S100A8 Induction in Keratinocytes by Ultraviolet A Irradiation Is Dependent on Reactive Oxygen Intermediates

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Cutaneous exposure to ultraviolet (UV) A (320–400 nm) results in the formation of damaging reactive oxygen intermediates, which are implicated as mediators of DNA damage, apoptosis, and photoaging. S100A8 is a low-molecular-weight calcium-binding protein, highly sensitive to oxidation. In this study, UVA-induced S100A8 expression by keratinocytes was investigated. UVA (50–100 kJ per m^2) strongly induced S100A8 in differentiated keratinocytes in the epidermis of BALB/c mice. Similarly, S100A8 mRNA and monomeric protein were significantly upregulated in PAM212 cells (a murine keratinocyte cell line) in response to 10 kJ per m^2 UVA 24 h after irradiation. Although S100A9 associates with S100A8 in neutrophils and abnormally differentiated keratinocytes (human psoriasis), in this study it was not coinduced with keratinocyte S100A8. Dorsal application of 4-hydroxy-tempo (a superoxide dismutase-mimicking agent) to mice concentration-dependently reduced UVA-induced S100A8 expression. Incubation of PAM212 cells with superoxide dismutase and catalase during UVA irradiation also abrogated S100A8 induction. These results suggest that UVA-induced S100A8 is expressed by keratinocytes in response to generation of reactive oxygen intermediates. *Key words: singlet oxygen/superoxide enzymes/S100 proteins. J Invest Dermatol 121:1168–1174, 2003*

Ultraviolet (UV) radiation triggers rapid induction of sets of genes (including those involved in oxidative defense) that are protective, particularly against UV-induced DNA damage. Responses to UVA irradiation (320–400 nm) of the skin are predominantly mediated by oxygen-dependent processes via the formation of reactive oxygen intermediates (ROI), including the superoxide anion ($O_2 \cdot^-$), the hydroxyl radical, hydrogen peroxide (H_2O_2), and singlet molecular oxygen. ROI oxidize a large range of biologic molecules, causing damage to cellular membrane lipids, proteins, and DNA (Vile and Tyrrell, 1995; Ryter and Tyrrell, 1998). UVA-induced ROI promote apoptosis and activate numerous proteins with antioxidant and free-radical-scavenging properties involved in the cellular stress response (Pourzand and Tyrrell, 1999). Keratinocytes, located in the outermost layer of the skin, contain higher than normal levels of antioxidants including glutathione and enzymes involved in antioxidant defense. These include superoxide dismutase (SOD), a radical scavenger of $O_2 \cdot^-$ that catalyzes dismutation of $O_2 \cdot^-$ to O_2 and H_2O_2 (Trenam et al, 1992), and catalase, which converts H_2O_2 to H_2O and O_2 (Liochev and Fridovich, 1993). Nevertheless, as may occur in inflammation, high levels of ROI generated by UVA could overwhelm normal defenses to oxidative damage so that additional protective responses are provoked.

S100 calcium-binding proteins are highly conserved, low-molecular-weight (9–14 kDa) acidic proteins with important regulatory functions including calcium buffering, regulation of kinases and phosphatases, cell proliferation, differentiation, energy metabolism, cytoskeletal–membrane interactions, embryogenesis, cell migration, and inflammation (Kerkhoff et al, 1998; Donato, 2001). To date, 18 human S100 family members have been described, 13 residing within a cluster located on chromosome 1q21 and several murine homologs on chromosome 3 (Ridinger et al, 1998). Expression of human S100A8 and A9 is associated with acute and chronic inflammatory diseases including infection, rheumatoid arthritis, cystic fibrosis, and Crohn's disease (Kerkhoff et al, 1998; Passey et al, 1999). These proteins comprise $\sim 40\%$ of the constitutively expressed cytoplasmic proteins of neutrophils (Harrison et al, 1999; Passey et al, 1999) and are upregulated in several cell types in a stimulus-specific manner. The S100A8/A9 complex may regulate leukocyte activation, has antimicrobial activity, and is a transporter of unsaturated fatty acids and arachidonate (Kerkhoff et al, 1999a; Passey et al, 1999). S100A9 may mediate leukocyte transmigration (Manitz et al, 2003), and murine S100A8 is a potent leukocyte chemoattractant (Cornish et al, 1996; Kerkhoff et al, 1999b). S100A8/A9 are not found in normal skin but are expressed by epidermal keratinocytes in a variety of inflammatory dermatoses (Kelly et al, 1991). Human S100A8/A9 expression has been associated with hyperproliferative responses such as occur in psoriasis (Gabrielsen et al, 1986) and in wound healing (Thorey et al, 2001). The human genes are overexpressed in psoriatic lesions of 1q-linked families and may be involved in pathways associated with the disease-susceptibility locus PSORS4 (Semprini et al, 2002). Although their exact roles are unclear, S100A8/A9 may be involved in Ca^{2+}-dependent reorganization of cytoskeletal filaments that affect keratinocyte proliferation and differentiation (Saintigny et al, 1992; Goebeler et al, 1995).

Our recent experiments led to the proposal that S100A8 may be involved in oxidative defense in acute inflammation. Large amounts of S100A8 are deposited at acute inflammatory sites and this protein is exquisitely sensitive to oxidants such as $O_2 \cdot^-$ and hypochlorite generated by activated neutrophils (Harrison *et al*, 1999; Raftery *et al*, 2001). Moreover, gene expression in elicited macrophages stimulated with lipopolysaccharide (LPS) or tumor necrosis factor (TNF) is mediated by interleukin (IL)-10 (Xu *et al*, 2001) and anti-inflammatory mediators such as prostaglandin E$_2$, cAMP (Xu *et al*, 2001), and glucocorticoids (C.L. Geczy, unpublished observations) markedly amplify TNF or LPS-mediated S100A8 induction, strongly supporting the notion that this protein may have an anti-inflammatory function by scavenging oxidants (Harrison *et al*, 1999; Raftery *et al*, 2001).

