Inhibitory Effects of Pentoxifylline on Ultraviolet B Light–Induced Cutaneous Inflammation

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It is now recognized that ultraviolet (UV) radiation is a potent environmental insult capable of interfering with immunity to skin cancers and modifying certain immunologic reactions within both locally irradiated skin and distant, unexposed sites. Exposure to UVB light (290–320 nm) induces a potent cutaneous inflammatory response that involves the infiltration of leukocytes into the dermis as well as the production of proinflammatory cytokines by both resident epidermal keratinocytes and dermal cells. Tumor necrosis factor-α (TNF-α) is a proinflammatory cytokine that has been shown to be a major mediator of UVB light effects on cutaneous immunity. Recent studies have demonstrated that pentoxifylline (PTX), a xanthine-derived phosphodiesterase inhibitor, has the ability to inhibit synthesis of TNF-α. To examine the effects of PTX on UVB-mediated cutaneous inflammation, Skh/hr hairless mice were injected intraperitoneally with either phosphate-buffered saline or 50 μg/g PTX 1 h before exposure to 2240 J/m^2 UVB. Reverse transcription–polymerase chain reaction and immunohistochemical techniques were used to demonstrate that 24 h to 1 wk after UVB-light irradiation, PTX inhibited UVB-induced *TNF-α* gene expression, inhibited the increase in epidermal TNF-α protein synthesis, blocked the increase in epidermal proliferation observed after exposure to UVB light, and decreased production of myeloperoxidase by neutrophils infiltrating into the dermis. These studies demonstrated that PTX modifies epidermal responses after acute UVB light exposure and suggest that PTX treatment may be used clinically to modulate the deleterious effects of long-term UVB-light irradiation. *Mol. Carcinog. 22:16–25, 1998.*

Key words: epidermis; cytokines; inflammation; tumor necrosis factor-α

INTRODUCTION

The dramatic increase in the incidence of skin cancer, resulting from the rapid and expanding loss of the earth's ozone layer, has highlighted research examining the link between exposure to ultraviolet (UV) light and epidermal malignancies. UV radiation is believed to facilitate the maintenance and growth of transformed cells by impairing immunosurveillance, by directly diminishing cell number and function, and by inducing soluble inflammatory mediators [1–5]. Epidermal keratinocytes, the specialized epithelial cells that make up most of the epidermal layer, produce a large number of immunologic and inflammatory mediators [6] whose levels are elevated after exposure to UV light. These soluble factors are believed to contribute significantly to the development and perpetuation of cutaneous inflammation [7–9] as well as to mediate the immunopathologic effects of UV radiation [10].

One of the most prominent cytokines involved in UV light–induced inflammation is tumor necrosis factor-α (TNF-α). TNF-α is a multifunctional cytokine that has diverse and potent effects on inflammatory processes and immunity [11,12]. Kock et al. [13] demonstrated that significant amounts of TNF-α are present in supernatants of keratinocytes irradiated in vitro with UVB light (290–320 nm) as well as in serum obtained from severely sunburned human volunteers after a single total-body UVB exposure. These findings indicated that upon stimulation, keratinocytes are able to synthesize and release TNF-α locally, which may then gain access to the systemic circulation. Studies have demonstrated that intradermal injection of TNF-α mimics the effects of UVB radiation and that injection of neutralizing anti–TNF-α antibodies abolishes the ability of UVB to suppress contact hypersensitivity (CHS) reactions [14–16]. These data suggest that TNF-α is one of several critical mediators of UVB's effects on cutaneous immunity.

One approach to blocking the deleterious effects of exposure to UVB is to inhibit the cytokine mediators that are synthesized and released after exposure to UVB light. Recent studies have revealed that pentoxifylline (PTX), a xanthine-derived phosphodi-

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Abbreviations: UV, ultraviolet; TNF-α, tumor necrosis factor-α; CHS, contact hypersensitivity; PTX, pentoxifylline; MPO, myeloperoxidase; i.p., intraperitoneal; PCNA, proliferating-cell nuclear antigen; DAB, 3,3′-diaminobenzidine; ABC, avidin-biotinylated horseradish peroxidase complex; RT, reverse transcription; PCR, polymerase chain reaction; HPRT, hypoxanthine phosphoribosyltransferase; PBS, phosphate-buffered saline.

esterase inhibitor [17] used clinically for the treatment of vascular insufficiency, has potent anti-inflammatory effects primarily due to its ability to inhibit cytokine synthesis [18–20]. PTX has been shown to reduce *TNF-α* mRNA transcription and TNF-α protein production in murine macrophages and human monocytes through a cAMP signal-transduction pathway [21,22]. PTX has also been shown to block the effector phase of CHS, which is believed to be mediated by TNF-α [23,24]. In addition, PTX inhibits superoxide anion production, phagocytosis, cytokine-mediated adhesion-integrin molecule interactions, and myeloperoxidase (MPO) activity in neutrophils, which ultimately results in fewer neutrophils with a diminished ability to produce oxidants at sites of inflammation [25–28]. However, the in vivo effects of PTX on the acute cutaneous inflammatory response and the cutaneous cytokine protein levels after UVB exposure have not been reported.

