in the biopsies taken 6 h after exposure (data not shown).

Neutrophils, MMP-9, and MMP-1 positive cells Large numbers of neutrophil elastase, MMP-9, and MMP-1 positive cells were detected in white skin that had been irradiated with 12,000–18,000 mJ per cm^2-Waldmann of SSR. None of these cells was detected in irradiated black skin (Fig 2 and Table I*A*). In the dose–response study conducted with a separate group of white skinned volunteers, neutrophil elastase, MMP-9, and MMP-1 positive cells were only detected in skin that had been exposed to equal or more than 1 MED of SSR (Table I*B*).

***In situ* elastase, gelatinase, and collagenase enzyme activity** Elastase, gelatinase, and collagenase activity was prominent in white skin that had been irradiated with 12,000–18,000 mJ per cm^2-Waldmann (Fig 3). No such enzyme activity was detected in irradiated black skin. In the dose–response study, elastase, gelatinase, and collagenase activity was only detected in skin that had been irradiated with at least 1 MED of SSR (data not shown).

Keratinocyte activation The cytokeratins 6, 16, and 17 were upregulated after 24 and 48 h in the epidermis of white skinned volunteers. Since these three cytokeratins showed identical staining patterns, only the results for cytokeratin 16 are shown here (Fig 4). The staining intensity (maximal after 48 h) varied between the white skinned volunteers, but increased expression following irradiation was consistent. Black skinned volunteers showed no upregulation of these cytokeratins.

IL-10 producing neutrophils In three of the six white skinned volunteers, IL-10 positive cells were found to infiltrate the epidermis 24 and 48 h after irradiation. Double staining for neutrophil elastase and IL-10 showed double staining positive cells (Fig 5). These cells were further characterized to be CD11b positive and CD36 negative (data not shown). No IL-10 positive/neutrophils elastase negative/CD11b positive/CD36 negative cells were found to infiltrate the epidermis of the black skinned volunteers at any time point.

Discussion

We compared skin responses in black and white skinned individuals after exposure to a fixed physical dose of SSR. Additionally, we performed a dose–response study with solar simulating radiation on white skinned subjects. Our results suggest that the observed differences found in black and white skin after SSR exposure are due to a difference in skin pigmentation. Figures 1, 2, and 4 demonstrate the

presence of abundant skin pigment (melanin) in black skin as compared with white skin. Melanin is concentrated at the apical pole of basal keratinocytes. Melanin appears to function as a barrier to SSR and protect basal keratinocytes and the dermis beneath from its damaging effects.

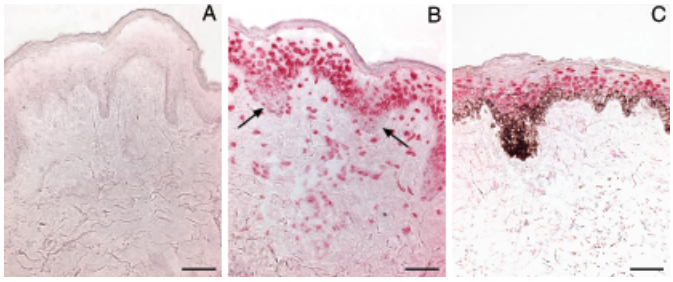

Figure 1
Distribution of SSR-induced DNA photo damage in black and white skin. Thymine dimer staining before exposure (white skin) (*A*) and 6 h after exposure to 18,000 mJ per cm^2-Waldmann of SSR in white (*B*) and black skin (*C*). Basal keratinocytes situated in the depths of the epidermal folds in white skin are relatively protected (→). In the upper dermis, thymine dimers are abundantly present in white but not in black skin. *Scale bars*: 50 μm.

Histological evidence to support a barrier function of melanin is provided by the distribution of SSR-induced DNA photodamage in black and white skin: supra-basal epidermal cells were equally damaged, whereas basal keratinocytes and cells in the dermis were relatively protected in black skin (Fig 1). In this way, proliferating cells in black skin appear to be better protected against the mutagenic effects of SSR and against malignant transformation.

Another indication that black skin is better protected against the damaging effects of SSR is the absence of a neutrophilic infiltrate and the absence of MMP-9 and MMP-1 positive cells in irradiated black skin. These cells were abundantly present in white skin irradiated with an equal, or even a lower physical dose of SSR (Table I*A* and Fig 2).

