Protease-Activated Receptor 2, a Receptor Involved in Melanosome Transfer, is Upregulated in Human Skin by Ultraviolet Irradiation

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Previous studies have shown that the protease-activated receptor 2 is involved in skin pigmentation through increased phagocytosis of melanosomes by keratinocytes. Ultraviolet irradiation is a potent stimulus for melanosome transfer. We show that protease-activated receptor 2 expression in human skin is upregulated by ultraviolet irradiation. Subjects with skin type I, II, or III were exposed to two or three minimal erythema doses of irradiation from a solar simulator. Biopsies were taken from nonexposed and irradiated skin 24 and 96 h after irradiation and protease-activated receptor 2 expression was detected using immunohistochemical staining. In nonirradiated skin, protease-activated receptor 2 expression was confined to keratinocytes in the lower one-third of the epidermis. After ultraviolet irradiation protease-activated receptor 2 expression was observed in keratinocytes in the upper two-thirds of the epidermis or the entire epidermis at both time points studied. Subjects with skin type I showed delayed upregulation of protease-activated receptor 2 expression, however, compared with subjects with skin types II and III. Irradiated cultured human keratinocytes showed upregulation in protease-activated receptor 2 expression as determined by immunofluorescence microscopy and Western blotting. Cell culture supernatants from irradiated keratinocytes also exhibited a dose-dependent increase in protease-activated receptor-2 cleavage activity. These results suggest an important role for protease-activated receptor-2 in pigmentation *in vivo*. Differences in protease-activated receptor 2 regulation in type I skin compared with skin types II and III suggest a potential mechanism for differences in tanning in subjects with different skin types. ***Key words: keratinocytes/melanosome/protease-activated receptor-2 (PAR-2)/UV irradiation. J Invest Dermatol 117:1412–1420, 2001***

Protease-activated receptor-2 (PAR-2) is a member of a novel G-protein-coupled seven transmembrane receptor family (reviewed by Dery *et al*, 1998). These receptors are irreversibly activated through proteolytic cleavage of the amino terminus. Subsequent to proteolytic cleavage, the newly exposed NH_2 terminus acts as a tethered peptide ligand, which binds and activates the receptor. Four protease-activated receptors have been cloned to date (Vu *et al*, 1991; Nystedt *et al*, 1994; Ishihara *et al*, 1997; Xu *et al*, 1998) and are characterized by their sensitivity to activation by thrombin and trypsin. PAR-1 and PAR-3 are activated by thrombin (Vu *et al*, 1991; Ishihara *et al*, 1997), whereas PAR-4 is activated by both thrombin and trypsin (Xu *et al*, 1998). In HaCaT cells PAR-2 is activated by trypsin, mast cell tryptase, and factor VIIa or factor Xa (Kong *et al*, 1997; Smith-Swntosky *et al*, 1997; Camerer *et al*, 2000). In neuronal cells PAR-2 can be activated by the neuronal growth-associated protein B-50/GAP-43 (Hollenberg *et al*, 2000).

PAR-2 is widely distributed in the body including gastrointestinal tract, pancreas, kidney, liver, lung, cardiovascular system, ovary, eye, and brain (Nystedt *et al*, 1995a; 1995b; 1994; Bohm *et al*, 1996a; D'Andrea *et al*, 1998). It is expressed by epithelial cells (keratinocytes, epithelial-derived tumor cells), endothelial cells, enterocytes, smooth muscle cells, neutrophils, neurons, and T cell lines (Mari *et al*, 1996). The endogenous activator for PAR-2 in the epidermis remains to be defined, but because tissue distribution of PAR-2 expression is not limited to the gastrointestinal system where luminal trypsin is present, it has been suggested that trypsin-like enzymes such as mast cell tryptase may activate PAR-2 (Molino *et al*, 1997). Mast cell tryptase has been shown to cleave and activate PAR-2 in cells that express the receptor (Mari *et al*, 1996; Corvera *et al*, 1997; Molino *et al*, 1997), including keratinocytes (Santulli *et al*, 1995; Molino *et al*, 1997; Schechter *et al*, 1998; Steinhoff *et al*, 1999). PAR-2 is also activated by a synthetic peptide SLIGRL, which corresponds to the cleaved N terminus, independent of receptor cleavage (Steinhoff *et al*, 1999). The mouse peptide (SLIGRL) is equipotent to the human peptide (SLIGKV) in activating the human PAR-2 (Nystedt *et al*, 1995b; Bohm *et al*, 1996a). In human keratinocytes, activation of PAR-2 results in Ca^{2+} mobilization (Bohm *et al*, 1997).

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Abbreviation: PAR-2, protease-activated receptor 2; PCNA, proliferating cell nuclear antigen.

PAR-2 is involved in a broad spectrum of physiologic processes, including growth and development, mitogenesis, injury responses, and cutaneous pigmentation. PAR-2 activation stimulates MAP kinase in some cell types, resulting in proliferation, whereas in keratinocytes PAR-2 inhibits growth and differentiation (Derian *et al*, 1997). Human dermal fibroblasts, which express PAR-2 or a PAR-2-like receptor, show concentration-dependent increases in proliferation in response to mast cell tryptase (Akers *et al*, 2000). In enterocytes PAR-2 activation induces arachidonic acid release, eicosanoid generation, and cyclooxygenase-2 expression (Kong *et al*, 1997). In neutrophils, bacterial proteinases activate PAR-2, which stimulates Ca^{2+} influx and increased expression of the CD11b adhesion molecule (Vu *et al*, 1991). In rat heart, PAR-2 activation protects against reperfusion injury (Napoli *et al*, 1999). This effect is coupled to elevation of PAR-2 and tumor necrosis factor α (TNF-α) expression. Studies have shown that PAR-2 mRNA is upregulated by TNF-α and interleukin-1 (IL-1) in vascular endothelial cells (Nystedt *et al*, 1996). PAR-2 deficient mice show delayed onset of leukocyte rolling in response to trauma due to absence of PAR-2-induced p-selectin expression (Lindner *et al*, 2000). In keratinocytes, PAR-2 activation stimulates secretion of IL-6 and granulocyte macrophage colony stimulating factor (GMCSF; Wakita *et al*, 1997). Furthermore, in several recent studies Seiberg *et al* (2000a) and Sharlow *et al* (2000) demonstrated that, in keratinocyte–melanocyte cocultures, PAR-2 activation induced melanosome transfer through increased keratinocytic phagocytosis of melanosomes. In addition, the same group provided evidence that PAR-2 affects pigmentation via modulation of melanosome uptake *in vivo*. They showed that serine protease inhibitors that interfere with PAR-2 activation induced a dose-dependent depigmentation of the skin of dark-skinned Yucatan swine. The inhibitors work by inhibiting melanosome transfer and distribution, which leads to skin lightening *in vivo* (Seiberg *et al*, 2000b). Moreover, inhibition of PAR-2 activation prevented ultraviolet B (UVB) induced pigmentation both *in vitro* and *in vivo* (Seiberg *et al*, 2000b; Paine *et al*, 2001). Interestingly, treatment of epidermal equivalents with trypsin, a serine protease that activates PAR-2, induced pigmentation to the same level as observed with UV irradiation (Seiberg *et al*, 2000b). These data suggest an important role for PAR-2 as a cytokine-inducible inflammatory mediator in the skin and as a protein involved in epidermal pigmentation.