The goal of the study reported here was to characterize the effects of acute UVB exposure on epidermal proliferation, inflammation, and cutaneous TNF-α gene expression and protein production and to examine the modulation of these effects by systemic administration of PTX in the Skh/hr hairless mouse model, which has been used as a model of UVB-induced squamous cell carcinoma growth. Shortly after UVB irradiation, PTX inhibited UVB-induced increases in epidermal TNF-α gene expression and protein synthesis, decreased neutrophil MPO levels, and blocked the increase in epidermal proliferation usually observed following exposure to UVB.

MATERIALS AND METHODS

Treatment of Skh/hr Mice

Initial studies were performed to determine the optimal dose of PTX (20 μg/g, 50 μg/g, or 100 μg/g) for inhibiting the UVB-induced inflammatory response. The dose of 50 μg/g PTX was chosen for further studies based upon its anti-inflammatory activity. Skh/hr hairless mice received intraperitoneal (i.p.) injections of either saline or PTX (50 μg/g) 1 h before UVB exposure. Control unirradiated animals received i.p. injections of saline or 50 μg/g PTX. Groups exposed to UVB were irradiated dorsally, three times weekly, with Phillips F40UVB lamps (American Ultraviolet Company, Lebannon, IN), which emit primarily UVB light (290–320 nm). The total UVB dose per irradiation was 2240 J/m^2, as measured with a UVX digital radiometer (UVP Inc., San Gabriel, CA), and in our hands is the equivalent of one minimal erythemic dose. Three separate experiments were performed (n = 12 mice per group). The inflammatory response induced by UVB exposure was quantitated by measuring with calipers the extent of skin swelling 24 h and 1 wk after treatment.

Detection of Infiltrating Neutrophils

Tissue samples (0.5 cm^2) were isolated from the dorsal skin of four Skh/hr mice per group at the time points described above. The experiment was repeated three times for a total of 12 tissues per treatment group, which were then stained for Gr-1 localization. (Gr-1 is a monoclonal antibody directed against a mouse myeloid differentiation antigen that is present on the surface of all neutrophils and to a lesser extent on myeloid cells but is not present on lymphoid or erythroid cells [29,30]). In brief, sections of tissue were placed in 10% neutral buffered formalin, washed in 0.2 M phosphate buffer, and then processed and embedded in paraffin blocks for microtome sectioning. Sections (5 μm) were cut and mounted onto ProbeOn Plus microscope slides (Fisher Scientific, Pittsburgh, PA). The sections were deparaffinized with Histo-Clear (National Diagnostics, Atlanta, GA) and rehydrated in a graded series of alcohols with a final wash in distilled water. The staining was performed with the Microprobe Manual Staining System (Fisher Scientific). Non-specific binding was blocked with 10% normal rabbit serum. For detection of infiltrating neutrophils within the dermis of Skh/hr mice, the primary monoclonal antibody used was rat anti–mouse Ly-6G (Gr-1) (Pharmingen, San Diego, CA) diluted 1:400. Incubation time was 1 h. The nonspecific antibody used for the Gr-1 studies was $IgG_{2b,\kappa}$ (Pharmingen). The biotinylated secondary antibody was rabbit anti-rat (Vector Laboratories, Burlingame, CA) diluted 1:400. After rinsing with buffer, avidin-biotinylated horseradish peroxidase complex (ABC Elite) (Vector Laboratories) was placed on the tissue for 30 min and then rinsed again. The tissue sections were then incubated for 5 min with the chromagen 3,3´-diaminobenzidine (DAB) (Sigma Chemical Co., St. Louis, MO). DAB development was stopped by rinsing with distilled water. The tissues were then counterstained with Harris' hematoxylin (Shandon, Pittsburgh, PA) for 1 min. The slides were then dehydrated, mounted, and viewed. The number of Gr-1–positive cells was quantitated with the Oncor V-150 Imaging System (Oncor, Gaithersburg, MD).

Detection of MPO in Cutaneous Tissue

To detect MPO activity in cutaneous tissue, skin punches were obtained from isolated dorsal skin of each mouse by using a 10-mm-diameter cork borer. The samples were weighed before they were minced with scissors in 1.25 mL of 0.5% hexadecyltrimethylammonium bromide in 50 mM potassium phosphate buffer, pH 6.0. The tissue punches were homogenized at 4°C, subjected to three cycles of sonication and freezing and thawing, and then centrifuged at 4°C in a minicentrifuge for 30 min at 14 000 rpm. The supernatants were then transferred to new tubes, and 10 μL of each sample was placed into in-

dividual wells of 96-well microtiter plates containing 290 μL substrate (0.167 mg/mL *o*-dianisidine dihydrochloride and 0.0005% H_2O_2 in 50 mM potassium phosphate buffer, pH 6.0). MPO activity was measured spectrophotometrically at 450 nm over 5 min by using a programmable Molecular Devices microplate reader (Menlo Park, CA). The data were expressed as the mean units of MPO activity of three 10-mm skin punches. One unit of MPO activity was defined as that degrading 1 μmol of peroxide/min at 25°C [30].