Neutrophil elastase was used as a marker to detect the presence of neutrophil infiltration, since neutrophils cannot be detected morphologically in frozen skin sections. Neutrophil elastase is a potent proteolytic enzyme (Doring, 1994; Shapiro, 2002) and *in situ* zymography strongly suggests that where it is immunohistochemically detected in our study, it is also enzymatically active. The same appears to apply to MMP-9 and MMP-1 detected in our

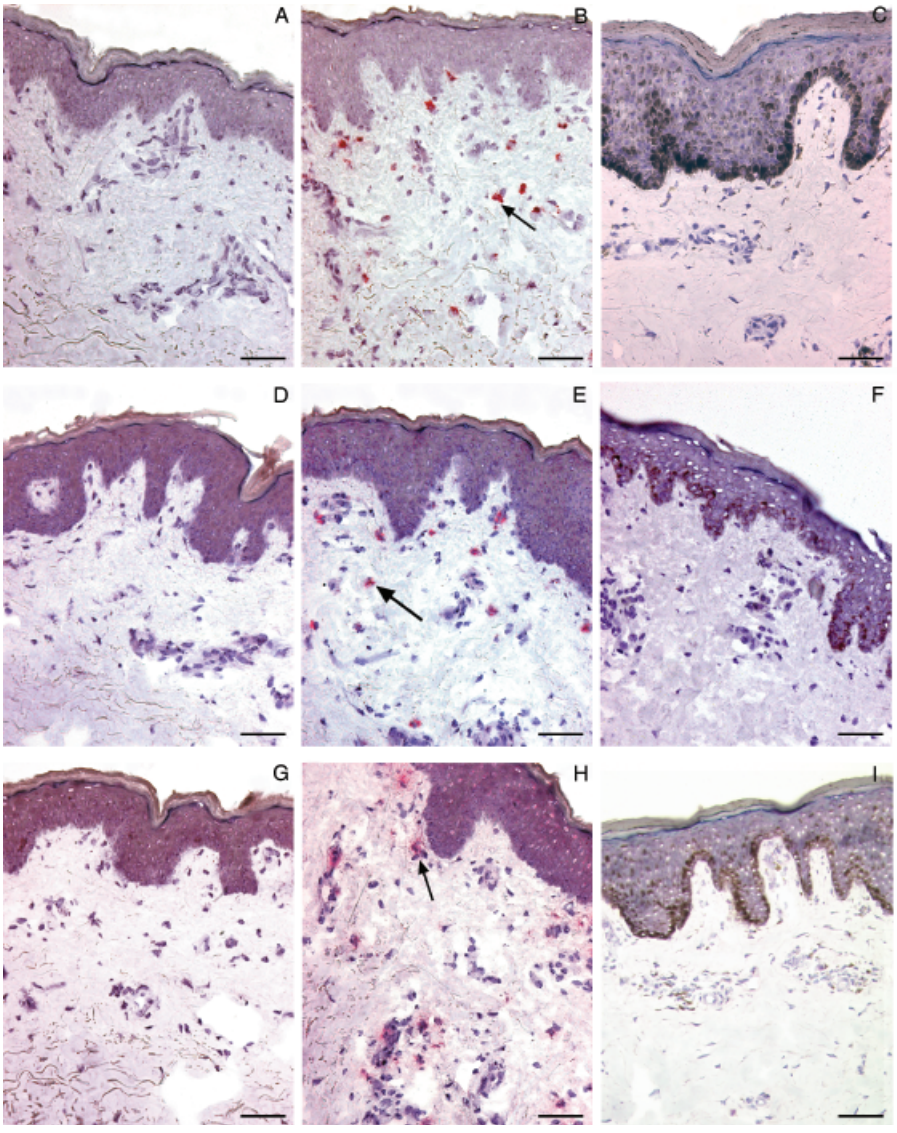

Figure 2
Detection of neutrophil elastase MMP-9 and MMP-1 in white but not in black skin following exposure to an equal physical dose of SSR. Neutrophil elastase (*B*), MMP-9 (*E*), and MMP-1 (*H*) positive cells in white skin six hours after exposure to 18,000 mJ per cm^2-Waldmann of SSR. No such staining is found in black skin exposed to an equal physical dose of SSR, as shown in *C* (neutrophil elastase), *F* (MMP-9), and I (MMP-1), respectively. *A*, *D*, and *G* are the white skin controls before exposure. *Scale bars*: 50 μm.