UV irradiation of keratinocytes stimulates pigmentation in human skin through multiple mechanisms including upregulation of key melanogenic enzymes, DNA photodamage, which directly results in melanogenesis, increased melanosome transfer to keratinocytes, and increased melanocyte dendricity (Pathak *et al*, 1965; 1978; Sturm, 1998; Gilchrest and Eller, 1999; Park and Gilchrest, 1999). A recent report suggests that UV radiation (UVR) may regulate skin pigmentation through downregulation of neprilysin, a neutral endopeptidase that degrades melanocortin (Aberdam *et al*, 2000). It is well known that individuals with skin type I tan poorly compared with individuals with skin types II and III. Because previous studies have shown that activation of PAR-2 induces melanosome transfer *in vivo*, because inhibition of PAR-2 activation prevents UVB-induced pigmentation, and because UVR is a potent stimulus for melanosome transfer *in vivo*, we hypothesized that UVR might upregulate PAR-2 expression *in vivo*. Furthermore, we hypothesized that differences in regulation of PAR-2 in response to UVR may contribute to differences in tanning in individuals with different skin types. We examined the effect of UVR on the expression of PAR-2 in epidermis in subjects with type I, II, or III skin *in vivo*. We show that UVR elicits an increase in PAR-2 protein expression in human skin, suggesting a role for PAR-2 in melanosome transfer *in vivo* in response to UVR. We also demonstrate differences in PAR-2 regulation among subjects with type I skin compared with types II and III skin. Irradiation of cultured human keratinocytes resulted in upregulation of PAR-2 protein by immunofluorescence microscopy and Western blotting, and induced a dose-dependent increase in secreted proteases with PAR-2 cleavage activity. These results support a mechanism for UVR-induced PAR-2 upregulation and activation in skin and suggest a potential mechanism for skin-type-related responses to UVR *in vivo*.

MATERIALS AND METHODS

Subject For analysis of the effects of UVR on PAR-2 expression, 36 subjects were used. The subjects ranged in age from 22 to 67 y and there were 12 men and 24 women. Each subject was evaluated by a dermatologist (AP) and subjects were divided into three groups based on skin type according to the Fitzpatrick standard criteria (Fitzpatrick, 1988). Twelve subjects for each skin type were examined for the work presented here. None of the female participants was pregnant or took oral contraceptives. To determine the minimum erythema dose (MED) 12 doses of UVA/UVB irradiation (290–400 nm) from 20 mJ to 300 mJ (the dose range was adjusted for the subject's skin type) were administered in graded doses to buttock skin using a 1000 W xenon arc lamp solar simulator. The solar simulator was equipped with a UVC WG320 and visible cutoff filter UG11, beam turner, and cooling fan. The output was monitored by an IL 1700 radiometer and SED400 sensor probe purchased from International Light (Newburyport, MA). Twenty-four hours after administration of light, biopsies of control and 3 MED irradiated skin were obtained. Ninety-six hours following administration of UVR, a second control biopsy and a biopsy from 2 MED irradiated skin were obtained. The nonirradiated control skin sites were 3 cm from the irradiated skin sites. The tissues were fixed immediately in 10% formalin and the four biopsies from each patient were embedded into the same paraffin block. In the second arm, subjects received either placebo or Celecoxib and 10 d later irradiation and measurement of erythema was repeated as above. Data from the second arm of the study are not analyzed here.

Antibodies and reagents Rabbit PAR-2 polyclonal antibody was raised against human PAR-2 peptide fragment (^{37}SLGKVDGTSH-VTGKG^{47}V) by Research Genetics (Huntville, AL). This sequence is present in both cleaved and uncleaved receptors and therefore the antibody recognizes both active and inactive PAR-2. Monoclonal antibody to human PAR-2 raised in goat was purchased from Research Diagnostics (Flanders, NJ). Mouse monoclonal antibodies to proliferating cell nuclear antigen (PCNA) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA; clone PC10; Ann Arbor, MI). Ki-67 (clone MIB-1) mouse monoclonal antibody was purchased from Biogenex (San Ramon, CA). The Ki-67 antigen is a nuclear nonhistone protein expressed by cells in the proliferative phases of the cell cycle. p53 mouse monoclonal antibody (clone 1801) was purchased from Oncogene Science (Cambridge, MA). Polyclonal antibodies to trypsin were purchased from Biogenesis (Kingston, NH). Tryspin, used as a positive control, was purchased from Gibco-BRL (Grand Island, NY). Keratinocyte serum free medium (SFM) was obtained from Gibco-BRL. Biotinylated goat-antirabbit IgG and rabbit-antigoat IgG were obtained from Vector Laboratories (Burlingame, CA) and permanox Laboratory-Tek II chamber slides were obtained from Nalge Nunc International (Naperville, IL). Texas Red-conjugated goat antirabbit antibodies were purchased from Molecular Probes (Eugene, OR). Horseradish peroxidase conjugated antibodies against goat immunoglobulin were purchased from Vector Laboratories. Normal goat serum was purchased from Sigma (St. Louis, MO). Streptavidin horseradish peroxidase was obtained from Jackson Laboratories (Westgrove, PA). Aminoethylcarbizol chromogen was obtained from Soy Tek (Utah). Centricon-10 filters were purchased from Amicon (Beverly, MA).

Immunohistochemistry staining For PAR-2 staining, paraffin blocks were sectioned at 4 μm and placed on slides coated with poly L-lysine (Sigma). Antigen unmasking with heat retrieval in citrate buffer (pH 6.5) was accomplished by placing the slides in a microwave pressure cooker (1500 W at power level 6) for 30 min. Normal goat serum (10% in Tris-buffered saline) was used to block the nonspecific antibody binding. PAR-2 polyclonal antibody (1:5000) in antibody stabilizer (Dako, Carpinteria, CA) was applied at 4°C overnight. Secondary antibodies were incubated at room temperature for 30 min. Streptavidin horseradish peroxidase was applied for 30 min at room temperature. The reaction was developed with aminoethylcarbizol and the slides were counterstained in Mayer's hematoxylin. Negative controls consisted of preimmune serum from the same rabbit that gave rise to the primary antibody.