Detection of Cell Proliferation Measured by Immunohistochemical Detection of Proliferating-Cell Nuclear Antigen

Tissues were processed as described above for Gr-1 staining with the following modifications. The primary antibody was mouse anti–proliferating-cell nuclear antigen (PCNA) (diluted 1:100) (Signet Laboratories, Dedham, MA). The biotinylated secondary antibody was horse anti-mouse (diluted 1:200) (Vector Laboratories). Nonspecific binding was blocked with 10% normal horse serum (Vector Laboratories). The sections were incubated with the primary antibody for 2 h at room temperature. The amount of cellular proliferation was quantitated with the Oncor V-150 Imaging System.

Reverse Transcription–Polymerase Chain Reaction Detection of TNF-α Gene Expression

Dorsal skin was isolated from Skh/hr mice 24 h and 1 wk after treatment and immediately frozen in liquid nitrogen. RNA was isolated from samples containing intact epidermis and dermis by using RNA STAT 60 reagent (guanidinium thiocyanate and phenol solution; TEL-TEST 'B' Inc., Friendswood, TX) with Phase Lock Gel (5′ → 3′ Inc., Boulder, CO). The RNA was quantitated spectrophotometrically based on the formula 1 unit A_{260nm} = 40 μg/mL RNA. All RNA samples isolated for reverse transcription (RT)–polymerase chain reaction (PCR) were analyzed for integrity of 18S and 28S rRNA by ethidium bromide staining of 2 μg of RNA/sample resolved by electrophoresis on a 1.2% agarose-formaldehyde gel.

RT of 2 μg of total RNA was performed in a volume of 40 μL for 1 h at 37°C, by using 100 U of Moloney murine leukemia virus reverse transcriptase (GIBCO BRL, Gaithersburg, MD) in 10 mM Tris, pH 8.3; 50 mM KCl; 5 mM $MgCl_2$; 1 unit of RNasin; 1 mM each dATP, dGTP, dCTP, and dTTP; and 2.5 μM oligo(dT) (U.S. Biochemicals, Cleveland, OH). The samples were then heated to 95°C for 5 min to terminate the RT reaction. The reverse-transcribed cDNA obtained from 0.25 μg of total RNA was added to a PCR mixture at 4°C to give final concentrations of 10 mM Tris, pH 8.3; 50 mM KCl; 2.5 mM $MgCl_2$; 0.2 mM each dATP, dGTP, dCTP, and dTTP; 0.2 μM each 5′ primer and 3′ primer (Baron Biologicals, Milford, CT); and 0.25 U of Taq polymerase with a final reaction volume of 50 μL. The primers used for PCR amplification were selected from sequences spanning two exons separated by one or more long intronic sequences, which allowed identification of amplification of contaminating genomic DNA. The primers used for RT-PCR detection of *TNF-α* were previously reported [32]. Hypoxanthine phosphoribosyltransferase (*HPRT*) was used as a control housekeeping gene because it has the same order of magnitude of amplification as many murine cytokines [33].

The PCR mixture was heated to 95°C for 30 s in a Perkin-Elmer GeneAmp Thermocycler 9600 (Perkin-Elmer, Branchburg, NJ). Amplification was performed using hot-start PCR with Ampliwax 50 beads (Perkin-Elmer) for 32 cycles for *TNF-α* at 95°C for 15 s, and 72°C for 60 s and 25 sequential cycles for *HPRT* at 95°C for 15 s, 60°C for 30 s, and 72°C for 30 s, followed by a final extension reaction for 7 min at 72°C. To ensure that there was no amplification of contaminating genomic DNA, control samples were run for each primer pair without the addition of reverse transcriptase. The specificity of PCR amplification was determined by visualizing a fragment of the expected size with molecular-weight markers on an agarose gel stained with 1.5% ethidium bromide in 0.5× TBE. The gels were photographed with Polaroid type 667 film (Fisher Scientific). Quantitation of *TNF-α* gene expression was performed using a Microtek ScanMaker II HR (Microtek, Great Falls, VA) interfaced with a Power Macintosh 7100 personal computer (Apple, Cuper-tino, CA). Scans were saved as eight-bit TIFF files and imported into NIH Image (version 1.54) for analysis. A standard baseline for samples was used, and the magnification of the image and width of the measurement window were kept constant [34–36]. The densities of the *TNF-α* bands in square pixels were normalized to the densities of the *HPRT* bands. Each measurement represented the average of two separate RT-PCRs from three separate experiments.

Immunohistochemical Localization of TNF-α

Tissue samples (0.5 cm^2) from dorsal epidermis of four Skh/hr mice per group at the time points described above were isolated, fixed, processed, embedded, sectioned, and stained. The primary antibody used for TNF-α immunostaining was rabbit anti–mouse TNF-α (diluted 1:400) (Genzyme, Cambridge, MA). The biotinylated secondary antibody was goat anti-rabbit (diluted 1:200). Rabbit IgG (Vector Laboratories) was used as the isotypic control to determine the extent of nonspecific antibody binding.