Because UVA irradiation is mediated largely through generation of ROI, we investigated the pattern of expression of S100A8/A9 in skin of UVA-irradiated mice and in cultured keratinocytes. We show that UVA induced strong expression of S100A8, but not S100A9, in both systems and H$_2$O$_2$-induced S100A8 *in vitro*. Conversely, SOD, a radical scavenger of $O_2 \cdot^-$, and catalase inhibited UVA-induced S100A8 upregulation *in vitro*. Furthermore, S100A8 was suppressed in the skin of mice topically treated with 4-hydroxy-tempo (Tempol; a SOD-mimicking agent) before UVA-irradiation.

MATERIALS AND METHODS

Animals Pathogen-free, female BALB/c mice 10 weeks of age were obtained from the Animal Resource Center of the South Australian Department of Agriculture, Adelaide, Australia. All experiments were performed according to the ethical guidelines of the National Health and Medical Research Council of Australia.

UV irradiation The UVA source was a bank of FL40SBL lamps (NEC, Japan) emitting a broad band of UV, 300–440 nm, with 85% of the output in the UVA range (320–400 nm) and peak emission at 360 nm. For UVA-irradiation, no filter was used, although in some specified experiments a Mylar filter (gift from E. De Fabo, The George Washington University, Washington, DC) was used to absorb wavelengths <330 nm (De Fabo and Kripke, 1980). The UVB source was a bank of FS40 sunlamps (Westinghouse, Pittsburgh, PA) emitting a broad band of UV, 250–360 nm, with 65% of the output in the UVB range (280–320 nm); a polyvinylchloride plastic sheet was used to screen out wavelengths <290 nm. Concentration rates were monitored using a UVX radiometer with a UVX-36 (UVA) or UVX-31 (UVB) sensor (Ultraviolet Products, San Gabriel, CA). For irradiation, mice were clean-shaven over a uniform dorsal area (approx. 8 cm^2) and housed in individual compartments of Perspex cages. Mice were exposed to a single concentration of 1, 10, 50, or 100 kJ per m^2 UVA or three consecutive concentrations, 24 h apart, of 100 kJ per m^2 UVA. For irradiation of PAM212 cells, culture medium covering glass slides or petri dishes was replaced with 0.2 or 3.5 mL of phosphate-buffered saline, respectively, and cells exposed to a single concentration of 1, 10, 50, or 100 kJ per m^2 UVA or 600 or 1000 J per m^2 UVB. The UVA and UVB sunlamps were located 20 cm above the cages or petri dishes.

Cutaneous application of test reagents Tempol (4-hydroxy-tempo; Sigma Chemical Co., St Louis, MO) dissolved in 1/1/2 (vol/vol/vol) propylene glycol/distilled water/ethanol was applied to the shaved dorsal skin of mice (Yuen *et al*, 2002). Fifty microliters of diluent containing 0.07, 0.14, or 0.2 mg Tempol was applied 45 min before and immediately after UVA irradiation. Skin irritation, illness, or fatalities were not observed.

Cell culture PAM212 cells (a murine keratinocyte cell line) obtained from S. Yuspa (National Cancer Institute, Bethesda, MD; Yuspa *et al*, 1980) were cultured in MEM (with Earle's salts, L-glutamine, and 25 mM HEPES; Sigma) supplemented with 2.2 g per liter NaHCO$_3$, 200 U per mL penicillin, 200 μg per mL streptomycin (Sigma) and osmolality adjusted to 290 + 10 mOsm per kg H$_2$O with 0.58 g per liter NaCl. Medium was also supplemented with 2 mM glutamine and 10% fetal calf serum (HyClone, Logan, UT).

For subculture, adherent cells were incubated with 0.06% trypsin (Sigma) for 10 to 15 min at 37°C. Trypsin was neutralized with fetal calf serum-containing medium, and cells were centrifuged, resuspended, and

plated at 10^5 cells per mL on sterile glass slides coated with Histo Grip (Zymed, San Francisco, CA) or plated (5 × 10^6 cells/5 mL) in 100-mm-diameter petri dishes (Falcon, Lincoln Park, NJ). Cells were allowed to adhere for 24 h and then UV-irradiated; stimulated with medium containing 10 ng per mL TNF (Genzyme, Cambridge, MA), 10 ng per mL IL-10 (Peprotech, Rocky Hill, NJ), and 1 μg per mL LPS (*Escherichia coli* 0111:B4, Sigma); or received no treatment (control). To confirm the role of oxidative pathways, PAM212 cells were incubated with 0.0001% H$_2$O$_2$ or the superoxide-scavenging enzymes SOD (30 μg/mL) and/or catalase (2 μg/mL) during and again after UVA-irradiation, only during irradiation, or only after irradiation. For northern and western blot analyzes, PAM212 cells were incubated with SOD (30 μg/mL) and catalase (2 μg/mL) during and again after UVA irradiation or only after irradiation.

Immunohistochemical detection of S100A8 and S100A9 Samples of dorsal skin (two per mouse) were rolled, fixed in 10% buffered formalin, and paraffin-embedded ensuring vertical orientation of the roll, and 4-μm sections were cut. PAM212 cells cultured on HistoGrip-coated slides were fixed in 10% buffered formalin for 15 min, immersed in 95% ethanol for 5 min, and washed twice in phosphate-buffered saline. After incubation at room temperature with 10% normal sheep serum (Silenus, Hawthorn, Australia) for 15 min, PAM212 cells and mouse skin sections were incubated for 16 h at 4°C with 1.4 μg per mL rabbit polyclonal anti-S100A8 IgG or 0.3 μg per mL rabbit polyclonal anti-S100A9 IgG (detailed in Harrison *et al*, 1999). The antibodies only recognized the relevant immunogen and anti-A8 does not cross-react with S100B, S100A1, or S100A9 by western blotting. Endogenous peroxide activity was quenched by immersion into 0.3% H$_2$O$_2$ in phosphate-buffered saline for 10 min at room temperature. After incubation with a 1/250 dilution of biotin-conjugated sheep anti-rabbit IgG (Silenus) for 45 min at room temperature and 1/500 dilution of peroxidase-conjugated streptavidin (Dako, Carpinteria, CA) for 30 min at room temperature, the reaction product was developed with 3,3'-diaminobenzidine tetrahydrochloride (Sigma). Sections were counterstained with a weak Lillie Mayers hematoxylin solution (1/1 vol/vol with distilled water). Control sections incubated with diluted normal rabbit serum (1/650 vol/vol) or no primary antibody showed no positive staining.