For Ki-67 staining, standard immunohistochemistry procedures were followed as described previously (Manne *et al*, 1997). Paraffin blocks were

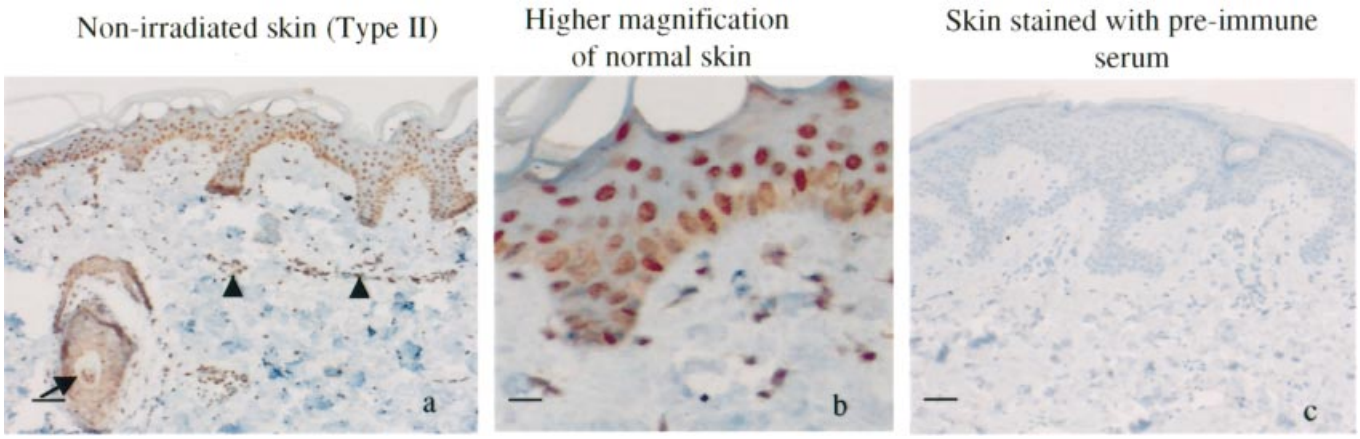


Figure 1. Distribution of PAR-2 in normal skin. (*a*) Biopsy from a subject with type II skin stained with polyclonal PAR-2 antibodies shows strong staining of the basal keratinocytes. Hair follicle keratinocytes are also stained (*arrow*) as are endothelial cells (*arrowhead*). Scattered dermal dendritic cells are also stained. (*b*) Higher magnification shows details of epidermal staining. Staining is predominantly cytoplasmic and is localized to the basal layer of the epidermis. (*c*) Biopsy from the same patient stained with preimmune serum. *Scale bars*: (*a*, *c*) 120 μm, (*b*) 70 μm.

sectioned at 4 μm and attached to SuperFrost/Plus slides (Fisher Scientific, Norcross, GA) by heating at 58°C for 1 h. After deparaffinization and rehydration the sections were transferred to a Tris-buffer bath (0.05 M Tris base, 0.15 M NaCl, 0.0002% Triton X-100, pH 7.6). High temperature antigen recovery was followed as described previously (Grizzle *et al*, 1998). Each section was treated with hydrogen peroxide to quench endogenous peroxidase activity and incubated with normal goat serum at room temperature for 1 h in order to reduce nonspecific immunostaining. Ki-67 primary antibodies were added at dilutions of 1:50. Staining for PCNA and p53 was identical to that described above for Ki-67 with the exception that the tissue was not heated. PCNA and p53 antibodies were used at a dilution of 0.03 μg per ml and 0.5 μg per ml, respectively. Negative controls remained in the goat serum (3%) at room temperature for 1 h. The primary antibodies were detected using a multispecies detection system from Signet Laboratories (Dedham, MA) according to the manufacturer's instruction with diaminobenzidine tetrachloride as chromogen. Each section was counterstained using hematoxylin.

Statistical analysis for immunocytochemical data For analysis of PAR-2 expression, the slides were examined by two pathologists (ACD and GS) independently and PAR-2 staining was graded as follows: level I, expression in the basal keratinocytes and first layer above the basal layer; level II, expression in the basal and suprabasal keratinocytes encompassing two-thirds of the epidermis; level III, staining throughout the entire epidermis including the granular layer. Intensity of staining was graded on a scale of 0 (no staining) to 4^+ (strongest intensity). Therefore, for each tissue section, two data points were obtained: level of staining (I, II, III) and intensity of staining ($0–4^+$). In the case of nonconcordance in grading of a biopsy, the biopsy was reviewed and a grade was assigned based on discussion between the two observers. Concordance in grading of the cases was 90% between the two observers. The data were analyzed with Wilcoxon signed rank and Kruskal–Wallis χ^2 tests. $p < 0.05$ was considered as statistically significant. For analysis of PCNA, p53, and Ki-67 immunostaining, slides were scored by a minimum of two evaluators (CRB, WB, or WEG). The cells were classified with respect to the intensity of immunostaining for each antigen and with respect to the percentage of cells stained at each level of intensity. Each evaluator estimated the intensity of the immunostaining on a scale of 0 (no staining) to 4^+ (strongest intensity). In addition, each evaluator estimated the absolute percentage of cells stained at each level of intensity. The percentage of cells at each level of intensity was multiplied by the appropriate intensity score to obtain a weighted average of the intensity score. The data were analyzed using the Wilcoxon signed rank test for paired comparisons. $p < 0.05$ was considered as statistically significant.

Cell culture, UV irradiation, immunofluorescence microscopy, and Western blotting Neonatal foreskins were obtained according to the University of Rochester Research Subjects Review Board and were the source of human keratinocytes. Epidermal suspensions were cultured in keratinocyte SFM as previously described (Scott and Haake, 1991). For immunofluorescence microscopy, keratinocytes were subcultured onto permanox slides (10^4 cells per well) and were irradiated 24 h later. The source of UVR was a 1000 W Oriel Xenon Arc Solar Simulator with UVC WG320 and Visible Cutoff Filter UG11, beam turner, and cooling fan. This produces electromagnetic irradiation from 295 nm (UVB) to 400 nm (UVA) and produces 3.75×10^{-3} W per cm^2. The output was monitored by an IL 1700 radiometer and SED400 sensor probe purchased from International Light. During UVR, medium was replaced with phosphate-buffered saline (PBS) containing Ca^{2+} and Mg^{2+}. The PBS was then replaced with culture medium and the cells were allowed to grow at 37°C with 5% CO_2 for 24 h before staining for PAR-2. The slides were fixed in 4% formalin in PBS for 15 min at room temperature and incubated with 10% normal goat serum to block nonspecific binding. Polyclonal PAR-2 antibody (1:500) was added to the slides and allowed to incubate overnight at 4°C. After washing with PBS, Texas Red-conjugated goat antirabbit antibody was placed on the slides for 60 min at room temperature, followed by three washes with PBS. Slides were cover slipped with DAPI mounting media (Vector Laboratories) and antifade reagent (Molecular Probes). The slides were then examined using a Nikon Eclipse 800 immunofluorescence microscope and digital photographs were taken with a SpotTM Cooled color digital camera (Diagnostic Instruments, Sterling Heights, MI). Negative controls consisted of cells incubated with preimmune serum instead of primary antibodies. Experiments on three separate foreskin cultures were performed.