After rehydration, the tissues were rinsed with Automation Buffer (Biomedia Corp., Foster City, CA) and then incubated for 30 min with 10% normal goat serum (Vector Laboratories) to block nonspecific binding. The tissues were incubated with the primary antibody in a humidified chamber for 1 h at 37°C, rinsed, and then incubated with the

biotinylated secondary antibody for 30 min at room temperature. After rinsing, ABC Elite (Vector Laboratories) was placed on the tissue for 30 min. The sections were then rinsed with buffer and incubated with the chromagen DAB (Sigma Chemical Co.) for 5 min. DAB development was stopped by rinsing with distilled water. The tissues were then counterstained with Harris's hematoxylin (Shandon) for 1 min. The slides were then dehydrated, mounted, viewed, and photographed.

Statistical Analysis

The data were analyzed for significance by using a one-tailed Student's *t*-test to compare experimental data with that of the appropriate controls. The significance of analyzed data was expressed in *P* values, with $P \leq 0.05$ being statistically significant.

RESULTS

Measurement of Skin Thickening

Skin thickening was used as a measure of vascular permeability and edema, two hallmarks of a cutaneous inflammatory response. The cutaneous inflammation induced by exposure to UVB light was initially assessed by caliper measurement of dorsal skin thickness. Exposure of the dorsal epidermis of Skh/hr hairless mice to UVB light resulted in a time-dependent increase in skin thickening. One week after exposure to 2240 J/m^2 UVB light, there was a threefold increase in skin thickness (Figure 1). Systemic i.p. administration of 50 μg/g PTX 1 h before UVB exposure had no significant effect on UVB-induced skin thickening.

Quantitative Assessment of the Effects of PTX on UVB-Induced Dermal Neutrophil Infiltration

Previous studies have demonstrated that neutrophils play a key role in early cutaneous inflammatory responses. Quantitation of the number of Gr-1–positive cells within 10 high-power (60×) fields revealed a significant increase in the number of infiltrating neutrophils within the dermis 24 h after exposure to UVB light as compared with the number of neutrophils within the dermis of nonirradiated skin (Figure 2). Injection of 50 μg/g PTX 1 h before UVB exposure had no significant effect on the number of neutrophils within the dermal microenvironment.

Biochemical Determination of the Inhibitory Effect of PTX on UVB-Induced Neutrophil-Associated MPO Enzyme Activity

The ability of injected PTX to inhibit MPO, which has been used as a marker for activation of dermal leukocytes, was evaluated. As shown in Figure 3, exposure to UVB light significantly increased the amount of MPO detected within the skin of mice 24 h after exposure ($P \leq 0.02$) as compared with levels detected in skin isolated from mice injected with either PBS or PTX. Injection of 50 μg/g PTX significantly inhibited the dermal MPO activity detected 24 h after UVB light exposure ($P \leq 0.03$) as compared

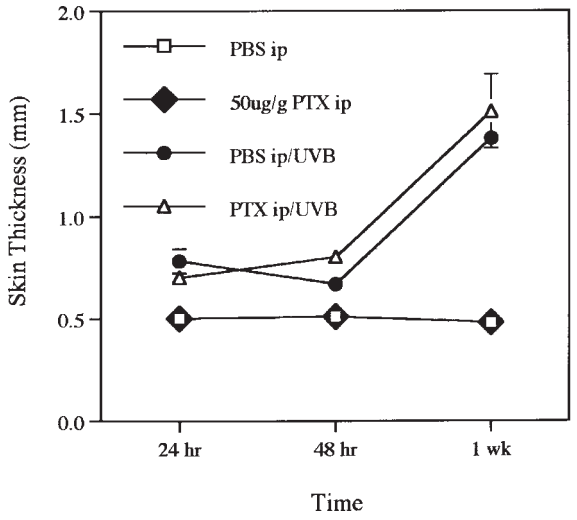

Figure 1. Skin thickening as a measure of the extent of UVB-mediated inflammatory response. I.p. injection of either 0.2 mL of phosphate-buffered saline (PBS) or 50 μg/g PTX in 0.2 mL PBS without UVB exposure had no effect on skin thickness. Exposure of the dorsal skin of Skh/hr hairless mice to UVB resulted in a time-dependent significant increase in skin thickening. Mice exposed to UVB light three times over a 1-wk period had a threefold increase in skin thickening as compared with control mice. Systemic i.p. administration of 50 μg/g PTX 1 h before UVB light exposure had no significant effect on UVB-induced skin thickening at any time point examined.

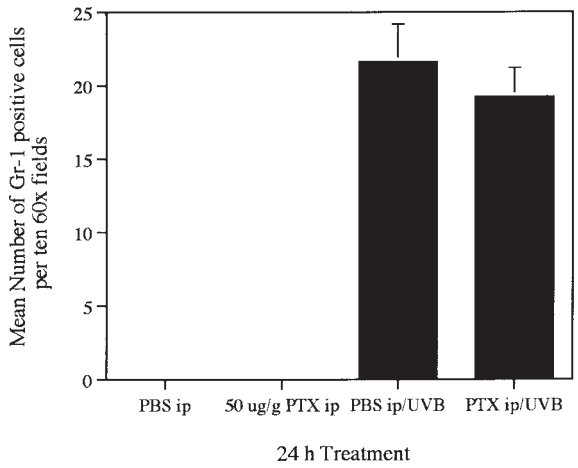

Figure 2. Effect of PTX on UVB-induced dermal neutrophil infiltration in Skh/hr mice. The neutrophils within the dermis were counted in sections of dorsal skin isolated from mice 24 h after treatment. Tissue sections were stained with rat anti-mouse monoclonal Gr-1 antibody, which stains cells that express a myeloid differentiation antigen that is restricted to mature neutrophils. The Gr-1–positive cells were counted with the Oncor V-150 Image Analysis System. Exposure to 2240 J/m^2 UVB light increased the number of neutrophils in the dermis of Skh/hr mice. I.p. injection of 50 μg/g PTX 1 h before UVB exposure had no statistically significant effect on the increased number of neutrophils induced by UVB light within the dermis.