Table I. Staining pattern of neutrophil elastase, MMP-9 and MMP-1

	Mean number of positive cells per mm^2*					
	Neutrophil elastase[a]		MMP-9[b]		MMP-1[c]	
Hours after exposure	SPT I–III	SPT VI	SPT I–III	SPT VI	SPT I–III	SPT VI
(*A*) 6, 24, and 48 h after exposing sun-protected white and black skin to 12,000–18,000 mJ per cm^2-Waldmann of SSR (1)						
Control	0	0	0	0	0	0
6	132 (23)	0	55 (18)	0	79 (18)	0
24	196 (23)	0	102 (32)	0	111 (29)	0
48	141 (30)	0	40 (19)	0	65 (12)	0
(*B*) 24 h after exposing white skin to increasing MED doses of SSR. 2 MED is equivalent to 12,000–18,000 mJ per cm^2-Waldmann of SSR (2)						
	Mean number of positive cells per mm^2†					
MED dose[d]	Neutrophil elastase		MMP-9		MMP-1	
0	0		0		0	
0.5	0		0		0	
1	77 (18)		13 (8)		12 (4)	
2	248 (46)		87 (32)		103 (25)	

Note: (1) *SEM values in between brackets (SPT I-III n = 6, SPT VI n = 6). [abc]When comparing the number of positive cells in black and white skin after 6, 24, and 48 h, a statistical difference was found ($p < 0.05$). (2) †SEM values in between brackets (SPT I-III n = 6). [d]Differences in numbers of positive staining cells between suberythemogenic and erythemogenic doses were statistically significant ($p < 0.05$). This also accounted for cell numbers in skin irradiated with 1 MED compared to skin irradiated with 2 MED ($p < 0.05$).

MMP, matrix metalloproteinase; SSR, solar-simulating radiation; SPT, skin phototype; MED, minimal erythemal dose.

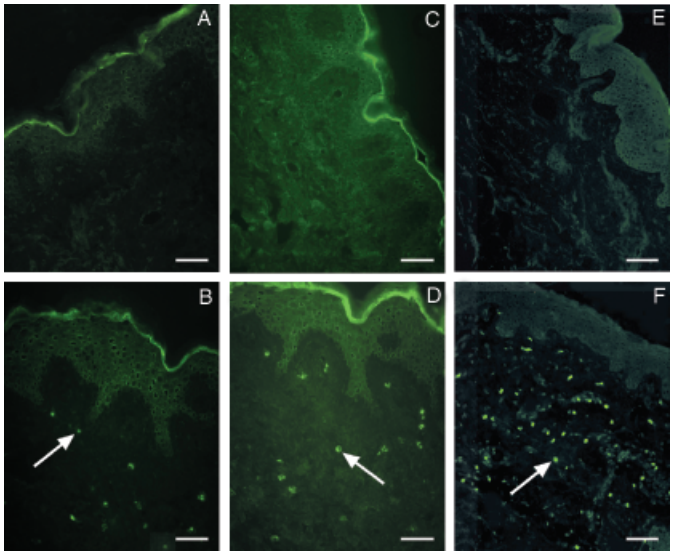

Figure 3
***In situ* elastase, gelatinase, and collagenase enzyme activity in white skin where neutrophil elastase, MMP-9, and MMP-1 positive cells had been immunohistochemically detected.** Enzyme activity was located in the upper dermis in a spot-like manner. *A* and *B* show collagenase activity, respectively, before and 6 h after SSR exposure. *C* and *D* show gelatinase activity before and after SSR exposure. *E* and *F* show elastase activity before and after SSR exposure. Compared with collagenase and gelatinase activity, elastase activity was most conspicuous. Elastase most likely reflects neutrophil elastase enzyme activity. Gelatinase probably reflects MMP-9 enzyme activity. Collagenase activity could reflect MMP-1 activity. *Scale bars*: *A–D*: 50 μm, *scale bars*: *E* and *F*: 80 μm.