Quantitation of S100A8- and S100A9-expressing PAM212 cells A video image analysis system (Video Pro 32, Leading Edge p/L, Adelaide, South Australia) was used to quantitate S100A8- and S100A9-stained keratinocytes. The system consists of an Olympus BX40 microscope with a 2.5 × camera eyepiece and a continuous interference filter monochromator for enhancement of hematoxylin (blue) and 3,3'-diaminobenzidine tetrahydrochloride (brown) contrast. Images were captured with a Panasonic color digital CCD video camera (Model GP-KR222, automatic gain disabled) interfacing an Intel 700 MHz MMX processor-based personal computer with a VCG multimedia 32-bit color video digitizer card and Video Pro 32, version 4, image analysis software.

For quantitation of S100A8 and S100A9 in PAM212 cells, the integrated optical density (IOD), representing the total amount of positive staining in a given field, and the area of the stained cells were measured. A positivity index based on expression of S100A8 was calculated as the percentage of positive staining in a given field divided by the total cell area (3,3'-diaminobenzidine tetrahydrochloride area plus counterstained area). For each slide, 100 images of consecutive fixed fields (image window, 512 × 512 pixels) were captured using a 40 × objective. Images of fields were captured at the center of the slide and across the width. A positive control section of UVA-irradiated mouse skin was included in each staining run, and this showed minimal differences in keratinocyte S100A8 and S100A9 staining intensity between runs. All measurements were standardized using a bright-field image of the background illumination to allow for correction of nonuniform illumination over a field of view.

Protein extraction and western blot analysis PAM212 cells (5 × 10^6) were lyzed with 1% Triton X-100 buffer supplemented with protease inhibitors (0.2 mM phenylmethylsulfonylfluoride, 20 μg/mL leupeptin, 20 μg/mL antipain, 20 μg/mL aprotinin; Sigma). After gentle mixing for 30 min at 4°C, cell debris was removed by centrifugation, and the supernatant frozen at −70°C. Protein (250 μL) from each sample was cleared using 100 μL of protein A–Sepharose (Pharmacia Biotech, Uppsala, Sweden) and 10 μg per mL rabbit IgG (Sigma) at 4°C overnight. The total protein of the cleared samples was determined using the Bio-Rad protein microassay kit (Bio-Rad Laboratories, Hercules, CA). Each sample (100 μg) or 500 ng recombinant mouse S100A8 was reduced with 10 mM dithiothreitol (Bio-Rad) and separated by 10% SDS–PAGE as previously described (Harrison *et al*, 1999). Following transfer of proteins, polyvinylidene difluoride

membranes were blocked for 2 h with 5% skim milk in Tris-buffered solution and incubated overnight at 4°C with 15 μg per mL preabsorbed primary rabbit anti-mouse S100A8 IgG or irrelevant rabbit IgG in 2% skim milk/Tris-buffered solution. Membranes were washed 4×10 min with $1 \times$ Tris-buffered solution with 0.1% Tween 20, incubated with 1 μg per mL horseradish peroxidase-conjugated goat anti-rabbit antibody (Bio-Rad) for 2 h, and washed 4×10 min with $1 \times$ Tris-buffered solution/ Tween 20, and then reactivity was detected with chemiluminescence as described (Harrison *et al*, 1999).

RNA extraction and northern analysis PAM212 cells (5×10^6) were lyzed in 1 mL of total RNA isolation reagent (Advanced Biotechnologies, Leatherhead, UK). RNA was isolated at 4°C using the manufacturer's protocol and precipitated with an equal volume of isopropanol for 10 min at 4°C and then centrifuged at $12,000 \times g$ (4°C) for 10 min. The RNA precipitate was washed twice with 75% ethanol by vortexing and subsequent centrifugation for 5 min at $7500 \times g$ (4°C) and air-dried to remove ethanol, and then samples were frozen at −70°C. RNA (20 μg) was resolved on 1% agarose gel and transferred onto nylon Hybond-N + membrane (Amersham, Buckinghamshire, UK) with alkali fixing and prehybridization performed at 55°C as detailed previously (Xu and Geczy, 2000). The membrane was hybridized with a ^{32}P-labeled mouse S100A8 riboprobe at 55°C overnight (Xu and Geczy, 2000) and, after being washed twice with 0.1% SDS/0.1% $1 \times$ SSPE for 30 min at 65°C, was exposed to a phosphoimager (GS-505 or GS-525, Bio-Rad) plate for 5 days. Blots were scanned using the Molecular Imager System, GS-525 (Bio-Rad), and analyzed with Multianalyst 1.0 software (Bio-Rad).

Expression of data and statistical analysis For measurement of IOD, the mean result from each slide (one per experiment) was used to calculate the mean value ± SD for three to six experiments. A multiple comparison procedure employing an ANOVA and Fisher's test was used to determine statistical significances in keratinocyte S100A8 expression between groups of varying treatments. Probabilities <0.05 were considered significant.