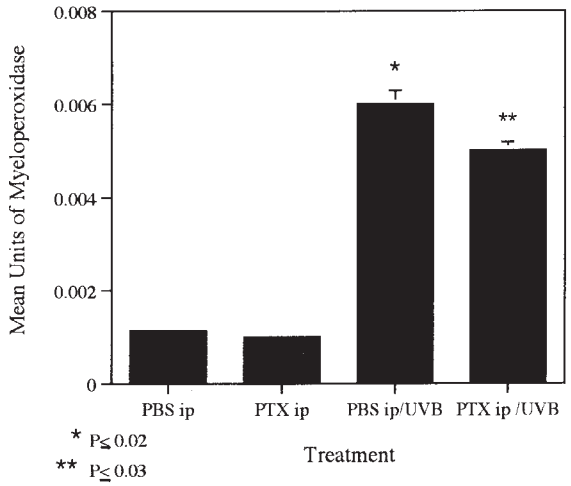

Figure 3. Effect of PTX on MPO activity in skin 24 h after UVB exposure. Exposure to 2240 J/m^2 UVB resulted in a significant increase in MPO levels in cutaneous skin as compared with nonirradiated controls ($P \leq 0.02$). Injection of 50 μg/g PTX 1 h before UVB exposure significantly decreased cutaneous MPO levels ($P \leq 0.03$).

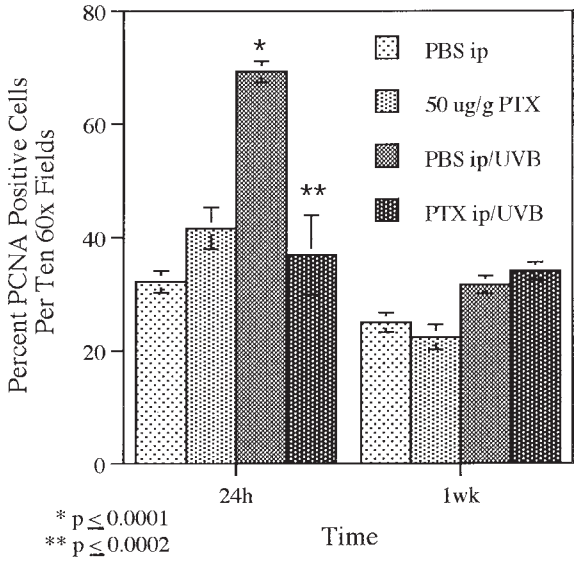

Figure 4. Quantitation of the effect of PTX on UVB light–induced increases in epidermal proliferation. Counting the PCNA-positive cells with the Oncor V-150 Image Analysis System revealed a significant increase in epidermal proliferation 24 h after exposure to UVB light as compared with treatment with either i.p. PBS or i.p. PTX only ($P \leq 0.0001$). Pretreatment of mice with 50 μg/g i.p. PTX 1 h before UVB exposure significantly inhibited UVB-induced proliferation 24 h after irradiation to the levels detected in control non–UVB-irradiated skin ($P \leq 0.0002$).

with the levels observed in control PBS-injected, UVB-exposed skin.

PCNA Analysis of Epidermal Proliferation

After PTX and/or UVB Treatment

In addition to causing a cutaneous inflammatory response, exposure of the skin to UVB light induces significant epidermal hyperplasia. Epidermal cell populations undergoing proliferation were identified and quantitated using anti-PCNA antibody and image analysis. Tissue isolated from Skh/hr mice exposed to UVB light and injected with PTX was examined for the extent of hyperplasia by immunohistochemical localization of anti-PCNA antibodies. Proliferation within the epidermis was quantitated by using the Oncor V-150 Image Analysis System to enumerate cells stained with anti-PCNA antibody. Quantitative analysis demonstrated that exposure to UVB light induced a significant hyperproliferative response within the epidermis ($P \leq 0.0001$) (Figure 4). I.p. injection of 50 μg/g PTX 1 h before UVB exposure significantly inhibited UVB-induced epidermal proliferation 24 h after treatment ($P \leq 0.0002$).