For Western blotting, equal numbers of keratinocytes were cultured in 60 mm dishes until 80% confluent in SFM. Cells were irradiated with 1 J per cm^2, 2 J per cm^2, or 4 J per cm^2, or sham irradiated with a solar simulator. In preliminary experiments, trypan blue exclusion studies were performed on irradiated cells to determine if irradiation resulted in toxicity. There was no evident cell death associated with any of the doses of UVR used. Twenty-four hours later culture supernatant was analyzed for protease and PAR-2 cleavage activity (see below). The cells were lyzed in RIPA buffer [150 mM NaCl, 1% Nonidet P-40, 0.5% deoxycholate (DOC), 0.1% sodium dodecyl sulfate (SDS), 50 mM Tris-HCl] with protease inhibitors (Boehringer Mannheim, Germany). Protein was quantitated using bovine serum albumen as standard (Bio-Rad Laboratories, Hercules, CA) and 50 μg was resolved on precast 10% SDS polyacrylamide gels (Jule, New Haven, CT). Following transfer to nitrocellulose membranes (Bio-Rad) and blocking overnight with 10% nonfat milk in Tris-buffered saline membranes were incubated with monoclonal antibodies to PAR-2 (1/5000) for 2 h at room temperature. Following washes, the reaction product was detected with an enhanced chemiluminescence reaction (Amersham Life Sciences) by exposing the membranes to Kodak XAR film (Rochester, NY). Assessment of lane loading was accomplished by staining the gel with Coomasie Brilliant Blue following transfer. Experiments on three separate foreskin cultures were performed.

Protease and PAR-2 cleavage assay Total protease activity was measured using the EnzChek protease assay kit, following the manufacturer's instructions (Molecular Probes). Samples were incubated with BODIPY fluorescent casein substrate at room temperature for 1 h

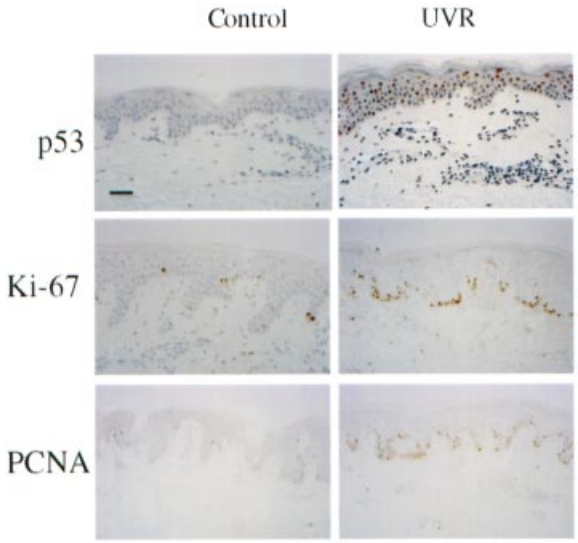


Figure 2. UVR upregulates key biomarkers in subjects' biopsies. To determine whether our subject population responded as expected to UVR, p53, Ki-67, and PCNA staining were assessed on all biopsies. Shown are representative examples of staining for these three markers in control and UVR-treated skin. The subject population showed upregulation of p53, Ki-67, and PCNA in a statistically significant manner. p53 is essentially absent in non-UVR-treated skin (*a*) but is induced in the nucleus at all cell layers following UVR (*b*, 2 MED/96 h). Ki-67 and PCNA showed scattered positive cells primarily in the basal layer of control skin (*c*, *e*) and strong induction in the basal layer following UVR (*d*, *f*, 2 MED/96 h). *Scale bar*: 120 μm.

and fluorescence was measured (excitation 485/emission 530) on a SpectraMax Gemini microtiter plate reader (Molecular Devices Corporation, Sunnyvale, CA) using Softmax Pro 3.0 software (Molecular Devices Corporation). Each experiment was performed in six replicates. The percent cleavage of the substrate by test samples was calculated and graphed using Microsoft Excel.

A synthetic peptide comprising the cleavage site of the human PAR-2 (SKGRSLIGK) (Lourbakos *et al*, 1998) was labeled with the fluorophore pair Edans/Dabsyl (Advanced Bioconcept, Montreal, Canada) and was used as a substrate for PAR-2 serine protease activators. Cleavage of this peptide with PAR-2 activators such as trypsin could be detected fluorescently (excitation 335 nm/emission 515 nm). 100 μM peptide were incubated with test samples for 1 h at room temperature, protected from light. Fluorescence was measured on SpectraMax Gemini microtiter plate reader using Softmax Pro 3.0 software. Each experiment was performed in six replicates. The percent of PAR-2 cleavage was calculated and graphed using Microsoft Excel.

RESULTS

Localization of PAR-2 in normal human skin In normal human skin PAR-2 expression was confined to keratinocytes in the basal layer and just above the basal layer in a cytoplasmic and peripheral membrane pattern. Keratinocytes above this layer, as well as in the granular layer and corneal layer, were devoid of PAR-2 expression (**Fig 1*a*, *b***). Nuclear staining was observed scattered throughout the epidermis within keratinocytes and in follicular root sheath keratinocytes and was absent in sections stained with preimmune serum (**Fig 1*c***). Approximately 50% of keratinocyte nuclei were labeled. To determine if the nuclear staining observed was due to nonspecific antigen unmasking from the antigen retrieval process, sections were stained with polyclonal PAR-2 antibody with and without antigen retrieval. In the absence of antigen retrieval, cytoplasmic and nuclear staining were retained but both were diminished in intensity (data not shown). PAR-2 was also expressed in smooth muscles of blood vessels and pili erector apparatus, endothelial cells, eccrine sweat glands, the basal layer of sebaceous glands, and fibroblasts within the dermis. Melanocytes did not express PAR-2 and there was no detectable difference in PAR-2 distribution or staining intensity among the three skin types (data not shown). Biopsies stained with preimmune serum showed no staining (**Fig 1*c***).