RESULTS

UVA-induced keratinocyte S100A8 expression No S100A8 was observed in the epidermis of untreated BALB/c mice (**Fig 1***A*), whereas UVA irradiation induced S100A8 in keratinocytes in a concentration-dependent manner (**Fig 1**). Expression was maximal 24 h after irradiation and remained maximal for a further 24 h. All UVA concentrations investigated caused thickening of the epidermis (hyperkeratosis) (**Fig 1***A–E*). A single concentration of 50 or 100 kJ per m^2 UVA induced S100A8 in differentiated keratinocytes in the stratum granu-losum (**Fig 1***C,D*), whereas three consecutive concentrations, 24 h apart, of 100 kJ per m^2 UVA were required to induce S100A8 in keratinocytes in the stratum spinosum (**Fig 1***E,F*). Cytoplasmic and nuclear S100A8 staining was heterogeneous in intensity, particularly in skin that received three consecutive concentrations, 24 h apart, of 100 kJ per m^2 UVA (**Fig 1***E*). There was no indication of extracellular localization of S100A8. This pattern of S100A8 expression in keratinocytes was similar in skins exposed to Mylar-filtered UVA radiation (**Fig 1***F*). A few infiltrating S100A8-positive neutrophils, monocytes, and macrophages were observed in subcutaneous fatty tissue vessels (**Fig 1***G*) and in the dermis after UVA irradiation (**Fig 1***E*). In marked contrast to S100A8, UVA irradiation (10, 50, or 100 kJ per m^2) did not induce epidermal S100A9 expression at any of the time points investigated (not shown). On the other hand, the anti-S100A9 antibody reacted with A9 in the limited number of neutrophils and macrophages infiltrating the dermis in response to UVA irradiation (not shown).

UVA induces S100A8 in PAM212 cells A few untreated PAM212 cells (5%–7%) expressed S100A8 in the cytoplasm (**Table I**), a finding consistent with other studies (Saintigny *et al*, 1992; Goebeler *et al*, 1995), showing basal expression of S100A8 in transformed keratinocytes but not in normal epidermis. In keeping with the *in vivo* experiments, UVA irradiation markedly upregulated immunoreactive protein levels (**Fig 2**). The numbers of PAM212 cells expressing S100A8, rather than amounts per

positive cell, significantly increased two- to threefold above baseline levels following UVA irradiation (**Table I**). Staining was diffuse within the cytoplasm and high levels were obvious in nuclei. Although some cells expressed S100A8 only in the cytoplasm, there were no cells positive for S100A8 only in the nucleus. With respect to IOD, **Fig 3***A* shows significant S100A8 expression in PAM212 cell incubated with the combination of TNFα, IL-10, and LPS (positive control), compared with untreated cells (negative control). Twenty-four hours after a single concentration of 1, 10, 50, or 100 kJ per m^2 UVA, only 10 kJ per m^2 UVA significantly increased S100A8 and after 24 h this had returned to normal levels. Concentrations of 50 or 100 kJ per m^2 UVA caused extensive cell loss. Compared with cell numbers of those given 0, 1, or 10 kJ per m^2 UVA, less than 50% (for 50 kJ per m^2) and 20% (for 100 kJ per m^3) of keratinocytes were adherent to culture slides 24 h after irradiation. Poor viability may account for the failure of these concentrations to induce significant amounts of S100A8. To determine whether UVB induced a response, and because the UVA lamps used had a small output of UVB, S100A8 expression by PAM212 cells irradiated with 600 or 1000 J per m^2 UVB was investigated. **Figure 3(*B*)** shows no significant differences in S100A8 expression between untreated cells and cells irradiated with 600 or 1000 J per m^2 UVB 24 h postirradiation. No cell loss occurred at these concentrations, whereas 1500 or 2000 J per m^2 UVB caused significant loss (not shown). Twenty-four hours after irradiation with different concentrations of UVA or UVB, no significant differences in S100A9 expression were observed between UV-irradiated cells and untreated control cells (**Fig 3***A,B*).

S100A8 induction may occur by an oxidative mecha-nism Nonirradiated skin of BALB/c mice treated topically with Tempol or diluent (**Fig 4***A*) was not different from untreated control skin (**Fig 1***A*). Administration of diluent 45 min before UVA irradiation (100 kJ per m^2) did not affect subsequent S100A8 expression in keratinocytes (**Fig 4***B,C*). In contrast, application of Tempol before and again immediately after UVA irradiation, reduced S100A8 expression in keratino-cytes in a concentration-dependent manner (**Fig 4***D–F*). The highest concentration of Tempol (0.4 mg in 2×50 μL vol/mouse) decreased epidermal hyperkeratosis and suppressed S100A8 expression in keratinocytes to levels apparent in untreated mice (**Fig 4***F*).

To further investigate whether the antioxidant enzymes could modulate UVA-induced expression of S100A8, PAM212 cells were incubated with SOD and/or catalase. **Figure 5** shows that SOD and catalase present during UVA irradiation (10 kJ per m^2) of PAM212 cells significantly reduced S100A8 expression to levels apparent in untreated cells although S100A8 expression was not altered if SOD and catalase were added to PAM212 cells immediately after irradiation. SOD and catalase did not affect S100A8 expression in nonirradiated PAM212 cells or cells treated with TNFα, IL-10, and LPS (positive control) (**Fig 5**). H$_2$O$_2$ (0.0001%) increased S100A8 expression twofold above baseline levels of untreated cells (**Fig 5**), confirming upregulation of S100A8 in response to an oxidative stimulation.

Northern blot analysis confirmed S100A8 mRNA up-regulation in PAM212 cells exposed to UVA (**Fig 6***A, lane 3*) and transcript levels were unchanged in cells treated with SOD and catalase after UVA irradiation (**Fig 6***A, lane 5*). In contrast, S100A8 mRNA levels were decreased in PAM212 cells incubated with SOD and catalase during and again after UVA irradiation (**Fig 6***A, lane 4*). PAM212 cells cultured with TNFα, IL-10, and LPS (**Fig 6***A,* positive control, *lane 2*) expressed high levels of S100A8 mRNA.