RT-PCR Assessment of Inhibition of UVB-Induced *TNF-α* Gene Expression by PTX

TNF-α is recognized as a critical cytokine in UVB-induced immunosuppression in the skin. The ability of PTX to inhibit UVB-induced *TNF-α* gene expression was determined by RT-PCR analysis. *TNF-α* gene expression in Skh/hr mouse skin increased significantly 24 h after exposure to UVB light as compared with that in non–UVB irradiated skin injected with PBS i.p. ($P \leq 0.02$) (Figure 5A). This elevation in gene expression persisted 1 wk after UVB light exposure ($P \leq 0.02$) (Figure 5B). Injection of 50 μg/g PTX 1 h before UVB exposure resulted in a significant decrease in *TNF-α* gene expression after 24 h ($P \leq 0.003$) and 1 wk ($P \leq 0.04$) as compared with levels detected in PBS-injected, UVB-irradiated skin (Figure 5A and B).

Immunochemical Localization of TNF-α After PTX and/or UVB Treatment

TNF-α–immunoreactive protein was localized within the epidermis of cutaneous tissue isolated from Skh/hr hairless mice. Immunohistochemical analysis was used to identify and localize cells within skin that produced TNF-α protein in response to UVB exposure and to examine the ability of PTX to inhibit cell-type–specific TNF-α protein production. Representative photographs depicting the TNF-α staining pattern observed within each of the 12 tissue selections isolated from the four treatment groups are shown in Figure 6. TNF-α protein was present within the epidermis of mice after i.p. injection of PBS only (Figure 6A). Similar results were observed in the epidermis isolated from mice treated only with 50 μg/g PTX (data not shown). Exposure of mice to 2240 J/m^2 UVB light resulted in an increase in TNF-α protein throughout the epidermis after 24 h (Figure 6B) and 1 wk. The TNF-α protein levels within the epidermis of mice injected with 50 μg/g PTX and exposed to UVB (Figure 6C) were significantly lower than the TNF-α protein levels present in tissue isolated from PBS-injected, UVB-irradiated mice (Figure 6B). The isotypic control