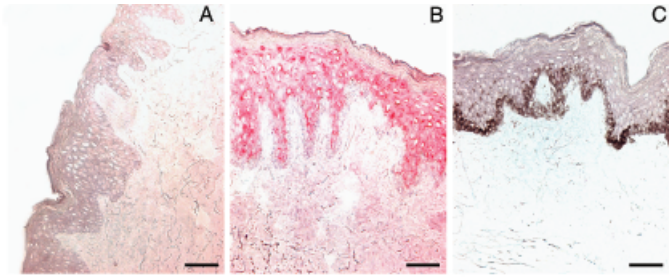

Figure 4
Cytokeratin 16 expression in irradiated white skin. The keratinocyte activation marker cytokeratin 16 was expressed in the epidermis of white skinned individuals 24 and 48 h after exposure to 18,000 mJ per cm^2-Waldmann of SSR. This cytokeratin was not upregulated in black skin after exposure to 18,000 mJ per cm^2-Waldmann of SSR. (*A*) White skin control before exposure. (*B*) White skin 48 h after exposure. (*C*) Black skin 48 h after exposure. *Scale bars*: 50 μm.

study (Fig 3). In the skin, MMP-9 has generally been associated with keratinocytes and fibroblasts. Our data, however, suggests that MMP-9 is in fact neutrophil derived. In an ongoing study (data not shown), an influx of neutrophils following SSR exposure was confirmed using paraffin-embedded skin sections (in which neutrophils can be detected morphologically). In the same study, additional frozen skin sections showed immunohistochemical double staining of neutrophil elastase and MMP-9. Our data are supported by studies that also show enzymatically active MMP-9 as a product of neutrophils (Golub *et al*, 1995; Choi *et al*, 2002). Although neutrophils and MMP-9 and MMP-1 positive cells were not detected in black skin, it is likely that these cells will appear when black skin is irradiated with higher doses of SSR. In the dose–response study, we have shown that erythemogenic doses are required to induce these cells (Table I*B*) and 18,000 mJ per cm^2-Waldmann

was not sufficient to induce erythema in black skin. This may explain why black skin is more resistant to photoaging. Higher absolute doses of SSR (and thus sunlight exposure) are necessary to induce infiltrating neutrophils, which, by releasing active proteolytic enzymes, can contribute to photoaging.

A third hallmark is the expression of the cytokeratins 6, 16, and 17, which was observed only in white skin (Fig 4), more specifically in white skin irradiated with $\geqslant$1 MED of SSR (data not shown). The expression of these activation markers is likely to precede epidermal hyperplasia that could serve as a protective mechanism by increasing the physical barrier to solar radiation. Whether an interaction between neutrophil elastase and keratinocytes, as recently described *in vitro* (Rogalski *et al*, 2002), plays a role in this activation process remains to be elucidated. Increased mitosis of basal keratinocytes may precede epidermal hyperplasia. The expression of Ki67 (a mitosis marker) was measured in our skin samples. A statistical difference in Ki67 positive cell numbers between black and white skin after SSR exposure was not detected. The expression of cytokeratins, however, was maximal after 48 h and expression of mitotic markers should therefore also be investigated at later time points.

An interesting finding was that in three of the six white skinned volunteers, IL-10/neutrophil elastase double-positive cells infiltrated the epidermis (Fig 5). Sporadic IL-10 positive/neutrophil elastase negative cells were also found in the epidermis, but the majority were double positive. Whether IL-10 positive neutrophils appear in the skin prior to IL-10 positive macrophages (Kang *et al*, 1994) still needs further investigation. Importantly, keratinocytes have also been shown to produce IL-10 following UV exposure (Curiel-Lewandrowski *et al*, 2003). IL-10 positive cells were not detected in the dermis of white skinned volunteers, nor were they detected in irradiated black skin. A variety of studies have reported that IL-10 is a product of neutrophils (Koller *et al*, 1998; Shimonkevitz *et al*, 2000; Glowacka *et al*, 2002; Spuck *et al*, 2003). Besides their destructive capabilities, neutrophils that have infiltrated the skin may thus also have an immunosuppressive function. This concept is supported by data describing an increased incidence of skin cancer in dermatological conditions, where (chronic) infiltration of neutrophils is involved (Mackie, 1998; Wallach, 2000).