UVR upregulates expression of PAR-2 in human skin Previous studies have shown that UVR induces the expression of multiple different proteins in human skin. Of these, p53, PCNA, and Ki-67 have been shown to be consistently induced in skin *in vivo* in response to UVR (Hall *et al*, 1993; Einspahr *et al*, 1996; Van der Vleuten *et al*, 1996). As a control for UVR, we examined the expression of p53, PCNA, and Ki-67 in irradiated subjects (**Fig 2**). Ki-67 was induced in the nuclei of cells in all layers of the epidermis in a statistically significant way in every case studied 96 h postirradiation ($p < 0.05$). p53 was induced in the nuclei of all cells in all layers of the epidermis at both doses of light and time points in a statistically significant way ($p < 0.05$). PCNA was induced in the nuclei of all cells in all layers of the skin 96 h following irradiation in a statistically significant way ($p < 0.05$). These data confirm the physiologic effect of UVR in the study subjects. Representative examples of data showing the effect of UVR on PAR-2 distribution for each skin type are demonstrated in **Fig 3(*a*)**. The data are summarized in **Table I**.

The primary effect of UVR on PAR-2 expression consisted of *de novo* expression of PAR-2 in keratinocytes in the middle and upper layers of the epidermis (**Fig 3*a***). In nonirradiated skin, PAR-2 is expressed only in the basal and suprabasal keratinocytes in a cytoplasmic and membranous pattern. Twenty-four hours following UVR with 3 MED, and 96 h following 2 MED, PAR-2 expression is distributed throughout the entire epidermis. Skin types II and III showed statistically significant changes in PAR-2 expression following UVR at both doses and time points, but subjects with skin type I only showed statistically significant changes in PAR-2 expression 96 h post-UVR (2 MED; **Table I**). Whereas subjects with type I skin responded to UVR 24 h after a dose of 3 MED with *de novo* expression of PAR-2 in keratinocytes above the basal layer, these data did not reach statistical significance ($p > 0.05$). **Figure 3(*b*)** shows a representative example of a subject with skin type I who showed upregulation of PAR-2 96 h following 2 MED UVR but failed to show upregulation of PAR-2 24 h following 3 MED UVR. Staining intensities for PAR-2 were also increased following exposure to UVR in most cases, although this did not reach statistical significance ($p > 0.05$, data not shown).

UVR upregulates PAR-2 protein expression in cultured human keratinocytes To determine if UVR regulates PAR-2 expression in cultured human keratinocytes *in vitro*, cultured keratinocytes were irradiated with a solar simulator at a dose of 2 J per cm^2, and PAR-2 expression was determined by immunofluorescence microscopy 24 h later. Keratinocytes showed uniform PAR-2 cytoplasmic staining in the absence of UVR (**Fig 4**). Unstained cells were identified as melanocytes by their characteristic dendritic morphology. PAR-2 immunoreactivity in the perinuclear area (presumed Golgi apparatus) was also noted. UVR induced a subjective increase in intensity in PAR-2 expression in cultured keratinocytes (**Fig 4*b***), 2-fold compared with nonirradiated cells (**Fig 4*a***). Cells stained with preimmune serum showed weak or no staining (**Fig 4*c***). By Western blotting, PAR-2 expression was increased in irradiated keratinocytes compared with nonirradiated controls (**Fig 5**).

Sham-irradiated cells showed a faint immunoreactive band of ≈55 kDa for PAR-2. In contrast, cells irradiated with 1 J per cm^2, 2 J per cm^2, and 4 J per cm^2 displayed a strong immunoreactive band of ≈55 kDa for PAR-2, which was clearly increased compared with control cells. Coomassie stain of the gel confirmed equal loading of the lanes.

Proteases with PAR-2 cleavage activity are induced in UVR-treated keratinocytes To determine if UVR induces the release of PAR-2-activating proteases from cultured keratinocytes, cells were irradiated with one of three different

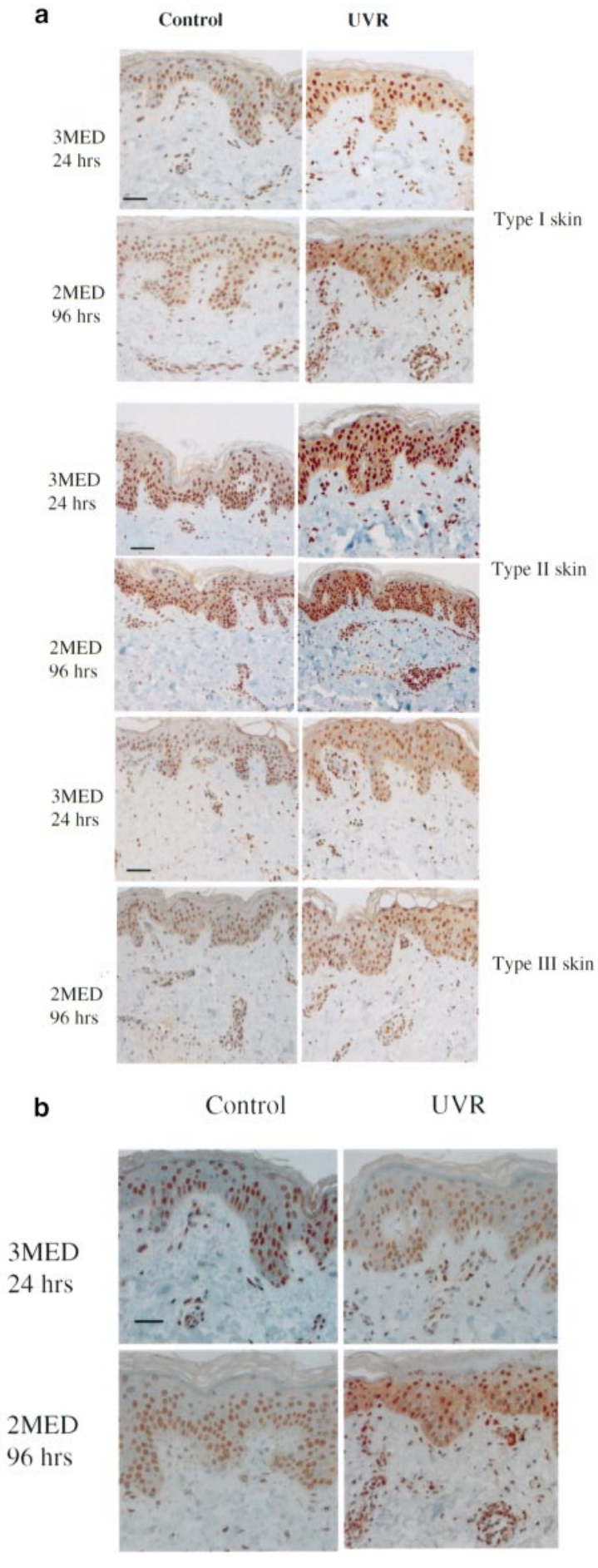


Figure 3. UVR upregulates PAR-2 expression *in vivo*. (*a*) Representative examples of control and UVR-treated skin stained for PAR-2 for each skin type and UVR dose are shown. Controls (non-UVR) show predominantly basal distribution of PAR-2 whereas postirradiation PAR-2 immunoreactivity is present throughout the epidermis. (*b*) Representative example of a subject with type I skin who demonstrated upregulation of PAR-2 96 h following 3 MED UVR, but failed to show upregulation of PAR-2 24 h following 2 MED UVR. *Scale bars*: (*a*) 120 μm, (*b*) 40 μm.