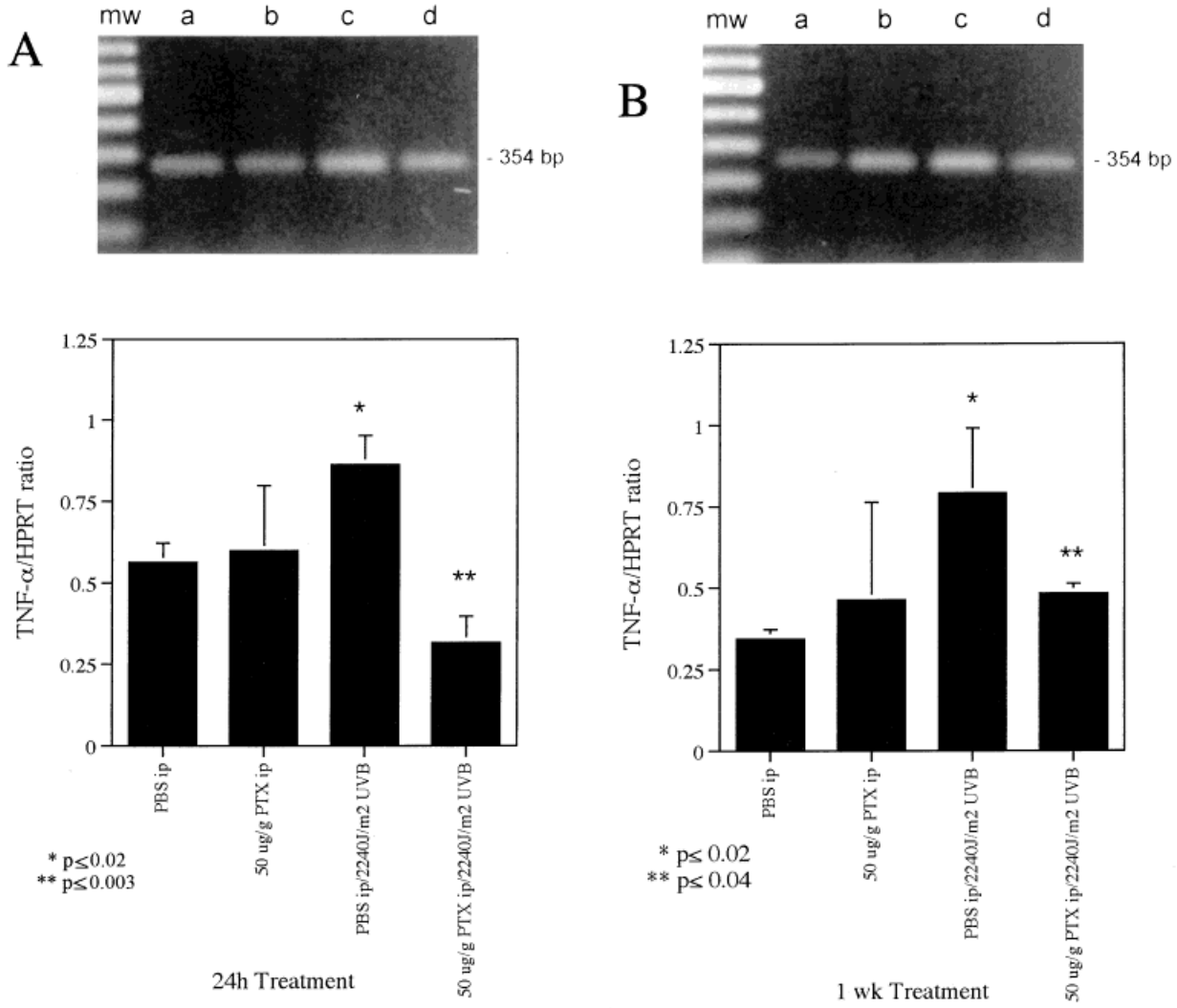

Figure 5. RT-PCR analysis of the effect of PTX injection on UVB-induced *TNF-α* gene expression. (Upper panels) The ethidium bromide–stained bands are the *TNF-α* cDNA PCR products (354 bp) detected in skin isolated from mice injected with 0.2 mL of sterile PBS (lane a), with 50 μg/g PTX (lane b), with 0.2 mL sterile PBS 1 h before exposure to 2240 J/m^2 UVB (lane c), and with 50 μg/g PTX 1 h before exposure to 2240 J/m^2 UVB (lane d). (Lower panels) Quantitation of *TNF-α* cDNA PCR product with NIH Image (version 1.47) demonstrated a significant increase in *TNF-α* gene expression both 24 h (A) and 1 wk (B) after exposure to UVB ($P \leq 0.02$). Injection of 50 μg/g PTX 1 h before UVB exposure significantly decreased the levels of *TNF-α* cDNA PCR product as compared with levels detected in PBS injected UVB irradiated skin.

antibody used to determine the levels of nonspecific background binding within the epidermal layer of tissue isolated from mice 24 h after exposure to 2240 J/m^2 UVB light in these studies did not bind above background levels (Figure 6D).

DISCUSSION

The importance of the skin as an immunologic organ second only to lymphoid organs and bone marrow is now recognized. In addition to being a mechanical barrier, the skin is critically involved in the regulation of immune responses that can affect the health of the entire organism. Our laboratory and others have demonstrated that there are a number of cell types within the skin that produce cytokines that regulate not only local cutaneous responses but also direct systemic responses after exposure to environmental and chemical insults [9,37–40]. In particular, keratinocytes, the major constituent of the epidermis, can synthesize, store, and release a number of cytokines, including TNF-α [9,38,41,42]. The constitutive production of these factors is usually very low but can be increased significantly by various stimuli such as endotoxin, viral infections, tumor promoters, or exposure to UV light [43,44]. It has become increasingly apparent that communication between epidermal cells of the skin and infiltrating inflammatory leukocytes through production of soluble mediators, including cytokines as well as reactive oxygen and nitrogen intermediates, plays an integral role in cuta-