In summary, the following skin changes were detected in white skin exposed to 12,000–18,000 mJ per cm^2-Waldmann of SSR: DNA photodamage in the epidermis and dermis; infiltrating neutrophils; the presence of enzymatically active neutrophil elastase, MMP-9 or MMP-1; activation of keratinocytes; and IL-10 positive cells in the epidermis. In black skin irradiated with 18,000 mJ per cm^2-Waldmann of SSR, DNA damage was limited to the supra-basal epidermis and none of the other skin changes

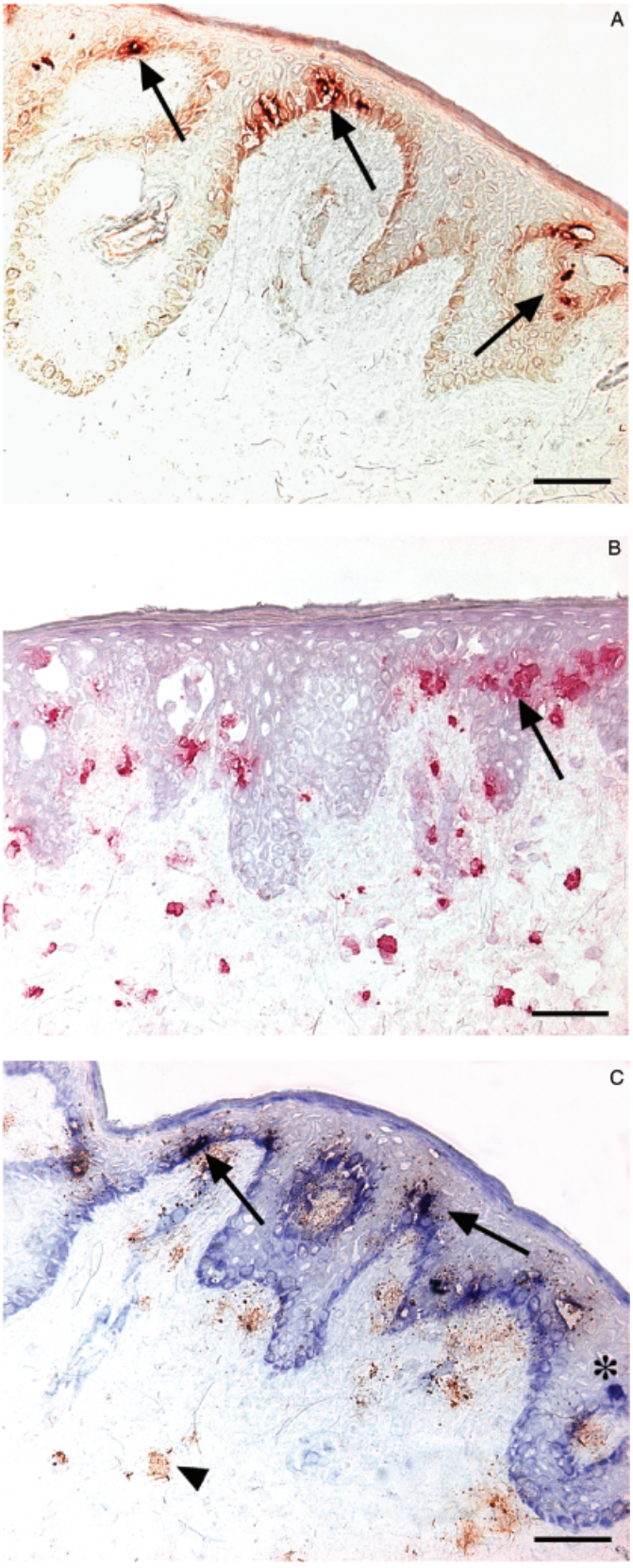

Figure 5
IL-10 positive neutrophils appear to infiltrate the epidermis of irradiated white skin. In three of the six white skinned volunteers, neutrophil elastase positive cells infiltrated the epidermis (*B*: →). In the same three individuals, IL-10 positive cells were found in the epidermis (*A*: →). Double staining revealed mostly neutrophil elastase/IL-10 double positive cells in the epidermis (*C*: →), a sporadic neutrophil elastase negative/IL-10 positive cell in the epidermis (*C*: *), and neutrophil elastase positive/IL-10 negative cells in the dermis (*C*: ◀). Note: In the single staining procedure, Il-10 was visualized using AEC and stains brown (*A*). In the double staining procedure, Fast bleu BB salt was used to visualize IL-10 and therefore stains bleu (*C*). *Scale bars*: 50 μm.

was observed. Although other genetic factors may be involved, skin pigmentation appears to be primarily responsible for the observed differences. Skin pigment appears to increase the threshold dose at which SSR induces an inflammatory response in the skin. This is confirmed by data that show that the MED of black skinned individuals is a factor 2–10 higher than that of white skinned individuals (SPT VI compared with SPT I–III) (Fitzpatrick, 1988). Our findings provide an explanation as to why black skinned individuals are less susceptible to sunburn, photoaging, and skin carcinogenesis.