Western blot analysis confirmed increases in UVA-induced S100A8 expression in PAM212 cells quantified by video image analysis and its modulation by SOD and catalase (**Fig 6***B*). A component with the same mass as recombinant S100A8 (~ 10
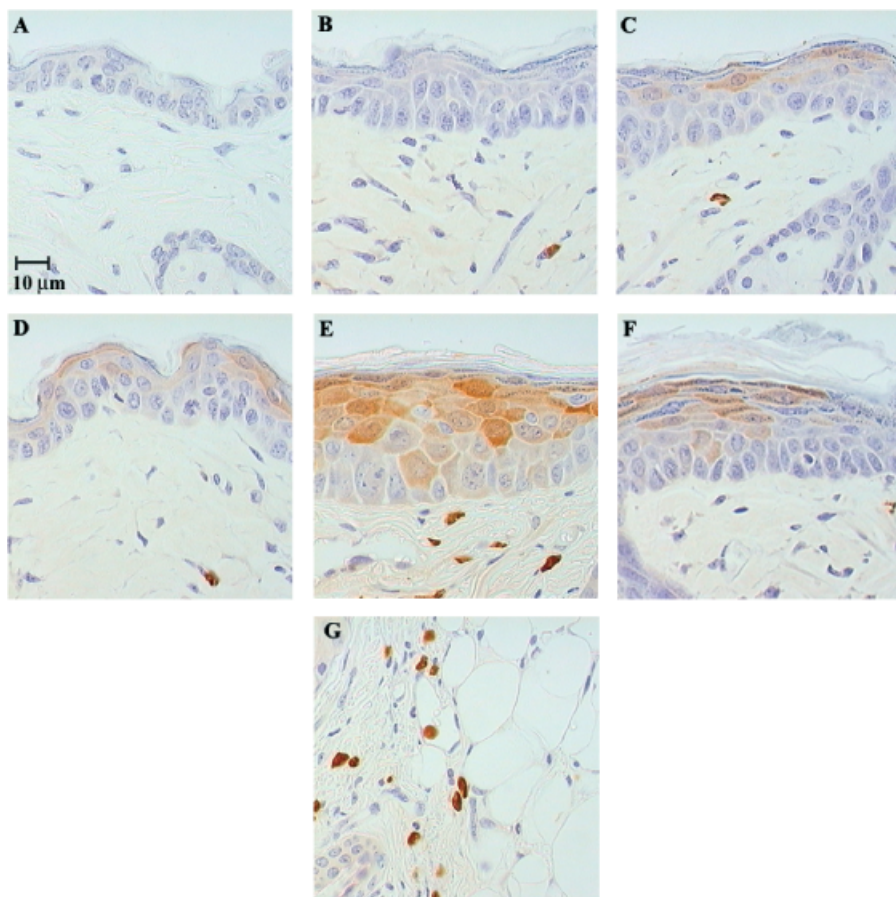
Figure 1. Skin sections showing S100A8 expression 24 h after UVA irradiation of BALB/c mice. (*A*) Nonirradiated skin; (*B*) 10 kJ per m^2 UVA; (*C*) 50 kJ per m^2 UVA; (*D*) 100 kJ per m^2 UVA; (*E*) three consecutive concentrations 100 kJ per m^2 UVA, 24 h apart; (*F*) three consecutive concentrations 100 kJ per m^2 Mylar-filtered UVA, 24 h apart; (*G*) granulocytes in dermal vessels after 100 kJ per m^2 UVA.
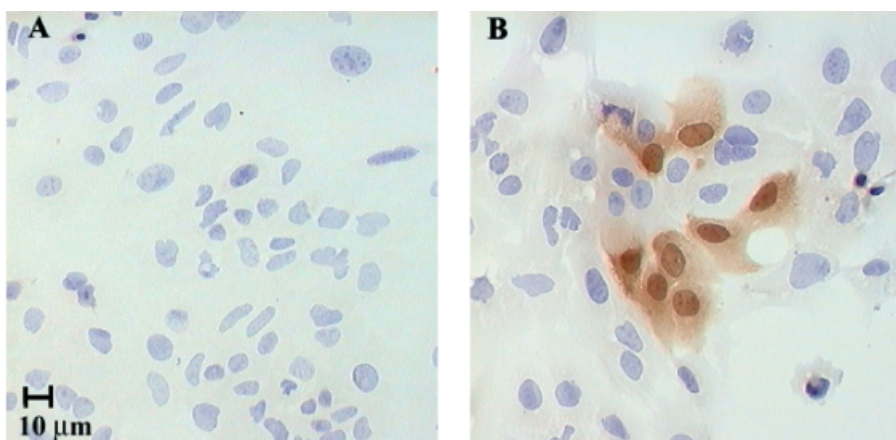

Figure 2. PAM212 keratinocytes express S100A8 24 h after UVA irradiation. (*A*) Nonirradiated cells; (*B*) cells irradiated with 10 kJ per m^2 UVA.

Table I. Expression (mean $\pm$ SD) of S100A8 by PAM212 cells 24 h after UVA irradiation

Treatment	Total cell area (μm^2)	3,3′-Diaminobenzidine tetrahydrochloride-stained area (μm^2)	Cells expressing S100A8 (%)
Control (n = 6)	3321 $\pm$ 768	160 $\pm$ 82	5 $\pm$ 2
1 kJ/m^2 UVA (n = 4)	2784 $\pm$ 891	256 $\pm$ 152	9 $\pm$ 2
10 kJ/m^2 UVA (n = 4)	4617 $\pm$ 2884	505 $\pm$ 244	12 $\pm$ 4[a]
TNF + LPS + IL-10 (n = 6)	3574 $\pm$ 799	555 $\pm$ 233	16 $\pm$ 5[a]

[a]Significant increase compared with control untreated cells (p < 0.05).