Table I. PAR-2 expression is upregulated in human skin epidermis after UVR[a]

	3 MED/24 h			2 MED/96 h		
Skin type (*N*)	I (12)	II (12)	III (12)	I (12)	II[b] (12)	III (12)
Increased	6	**8**[c]	**8**	**10**	**7**	**11**
Unchanged	6	4	4	2	3	1

[a]The data are presented as changes in PAR-2 distribution in UVR skin compared with nonirradiated skin from the same patient taken at the same time. Therefore, cases in which *de novo* expression of PAR-2 into the upper layers of the epidermis was seen compared with control sections are designated as "increased". Similarly, if no change in expression of PAR-2 in response to UVR compared with control skin was observed this is represented as "unchanged".

[b]Two subjects in this group at this data point were uninterpretable due to technical problems.

[c]Bold, statistically significant ($p < 0.05$).

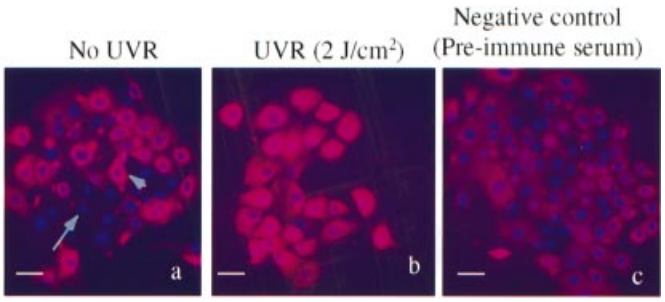


Figure 4. PAR-2 is upregulated by UVR in human keratinocytes *in vitro*. (*a*) Immunofluorescence microscopy of cultured human keratinocytes stained for PAR-2, counterstained with DAPI, and viewed with a filter to detect Texas Red and DAPI. Keratinocytes are uniformly stained with antibodies to PAR-2 with concentration around the Golgi apparatus (*arrowhead*). Unstained cells (*arrow*) were determined to be melanocytes by their characteristic dendritic morphology. (*b*) Twenty-four hours following UVR PAR-2 staining intensity has increased at least 2-fold. (*c*) Cells stained with preimmune serum show little or no staining. *Scale bars*: 40 μm.

doses of UVR and 24 h later the culture media were analyzed for protease activity and for PAR-2 cleavage activity. **Figure 6** summarizes the results for analysis of secreted protease activity and PAR-2 cleavage activity. There was a dose-dependent increase in secreted protease activity with increasing doses of irradiation (**Fig 6*a***). Nonirradiated keratinocytes exhibited a constitutive level of secreted protease activity, which showed a 50% increase after a dose of 4 J per cm^2. To determine if UVR-induced secreted proteases have PAR-2 cleavage activity, culture supernatants from irradiated cells were tested for their ability to cleave a synthetic peptide of human PAR-2 containing the PAR-2 cleavage site using a fluorometric assay (**Fig 6*b***). Non-irradiated keratinocytes showed constitutive levels of secreted PAR-2 protease cleavage activity, which showed a dose-dependent increase with increasing doses of UVR. Twenty-four hours following 1 J per cm^2 and 2 J per cm^2 there was a 41% and 42% increase, respectively, in PAR-2 peptide cleavage. Twenty-four hours following 4 J per cm^2 PAR-2 peptide cleavage rose to 82% over control levels. In an effort to determine the protease responsible for PAR-2 cleavage in UVR-treated keratinocytes, Western blotting for trypsin, a serine protease known to cleave PAR-2, was performed on cell lysates and supernatants from irradiated keratinocytes. Culture supernatants were concentrated using Centricon-10 filters and for each condition the entire supernatant was resolved by SDS polyacrylamide gel electrophoresis (SDS-PAGE) and probed for trypsin. No detectable band was identified in human keratinocyte cell lysates or culture supernatants by Western blotting with the antibody used in this study. A positive control (tryspin ethylenediamine tetraacetic acid) was strongly reactive (data not shown).

DISCUSSION

In this report we studied the response of 36 subjects with either type I, II, or III skin for the effect of UVR on PAR-2 expression. For each subject, a careful history of sun exposure, reaction to sun, and prior history of skin cancers was elicited, and control skin was compared with irradiated skin for expression and distribution of PAR-2, a novel receptor implicated in melanosome transfer and inflammation. In nonirradiated normal skin PAR-2 was weakly expressed in the basal and lower third of the epidermis, as well as in follicular root sheath cells, smooth muscle of erector pili and vessels, endothelial cells, eccrine sweat glands, the basal layer of sebaceous glands, and some dermal dendritic cells. These results are similar to most published observations (D'Andrea *et al*, 1998; Hou *et al*, 1998; Seiberg *et al*, 2000a), in which the predominant PAR-2 staining occurs in the lower epidermis. In contrast, Steinhoff *et al* (1999), using immunofluorescence microscopy, reported that PAR-2 predominantly stains the granular cell layer of normal skin. They detected staining of the basal layer and stratum Malphigi using the more sensitive avidin–biotin peroxidase technique. The difference between the staining observed in that report and ours may reflect differences in specimen preparation, differences in antibodies used, or both. In addition, specimens used in prior studies came from sun-exposed areas, which may account for some of the staining differences observed. The nuclear staining we observed has not been reported previously and may be due to the use of antigen retrieval. Antigen retrieval has been applied in immunohistochemical studies to enhance antigen detection (Cuevas *et al*, 1994). Although the mechanism of enhanced antigen retrieval via microwave irradiation is not clear, it is believed to be mediated via disruption of cross-linking proteins in a similar way to that achieved by enzymatic predigestion. It may also result in nonspecific staining through cross-reactivity of the antibody to unmasked nonspecific epitopes. Nuclear staining was absent in sections stained with preimmune serum and was present, albeit diminished, in sections stained without antigen retrieval. Although we cannot exclude the possibility that the nuclear staining observed is nonspecific, more studies are required to better understand this staining pattern.