Materials and Methods

Volunteers, irradiation procedures, and biopsy processing The skin characteristics of six healthy white- (SPT I–III: mean age 24.5 y, SEM 1.06) and six healthy black-(SPT VI: mean age 24.3 y, SEM 0.99) skinned volunteers are summarized in Table II. Six other white skinned volunteers (SPT I–III: mean age 25.3, SEM 3.21) were additionally recruited to study skin responses after exposure to different erythema effective doses of SSR (Table II, volunteers 13–18). All white subjects were of Dutch origin. Black subjects were of West-African and Afro-(South) American descent. The medical ethical committee of the University Medical Center Utrecht approved the study. Informed consents were obtained from all volunteers prior to commencing the experimental procedures.

For the irradiation protocols, CLEO Natural lamps (Philips, Eindhoven, the Netherlands) were used. This solar simulator emits predominantly UVA and 3% UVB. The ratio of UVB/UVA is comparable to mid-day sunlight during summer at 52°N, Amsterdam. The spectral energy distribution shows a continuum ranging from 290 to 420 nm, with a maximum around 355 nm. Relative spectral distribution measurements were performed with a calibrated standard UV–visible spectrometer (model 742, Optronic Laboratories, Orlando, FL, USA).

Throughout the experiments irradiation of the skin was monitored with a UV detector device (Waldmann, Schwenningen, Germany). The output at the surface of the skin was kept at 6.0 mW per cm^2 by adjusting the distance of the source to the exposed skin area. Applied physical doses were calculated by multiplying the irradiance measured with the time of exposure.

The same experienced investigator determined the minimal erythemal doses (MED) of the white skinned volunteers. A specially constructed test device (Boonstra *et al*, 2000) with nine windows (each 3×10 mm), which open consecutively in a geometrical time series (up to 50 min exposure time) exposing the underlying skin, was used for this purpose. The MED was taken as the 3×10 mm patch of irradiated buttock skin, which showed only just perceptible erythema and defined borders. To allow inter-study comparisons, the wavelengths adjusted erythema effective doses were calculated using the CIE erythema action spectrum (McKinlay and Diffey, 1987). These values are given in Table II.

Following MED determination, the first group of white skinned volunteers (1–6, Table II) was irradiated with a dose of 2 MED. Three areas of approximately 2×2 cm on the buttock were exposed and 4 mm punch biopsies were taken from the irradiated sites after 6, 24, and 48 h. A control biopsy was taken from a patch of non-irradiated buttock skin. The six other white skinned volunteers

Table II. Volunteer characteristics

No.	SPT	Skin color	Gender	Age (years)	MED (mJ per cm^2-Waldmann)	MED (mJ per cm^2-CIE eff)	PD[a] (mJ per cm^2-Waldmann)
1	II	White	Female	21	8700	50.8	17,400
2	II	White	Male	26	6300	36.8	12,600
3	I	White	Female	22	6000	35.0	12,000
4	II	White	Male	24	9000	52.8	18,000
5	II	White	Male	26	6300	36.8	12,600
6	III	White	Male	28	9000	52.8	18,000
7	VI	Black	Male	28	—	—	18,000
8	VI	Black	Male	22	—	—	18,000
9	VI	Black	Male	26	—	—	18,000
10	VI	Black	Male	22	—	—	18,000
11	VI	Black	Male	24	—	—	18,000
12	VI	Black	Female	24	—	—	18,000
13	II	White	Female	30	6300	36.8	[b]
14	II	White	Female	19	6300	36.8	[b]
15	I	White	Female	39	4500	26.3	[b]
16	II	White	Female	21	6300	36.8	[b]
17	III	White	Male	24	9000	52.8	[b]
18	II	White	Male	19	6300	36.8	[b]