kDa) was found in PAM212 cells treated with TNFα, IL-10, and LPS (**Fig 6B,** positive control, *lanes 3 and 4*) but was not detected in untreated cells (**Fig 6B,** *lanes 1 and 2*). S100A8 was detected in cells treated with UVA (**Fig 6B,** *lanes 5 and 6*), whereas incubation of PAM212 cells with SOD and catalase during and again after UVA irradiation (10 kJ per m^2) for 24 h downregulated S100A8 protein levels (**Fig 6B,** *lanes 7 and 8*). No differences in protein expression were observed between UVA-irradiated PAM212 cells (**Fig 6B,** *lanes 5 and 6*) and PAM212 cells incubated with SOD and catalase after irradiation of 10 kJ per m^2 UVA (**Fig 6B,** *lanes 9 and 10*). Increased S100A8 production by UVA-irradiated PAM212 cells has also been verified using an ELISA specific for mouse S100A8 (Hu *et al*, 1996). Twenty-four hours after exposure of PAM212 cells to 10 kJ per m^2 UVA, extracellular (culture
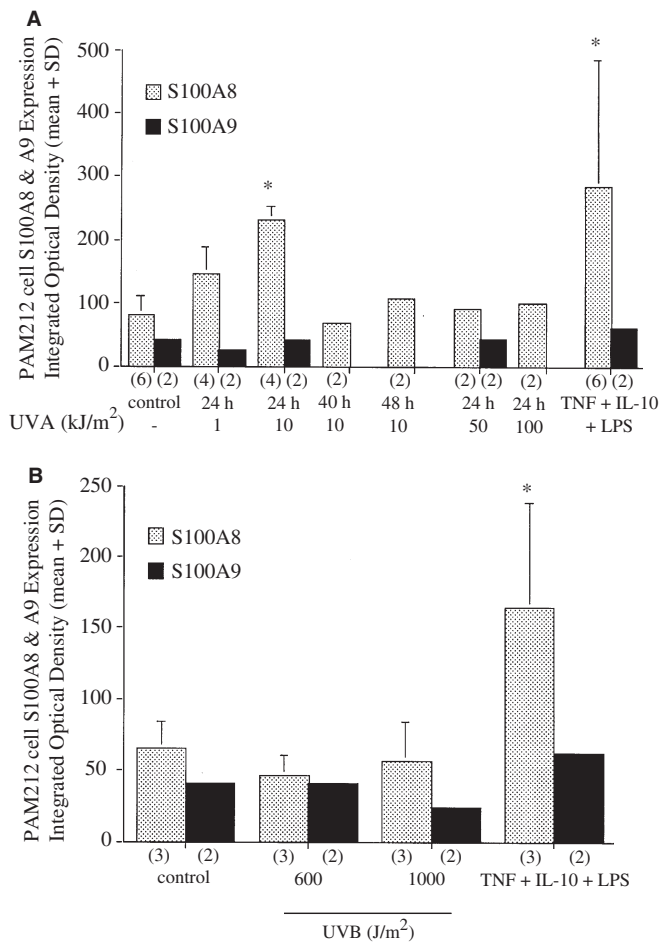
Figure 3. S100A8 and A9 in PAM212 cells after UVA and UVB irradiation. (*A*) PAM212 cells were irradiated with 1, 10, 50, or 100 kJ per m^2 UVA or 600 and 1000 J per m^2 UVB or stimulated with TNF (10 ng/mL), IL-10 (10 ng/mL), and LPS (1 μg/mL). Twenty-four hours after 1, 10, 50, or 100 kJ per m^2 UVA or 24, 40 and 48 h after 10 kJ per m^2 UVA (S100A8), the mean + SD IOD for S100A8 or S100A9 expression on 100 consecutive fields was recorded (n = 2–6 experiments per group). (*B*) PAM212 cells irradiated with 600 or 1000 J per m^2 UVB or stimulated with TNF (10 ng/mL), IL-10 (10 ng/mL), and LPS (1 μg/mL). The mean IOD for S100A8 or S100A9 expression 24 h after UVB-irradiation on 100 consecutive fields was recorded (n = 3 experiments per group for S100A8; and n = 2 experiments per group for S100A9). Data expressed as mean + SD for groups with n > 2. An *asterisk* indicates significant induction of S100A8 expression compared with that expressed by control (untreated) cells (p < 0.05).

supernatant) and cell-associated (cell lysate) levels of S100A8 were increased to 0.7 and 4.6 nM, respectively.

DISCUSSION

A variety of cell types including keratinocytes express S100A8 and A9 to fulfill both intra- and extracellular functions (reviewed by Passey *et al*, 1999; Donato, 2001). Depending on the stimulus, when expressed in the cytosol these proteins may aid cytoskeletal reorganization, migration (Harrison *et al*, 1999; Manitz *et al*, 2003), signal transduction (Kerkhoff *et al*, 1999a), and modulation of intracellular calcium (Schäfer and Heizmann, 1996). Alternatively, when S100A8 and A9 are released into the extracellular space following cell activation via a tubulin-dependent pathway, they affect local inflammation, leukocyte recruitment, and transendothelial migration (Rammes *et al*, 1997; Kerkhoff *et al*, 1999b;

Passey *et al*, 1999). Nevertheless, recent studies indicate that S100A8 is a potent scavenger of hypochlorous acid and other oxidants such as H_2O_2 (Harrison *et al*, 1999; Raftery *et al*, 2001) and upregulation of the murine A8 gene by anti-inflammatory mediators such as IL-10 and prostaglandin E_2 (Xu *et al*, 2001) suggest a protective role. In this study, we demonstrate that S100A8, but not S100A9, is upregulated in keratinocytes in response to UVA irradiation.