We show that UVR significantly changes the distribution of PAR-2 protein expression in human skin *in vivo*. We observed *de novo* expression of PAR-2 in keratinocytes in the upper layers of the epidermis following irradiation, which contrasts with its normal distribution to the lower third epidermal layer in nonirradiated skin. PAR-2 upregulation was observed at both the 2 MED and 3 MED doses of irradiation, and at both time points. These doses and time points were chosen because many acute effects of UVR on the skin appear in 3–6 h, reach a peak between 12 and 24 h (such as erythema and cytokine release), and proliferation effects are readily evident at 96 h (Soter, 1990). Whereas both doses of irradiation increased PAR-2 expression in the epidermis in a statistically significant way in type II and type III skin, PAR-2 upregulation was more pronounced in the 2 MED/96 h samples compared with the 3 MED/24 h samples. This may reflect the time necessary for new mRNA synthesis and translation. Further, we show that subjects with type I skin displayed statistically significant differences in UVR-related induction of PAR-2 expression compared with subjects with skin types II and III. Specifically, subjects with type I skin failed to show a statistically significant upregulation in PAR-2 expression 24 h following 3 MED irradiation. These observations suggest that differences in PAR-2 regulation in response to UVR may be one factor in determining a person's ability to tan.

By immunofluorescence microscopy on cultured human keratinocytes, PAR-2 was distributed in a cytoplasmic pattern with prominent localization to the presumed Golgi apparatus without detectable nuclear staining. These results are similar to those reported by Bohm *et al* (1996a) who also demonstrated large pools of PAR-2 in the Golgi apparatus in an epithelial cell line

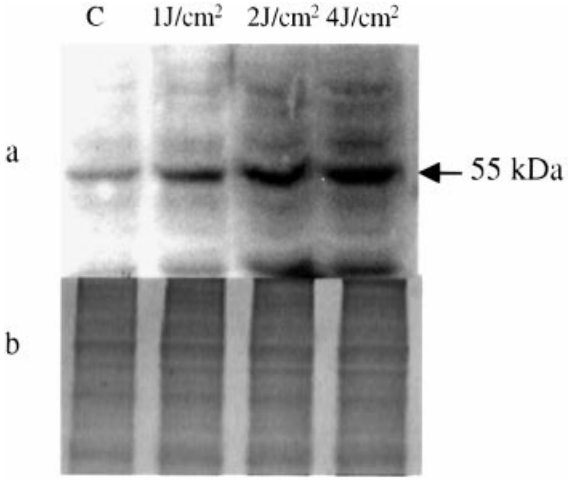
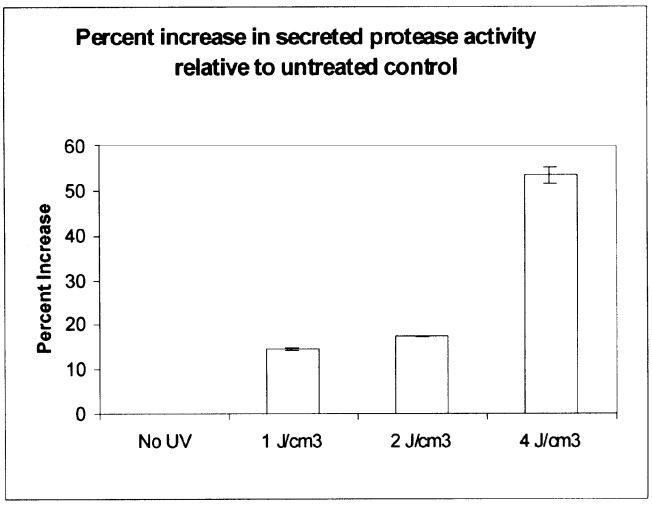
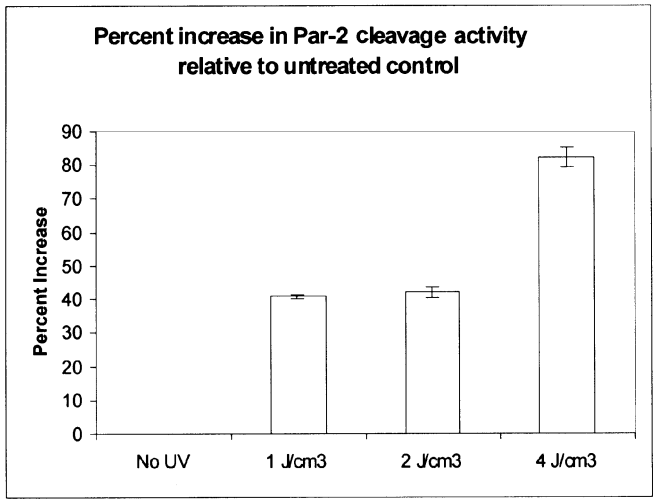


Figure 5. UVR of cultured human keratinocytes results in a dose-dependent increase in PAR-2 protein expression by Western blotting. (*a*) Lysates of irradiated keratinocytes were separated by SDS-PAGE and probed for PAR-2 with monoclonal antibodies against human PAR-2. A 55 kDa band corresponding to the expected molecular weight of PAR-2 was detected in sham-irradiated cell lysates. A clear increase in PAR-2 was observed at 1 J per cm^2 and was further increased at 2 J per cm^2. Levels of PAR-2 in cells irradiated with 4 J per cm^2 were similar to that seen in cells irradiated with 2 J per cm^2. (*b*) The gel was stained with Coomassie Brilliant Blue to confirm equal loading of the lanes.



a



b

Figure 6. UVR-treated cultured human keratinocytes secrete a protease with PAR-2 cleavage activity. (*a*) Culture supernatants of UVR-treated keratinocytes were assessed for protease activity using the EnzChek protease assay kit as described in *Materials and Methods*. Results are expressed as percent increase in protease activity compared with control (no UVR) ± standard error of the mean. Control cells showed baseline protease activity, which increased over 50% 24 h following a single dose of 4 J per cm^2 from a solar simulator. (*b*) Culture supernatants from UVR-treated keratinocytes were assessed for PAR-2 cleavage activity using a fluorolabeled peptide containing the cleavage site of PAR-2. Results are expressed as percent increase in PAR-2 cleavage activity compared with control (no UV) ± standard error of the mean. Control cells express a protease with PAR-2 cleavage activity, which increased over 80% following a single dose of 4 J per cm^2 from a solar simulator.

engineered to over-express human PAR-2. Because keratinocytes were cultured in low Ca^{2+} medium, they were expected to be proliferative rather than differentiated, as reported by others (Hennings *et al*, 1980a, b). Therefore, it is not surprising that all cultured keratinocytes expressed PAR-2 as these cells mimic in many ways the phenotype of basal keratinocytes *in vivo*. We detected a clear increase in staining intensity for PAR-2 following irradiation of cultured keratinocytes within 24 h of a single 2 J per cm^2 dose of UVR. These results were confirmed by Western blotting of cell lysates of irradiated cells with a monoclonal antibody to PAR-2. A clear increase in immunoreactive PAR-2 was detected in cells irradiated with 1 J per cm^2, 2 J per cm^2, and 4 J per cm^2. Because we demonstrated that UVR induces PAR-2 expression *in vivo* and *in vitro*, we next determined whether UVR of cultured keratinocytes would induce the secretion of a protease with PAR-2 cleavage activity. UVR did indeed induce a dose-dependent increase in secreted protease in cultured keratinocytes. Further, a dose-dependent increase in PAR-2 cleavage activity was also observed, strongly suggesting that UVR induces both increased expression of PAR-2 and PAR-2 activation through upregulation of a protease with PAR-2 cleavage activity.