White skinned volunteers (nos. 1–6) were exposed to 2 MED of SSR. This was equivalent to 12,000–18,000 mJ per cm^2-Waltmann. All black skinned volunteers (nos. 7–12) were exposed to 18,000 mJ per cm^2-Waltmann. Volunteers 13–18 participated in a dose–response study.
[a]PD: applied physical dose: irradiance measured with a Waldmann UV detector device multiplied by the time of exposure.
[b]Sun-protected buttock skin was exposed to 0, 0.5, 1, and 2 MED.
SPT, skin phototype; MED, minimal erythemal dose; SSR, solar-simulating radiation.

were irradiated with increasing doses of SSR (0, 0.5, 1, and 2 MED) and biopsies were taken after 24 h.

Black skinned volunteers were irradiated with a fixed physical dose of SSR (18,000 mJ per cm-Walmann) corresponding to 2–3 MED of the white skinned volunteers. As with the first group of white skinned volunteers, three 2×2 cm^2 areas on the buttock were exposed, and biopsies were taken after 6, 24, and 48 h including a control biopsy.

The biopsies were snap-frozen in liquid nitrogen, embedded in Tissuetek (Sakura,Torrance, CA,USA), and stored at $-80^{\circ}C$. In order to stain the skin immunohistochemically and evaluate enzymatic activity *in situ*, biopsies were cut into 6 μm thick skin sections with a freezing microtome and mounted on 3-aminopropyl tri-ethoxy silane (A3648, Sigma, St Louis, Missouri)-coated slides. Subsequently, the slides were left to dry and either put on Silicagel (MERCK, Darmstadt, Germany) to be used the following day or temporarily stored at $-80^{\circ}C$.

Immunohistochemistry To investigate UV-induced DNA photodamage thymine dimers were analyzed. The following steps (1–8) summarize the immunohistochemical staining procedure: skin sections were fixed, respectively, in (1) acetone containing 0.06% hydrogen peroxide, (2) 25% acetic acid-PBS solution, and (3) 0.07 M sodium hydroxide–70% ethanol–phosphate-buffered saline (PBS) solution. Next, the sections were consecutively incubated with (4) 10% normal rabbit serum (NRS) (5) monoclonal mouse anti thymine dimers (Kamiya Biomedical Company, Seattle, Washington; diluted 1:2000 in 1% NRS) (6) biotinylated rabbit anti mouse IgG1 (ZYMED, San Francisco, California; diluted 1:200 in 1% NRS), and (7) alkaline phosphatase (AP)-conjugated avidin–biotin complex (DAKO A/S, Glostrup, Denmark; diluted 1:50 in PBS). (8) AP activity was demonstrated using naphthol ASBI (Sigma) as a substrate and new fuchsine (Sigma) 10 mg per 100 ml as chromogen dissolved in 0,1 M TRIS-HCL + 0,05 M MgCl (pH 8.5) resulting in a pink staining. Endogenous AP activity was inhibited by adding levamisole (35 mg per 100 mL) to the above reaction mixture.

The presence of neutrophils in skin sections was demonstrated by staining cells using monoclonal mouse anti-human neutrophil elastase (NP57, DAKO A/S; diluted 1:50 in 1% normal human serum (NhuS)/1% normal horse serum (NhoS)). Sections were fixed in dry acetone and pre-incubated with 10% NhuS/10% NhoS. Subsequently, the sections were incubated with mouse anti-human neutrophil elastase (primary antibody). This was followed by incubation with biotinylated horse anti-mouse immunoglobulin (secondary antibody obtained from Vector, Burlingame, California; diluted 1:800) and AP-labelled streptavidine (Boehringer Mannheim GmbH, Mannheim, Germany; diluted 1:300). Both primary and secondary antibodies were diluted with 1% NhuS/1% NhoS. AP activity was detected as described above. Sections were counterstained with Mayer's hematoxylin.

MMP-9 and -1, cytokeratins 6, 16, and 17 were detected by performing practically identical staining procedures as described above for neutrophil elastase, differing only in the primary antibodies used. These were (all monoclonal antibodies): mouse anti-human MMP-9 (Neomarkers, Fremont, California; diluted 1:50), mouse anti-human MMP-1 (Oncogene, Boston, Massachusetts; diluted 1:50) and mouse anti-human cytokeratin 6, 16, and 17 (all three purchased from Novocastra, Newcastle, UK; diluted 1:50).