The monomeric form of S100A8 (10 kDa) was strongly induced in keratinocytes. Although western blotting did not indicate generation of S100A8 homodimers intracellularly, mild oxidation of Cys_{41} in S100A8 to forms containing sufenic and sulfonic acids, and possibly intermolecular sulfinamide bonds (Raftery *et al*, 2001; Raftery and Geczy, 2002) would not be obvious by this methodology. More precise analysis using mass spectroscopy of derived peptides from isolated protein would be required. SOD and catalase may inhibit UVA-induced S100A8 by scavenging ROI generated at the cell membrane, because these enzymes do not readily pass into the cell. Elevated levels of UVA-generated ROI in the cell membrane may induce S100A8 via calcium-dependent protein kinase C activity (Matsui *et al*, 1994). Our data suggest an intracellular role of S100A8 although this requires clarification. Further studies analyzing potential oxidation products of S100A8 in PAM212 cells and culture supernatants are currently being undertaken. Experiments in other cell systems indicate that overexpression of S100A8 can be toxic (Hsu and Geczy, unpublished observations) and suppression of S100A8 induction by antisense RNA may provide more understanding of its function in conditions of oxidative stress.

Downregulation of keratinocyte S100A8 by the topical application of a superoxide enzyme-mimicking agent, Tempol, to UVA-irradiated mouse skin or incubation *in vitro* with superoxide enzymes during UVA irradiation (**Figs 4–6**) support a role for S100A8 in oxidative stress responses. UVA-induced keratinocyte S100A8 in mouse skin increased concentration-dependently to a single exposure of UVA, and particularly to multiple exposures, possibly in the latter in response to cumulative ROI formation. The concentrations of UVA (10 × 100 to 3 × 100 kJ per m^2) used in our study are physiologically relevant, approximating to 0.1 to 1.5 of the minimal erythma concentration of sunlight in humans (Diffey, 1992).

Another S100 protein may also protect normal human keratinocytes against oxidative stress. S100A2 is p53-inducible, markedly increased in ERB-driven reactive epidermal hyperplasia, and, like S100A8 (Harrison *et al*, 1999), readily forms intermolecular disulfide bonds in response to H_2O_2 (Deshpande *et al*, 2000; Zhang *et al*, 2002). Treatment of normal and immortalized keratinocytes with H_2O_2 and the calcium ionophore A23187 reduced nuclear S100A2 staining but concomitantly increased cytoplasmic S100A2 disulfide dimers. We showed high levels of S100A8 in the nucleus of PAM212 cells and cytoplasmic levels appeared to increase in response to H_2O_2 and UVA irradiation. Translocation of S100A8 and A9 from the cytosol to the plasma membrane in leukocytes (Kerkhoff *et al*, 1999b; Donato, 2001), and to keratin intermediate filaments in epithelial cells (Mischke *et al*, 1996), occurs in response to elevated $[Ca^{2+}]_i$ concentrations. High cytosolic Ca^{2+} levels lead to reorganization of the epithelial cytofilament network (Kelly *et al*, 1991; Goebeler *et al*, 1995). In hyperproliferative dermatoses, such as human psoriasis, there is an association between S100A8 and S100A9 expression and restructuring of keratin filaments (Saintigny *et al*, 1992). In response to UVA-induced oxidative stress, S100A8 may be involved in the reorganization of keratin intermediate filaments and thereby regulate keratinocyte proliferation and differentiation. Topical application of Tempol (0.04 mg/100 μL/mouse) significantly reduced UVA-induced hyperkeratosis and S100A8 expression (**Fig 4F**). A possible functional role for S100A8 in keratinocyte differentiation is supported by the colocalization of the S100A8 gene with other epidermal structural genes (Mischke *et al*, 1996; Schäfer and Heizmann, 1996; Krieg *et al*, 1997).

Figure 4. Topical application of Tempol reduces S100A8 expression 24 h after UVA irradiation of BALB/c mice. (*A*) Diluent-treated nonirradiated skin; (*B*) 100 kJ per m^2 UVA; (*C*) diluent (50 μL) before and after 100 kJ per m^2 UVA; (*D*) Tempol (0.07 mg/50 μL) before and after 100 kJ per m^2 UVA; (*E*) Tempol (0.14 mg/50 μL) before and after 100 kJ per m^2 UVA; (*F*) Tempol (0.2 mg/50 μL) before and after 100 kJ per m^2 UVA.
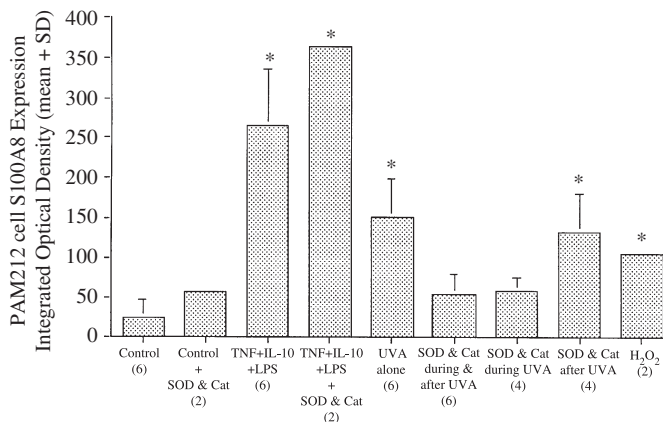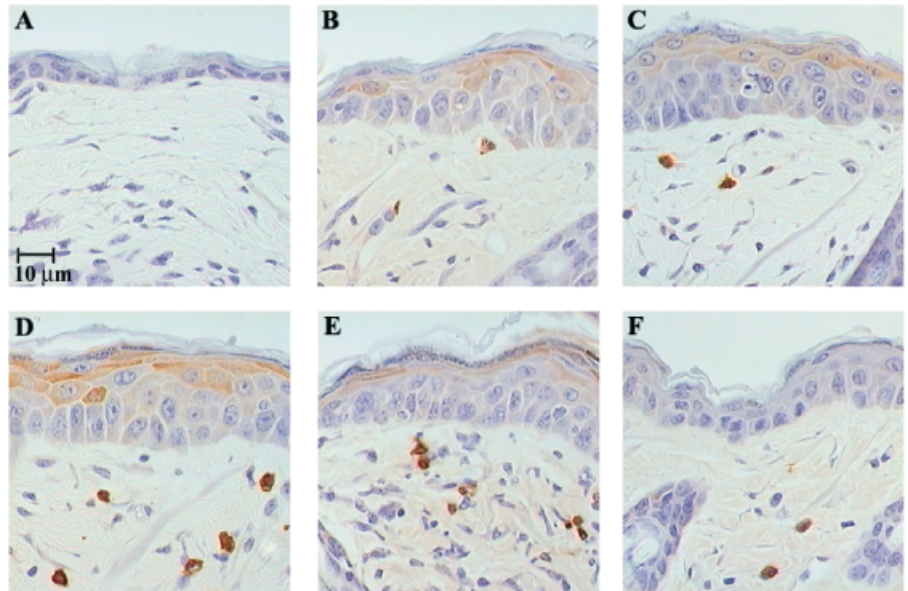