Mast cell tryptase has been shown to activate PAR-2 in keratinocytes *in vitro* and tryptase-containing mast cells are increased in inflammatory skin conditions, which show upregulated PAR-2 expression (Steinhoff *et al*, 1999). Previous studies have shown that dermal mast cells are activated after irradiation resulting in degranulation (Gilchrest *et al*, 1981). We observed increased numbers of mast cells in subjects' skin biopsies following irradiation and therefore it is likely that mast cell tryptase plays an important role in PAR-2 activation in response to UVR (unpublished observations). Based on our observation that UVR upregulates a PAR-2 activating protease with PAR-2 cleavage activity in cultured keratinocytes, it is likely that other proteases within the epidermis are involved in UVR-induced PAR-2 activation. Other tryspin-type serine proteases have been detected in human keratinocytes, which may prove to cleave PAR-2. Potential candidates include TLSP, a novel serine protease that is expressed in keratinocytes (Yoshida *et al*, 1998), and squamous cell chymotyrptic enzyme, both of which exhibit trypsin-like activity (Sondell *et al*, 1994). Whereas UVR may upregulate the expression or activity of a serine protease with PAR-2 cleavage activity, an alternative possibility is that UVR-induced inhibition of a serine protease inhibitor may account for the ability of UVR to activate PAR-2 in our culture system. Abts *et al* (1997) identified a UVR repressible gene in HaCaT and human keratinocytes from psoriatic epidermis. The gene product, termed hurpin (protease inhibitor

13), is a putative serine protease inhibitor in the ovalbumin family of serpins. Although the target protease for hurpin is currently unknown, it is possible that this or some other UV-regulated serine protease inhibitor may play a role in PAR-2 activation in the skin in response to UVR.

UVR modulates the function of a variety of epidermal and dermal cells through induction and release of a complex network of cytokines, chemokines, neuropeptides, adhesion molecules, and other inflammatory mediators, resulting in diverse biologic effects including erythema, edema, and pigmentation. It is interesting to speculate that upregulation of PAR-2 levels by UVR may contribute to the cytokine cascade observed in keratinocytes in response to UVR *in vivo*. Hou *et al* (1998) has shown that activation of PAR-2 stimulates interleukin-8 (IL-8) secretion by keratinocytes *in vitro*. Wakita *et al* (1997) showed that activation of PAR-2 by a murine agonist peptide induces gene expression for IL-6 and GMCSF in cultured human keratinocytes. Therefore it is possible that UVR upregulation of PAR-2 may in turn modulate synthesis and secretion of cytokines such as IL-8, IL-6, and GMCSF by keratinocytes *in vivo*.

The mechanism of UVR-induced PAR-2 upregulation remains to be determined. Recent reports showing that IL-1α and TNF-α upregulate PAR-2 expression *in vitro*, however, suggest that these cytokines may be responsible for PAR-2 induction in response to UVR *in vivo*. Human endothelial cells respond to treatment with IL-1α or TNF-α by upregulation of PAR-2 mRNA 5–10-fold, which occurs within 20 h and persists for up to 96 h in the continued presence of the cytokine (Nystedt *et al*, 1996). This time course is identical to that observed in PAR-2 upregulation in the skin, in which upregulation occurred 24 and 96 h following UVR. It is well documented that UVR induces keratinocytes to produce and secrete cytokines including IL-1, IL-6, IL-8, and TNF (reviewed in Kondo, 1999). The time course for secretion of these cytokines precedes the peak induction of PAR-2 noted here. Therefore it is possible that UVR-induced synthesis and secretion of keratinocyte-derived IL-1 and TNF-α may participate in upregulation of PAR-2. In support of this hypothesis are data showing that PAR-2 is upregulated in inflammatory conditions in the skin *in vivo* including lichen planus, where enhanced PAR-2 immunoreactivity was observed in the granular layer, and atopic dermatitis and psoriasis vulgaris, in which PAR-2 immunoreactivity was detected throughout the entire epidermis (Steinhoff *et al*, 1999). Another potential role for PAR-2 may be in mediating postinflammatory pigmentation, which occurs following a variety of inflammatory skin diseases such as dermatitis and psoriasis. It is possible that disordered regulation of PAR-2 in response to cytokines released during the inflammatory process may result in either increased uptake of melanosomes in neighboring keratinocytes, or even delayed or absent melanosome uptake. In the latter case, melanosomes may be released into the dermis and phagocytized by melanophages, resulting in the characteristic histologic picture of postinflammatory pigmentation.

UVR has profound effects on skin pigmentation through a variety of mechanisms. In reconstructed human epidermis, UVR results in increased presence of melanosomes around keratinocyte nuclei through increased melanocyte dendricity, melanin production, and transport to keratinocytes (Murli *et al*, 2000). One model of melanosome transfer is cytophagocytosis, in which the melanocyte dendrite tip is phagocytized by the keratinocytes (Cohen and Szabo, 1967; Wolff, 1973; Yamamoto and Bhawan, 1994). Following phagocytosis, melanosomes are taken up by autophagic vacuoles, transported to the perinuclear space, and degraded. PAR-2 has recently been shown to increase phagocytosis of melanosomes *in vitro* and to induce skin darkening *in vivo* through increased uptake of melanosomes (Seiberg *et al*, 2000a, b; Sharlow *et al*, 2000). Because the antibody we used for immunocytochemical staining of skin biopsies recognizes both the active and the inactive form of the PAR-2 receptor, it is unclear what proportion of newly expressed PAR-2 represents active receptor. Our *in vitro* data showing that UVR induces protease secretion with PAR-2 cleaving activity, however, suggests that UVR both upregulates and activates PAR-2. UVR-induced *de novo* expression and activation of PAR-2 may result in increased uptake of melanosome-containing dendrite tips, accounting in part for the well-known effects of UVR on skin pigmentation. As PAR-2 has been shown to play an important role in both cutaneous pigmentation and inflammation, it is possible that PAR-2, in concert with other keratinocyte-derived factors, is involved in the final common pathway of post-UVR inflammation and hyperpigmentation of the skin.

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