IL-10 expression was analyzed using monoclonal mouse anti human IL-10 (Instruchemie, Delfzijl, the Netherlands; diluted 1:300). Frozen skin sections were dried in air and fixed in 100 mL dry acetone containing 0.06% hydrogen peroxide. After pre-incubating with 0.1% bovine serum albumin (BSA)—PBS, the sections were incubated overnight at $4^{\circ}C$ with the IL-10 primary antibodies (diluted 1:300 in PBS–0.1% BSA). The following day, the sections were incubated with (step 1) biotinylated horse anti-mouse immunoglobulin (diluted 1:800 in 1% BSA–PBS) and (step 2) horseradish peroxidase-conjugated avidin-biotin complex (DAKO A/S; diluted 1:50 in 1% BSA–PBS). Staining was executed with 3-amino-ethyl-carbazole (AEC) purchased from Sigma (20 mg AEC in 5 mL DMF added to 95 mL of acetate buffer and 100 μL hydrogen peroxide). Finally, counterstaining was performed with Mayer's hematoxylin.

To stain both IL-10 and neutrophil elastase on the same slide, skin sections were first incubated with anti IL-10 antibodies as described above. Following incubation with biotinylated horse anti-mouse immunoglobulin (see above: step 1 of the second day in the IL-10 single staining protocol), sections were incubated with alkaline phosphatase-conjugated avidin–biotin complex (diluted 1:50 in 1% BSA–PBS). Next, the skin sections were incubated with 10% normal mouse serum (NMS) followed by incubation with horseradish peroxidase-conjugated neutrophil elastase horseradish NP57 (DAKO A/S; diluted 1:4 in 1% NMS) and ultimately staining was performed with Fast Bleu BB salt (Sigma) and AEC. Fast Bleu BB salt staining solution was prepared by adding 35 mg of levamisol, 25 mg of fast blue BB Salt, 12,5 mg of naphthol-ASMX (Sigma) dissolved in 1 mL of DMF to 100 mL of 0,05 M magnesiumchloride Tris-HCl.

(*Note*: The times of incubation and washing steps with PBS–Tween (0.05%) have been omitted in the descriptions for the purpose of clarity. New fuchsine, AEC, and fast bleuBB salt enzymatic activity was halted with distilled water.)

Microscopic evaluation Immunohistochemically stained skin sections were examined by light microscopy at $\times 200$ and $\times 400$ magnification. For quantification of cell numbers, stained cells in an area below and parallel to the dermal–epidermal junction (band-form; 0.445 mm broad) were counted. Results are expressed as mean number of positive cells per mm^2.

***In situ* zymography (Oh *et al*, 1999)** *In situ* zymography was performed using the following products: EnzChek Elastase Assay Kit (E-12056), DQ-collagen (D-12060), and DQ-gelatin (D-12054) all purchased from Molecular Probes, Eugene, Oregon.

To determine elastase enzyme activity, skin sections stored at $-80^{\circ}C$ were left to dry at room temperature. Thereafter, a reaction buffer (0.05 M TRIS-HCl, 0.15 M NaCl, 5 mM $CaCl_2$, and 0.2 mM NaN3, pH 7.6) containing 40 μg per mL DQ Elastin was transferred onto the skin sections and these were allowed to incubate for a short period of time. Enzyme activity was subsequently detected using fluorescence microscopy (FITC-channel, ex/em 505/515) and microphotographs were promptly taken. Skin sections incubated with a specific elastase inhibitor (supplied with the elastase assay kit) were used as negative controls.

To determine collagenase and gelatinase activity a similar procedure was followed: the same reaction buffer contained 40 μg per mL DQ-collagen type I or DQ-gelatin (both fluorescein conjugated). After transferring the solution onto the skin sections and covering them with coverslips, a short incubation period followed. Enzyme activity was detected by fluorescence microscopy using a different wavelength (FITC channel, ex/em 495/515).

Statistical analysis After logarithmic transformation of cell numbers (as the distribution of cell numbers per stained cross-sectional area is skewed to the right), a Student's *t* test was performed to compare stained cell numbers in non-irradiated and irradiated skin. Correlations were calculated with Pearson's test. Statistical analyses were performed using GraphPad Prism software (GraphPad Software, V.2.04a, San Diego, CA). p values <0.05 were considered significant.

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