Figure 5. SOD and catalase during UVA irradiation abrogate S100A8 induction in PAM212 cells. PAM212 cells were stimulated with TNF (10 ng/mL), IL-10 (10 ng/mL), and LPS (1 μg/mL) or H$_2$O$_2$ or irradiated with 10 kJ per m^2 UVA and incubated with SOD (30 μg/mL) and catalase (2 μg/mL) during and again after irradiation, only during irradiation, or only after irradiation. The mean IOD for S100A8 expression 24 h after UVA irradiation on 100 consecutive fields was recorded (n = 4–6 experiments per group). Data are expressed as means + SD. An *asterisk* indicates significant induction of S100A8 expression compared with control (untreated) cells (p < 0.05).

Figure 6. Effect of SOD and catalase on the induction of S100A8 mRNA and protein in PAM212 cells in response to UVA irradiation. (*A*) S100A8 mRNA expression by PAM212 cells untreated (*lane 1*); stimulated with TNF (10 ng/mL), IL-10 (10 ng/mL), and LPS (1 μg/mL) (*lane 2*); irradiated with 10 kJ per m^2 UVA (*lane 3*); or incubated with SOD (30 μg/mL) and catalase (2 μg/mL) during and again after 10 kJ per m^2 UVA irradiation (*lane 4*) or only after UVA irradiation (*lane 5*). The experiment presented in this figure was repeated with very similar results. (*B*) Western blot analysis of S100A8 expression by PAM212 cells. Untreated (*lanes 1, 2*); stimulated with TNF (10 ng/mL), IL-10 (10 ng/mL), and LPS (1 μg/mL) (*lanes 3, 4*); irradiated with 10 kJ per m^2 UVA (*lanes 5, 6*); or incubated with SOD (30 μg/mL) and catalase (2 μg/mL) during and again after 10 kJ per m^2 UVA irradiation (*lanes 7, 8*) or only after UVA irradiation (*lanes 9, 10*). The experiment represented in this figure was repeated with very similar results.

In contrast to UVA, UVB irradiation of PAM212 cells did not induce S100A8 or S100A9 expression (**Fig 3*B***). Although UVB radiation causes some oxidative damage to cells, this is thought to be minor compared with UVA-mediated oxidative stress (Tyrrell, 1996) and strengthens our proposal that UVA induction of S100A8 is via a ROI-dependent mechanism. It is interesting that S100A9 was not upregulated with S100A8 in response to UVA, either *in vivo* or *in vitro* (**Fig 3*A***). Selective expression of monomeric S100A8 occurs in elicited murine macrophages stimulated with numerous pro- and anti-inflammatory stimulants (Hu *et al*, 1996; Xu and Geczy, 2000; Xu *et al*, 2001) and in microvascular endothelial cells activated with LPS or IL-1 (Yen *et al*, 1997), supporting the notion that coexpression with S100A9 is not essential for S100A8 function or secretion (Rammes *et al*, 1997). A few infiltrating S100A8-positive neutrophils and macrophages were evident in the dermis directly below keratinocytes
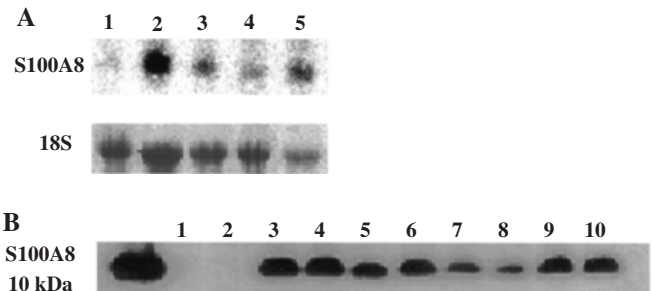
expressing S100A8 after UVA-irradiation suggesting limited secretion and that S100A8 was unlikely to be acting as a leukocyte chemoattractant as injection of low levels of the pure protein into mouse skin causes marked leukocyte infiltration (reviewed in Passey *et al*, 1999). Alternatively, secreted S100A8 may have been oxidized to an inactive form (Harrison *et al*, 1999; Raftery *et al*, 2001), although immunohistochemistry did not detect extracellular deposits in UVA-treated skin. S100A8 can be secreted and/or cell-associated. Preliminary results with PAM212 cells suggest that only about 15% of UVA-induced S100A8 is secreted. Thus, keratinocytes are similar to microvascular endothelial cells in which S100A8 is predominantly cell-associated (Yen *et al*, 1997).

In conclusion, S100A8, but not S100A9, is strongly induced in keratinocytes by UVA irradiation. Inhibition of S100A8 protein expression by application of Tempol *in vivo* and of mRNA

and protein in UVA-treated keratinocytes incubated with the superoxide-scavenging enzymes SOD and catalase *in vitro* strongly suggests that ROI mediate S100A8 induction in response to UV-induced oxidative stress. Nevertheless, whether this calcium-binding protein is functionally involved as a scavenger of oxygen radicals and/or is expressed along with other epidermal structural genes to aid cytoskeletal reorganization after UVA-mediated cellular changes is yet to be elucidated.

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