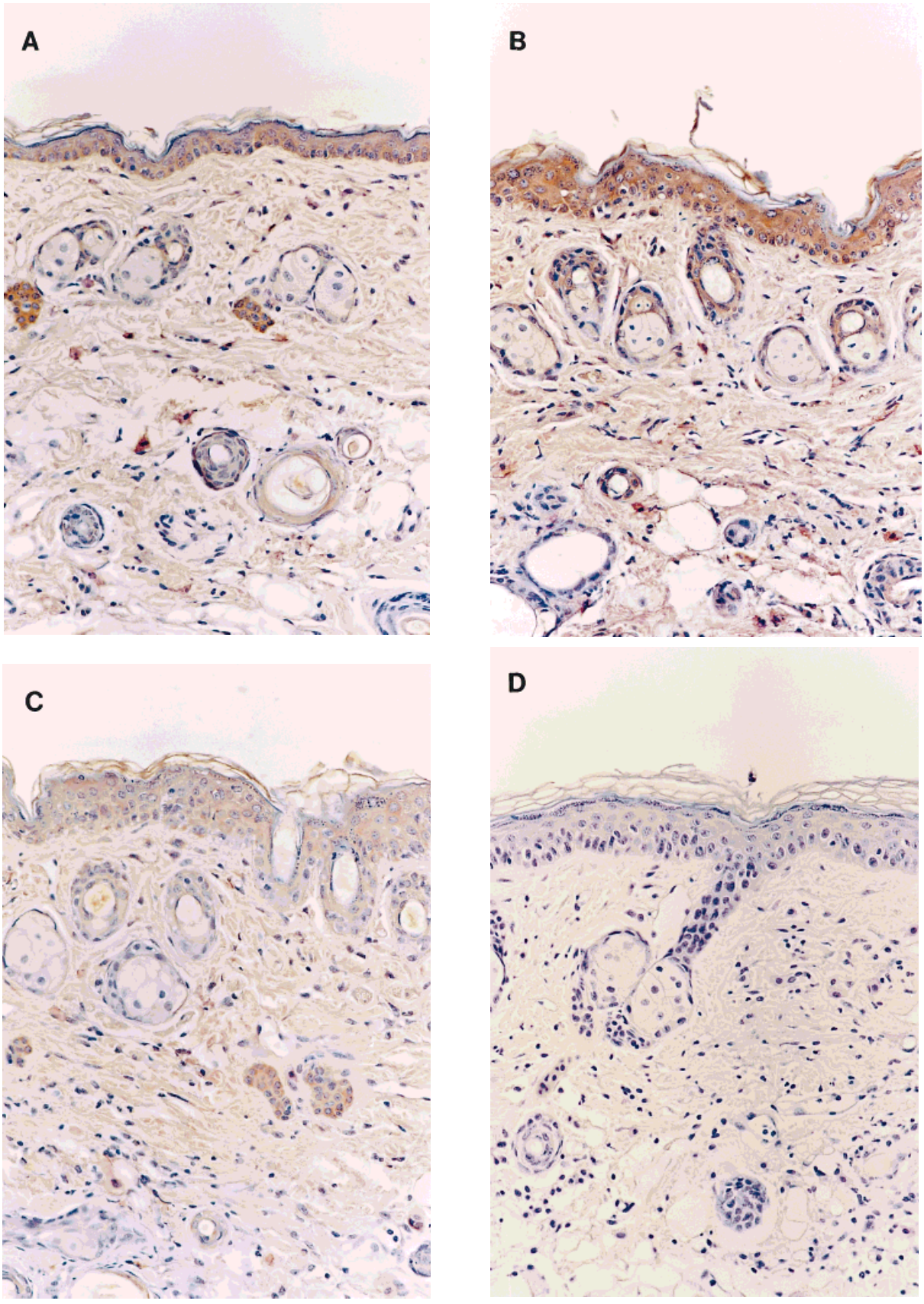

Figure 6.

neous homeostasis [45,46]. Consequently, these soluble mediators not only are involved in mediating local inflammatory reactions within the epidermis but also may enter the circulation and cause systemic effects. Thus, keratinocytes may regulate immune reactions both locally within the cutaneous microenvironment as well as systemically.

The depletion of the ozone layer and the concomitant increase in the amount of UVB light reaching the earth's surface has significantly increased the incidence of skin cancer. UVB light is responsible for most cutaneous damage after both acute and long-term exposure and is believed to be the single most important etiologic agent in human skin cancer. The goal of the studies reported here was to examine the in vivo ability of PTX, which has immunomodulatory activities as well as anti-proliferative activity, to abrogate or diminish acute UV light–induced cutaneous alterations that may ultimately lead to the growth of tumors. Initial manifestations of UVB-mediated damage include skin thickening and an increase in epidermal hyperplasia. Studies by Berman and Duncan [47] have shown that in vitro PTX inhibits proliferation of human fibroblasts from normal, keloidal, and sclerodermatous skin as well as their production of collagen, glycosaminoglycans, and fibronectin. In addition, PTX has been shown to inhibit 12-*O*-tetradecanoylphorbol-13-acetate (TPA) induced cutaneous *TNF-α* gene expression and also inhibit papilloma growth induced by a single topical application of 25 nmol of 7,12-dimethylbenz[*a*]anthracene followed at 1 wk by twice-weekly application of 2 μg of TPA [48]. In the study reported here, we demonstrated that systemic i.p. administration of PTX had different effects on UVB-induced cutaneous inflammatory responses that were dependent upon the parameters examined. Systemic i.p. administration of 50 μg/g PTX 1 h before UVB exposure had no significant effect on UVB-induced skin thickening, suggesting that PTX had no effect on the edema component of the UVB-induced cutaneous inflammatory response; however, PTX did decrease the UVB-induced epidermal hyperplasia observed 24 h after treatment.

UVB light has been demonstrated to have important effects on the immune system. In animal models originally developed by Kripke [49] and Daynes et al. [50], chronic exposure of laboratory mice to high doses of UVB light was shown to produce a selected set of immune abnormalities, including the inability to exhibit CHS after application of haptens to the skin and the inability to reject immunogenic tumors induced by UVB light in syngeneic mice [51–55]. Although it is believed that cytokines mediate the pathologic effects of UV light exposure, the exact cytokines that do so and their roles in the growth of UV-induced carcinomas remain unclear. One cytokine that is believed to play a key role in the damaging effects seen after UVB light exposure is TNF-α. This cytokine has become increasingly recognized as an important mediator of diverse physiological and immunological processes. It is an important cytokine orchestrating a variety of local immune responses that have pleomorphic effects on the cellular constituents of inflammation. Intradermal injection of TNF-α mimics the effects of UVB radiation, including blocking CHS responses, and injection of neutralizing anti–TNF-α antibodies abolishes the deleterious effects of UVB on induction of CHS [16,56]. TNF-α has also been found to be cytostatic for cultured normal epidermal keratinocytes and thus may play a part in downregulating the epidermal proliferation that occurs under homeostatic conditions [57]. These observations suggest that TNF-α is a major mediator of UVB effects on cutaneous immunity [58].

While most studies have focused on the beneficial role of PTX in endotoxin-induced shock in humans and have implicated TNF-α derived from macrophage/monocytes as the crucial effector molecule, the in vivo effects of PTX on UVB-induced TNF-α synthesis and its resulting cutaneous effects have only begun to be examined. Using a murine ear-swelling model as a measure of adverse effects of UVB light, Anderson and Elmets [59] found that pretreatment with anti–TNF-α antibodies or PTX significantly inhibits UVB light–induced ear swelling. In the studies reported here, we found that in vivo UVB exposure resulted in significant upregulation of *TNF-α* gene expression and protein production within skin and that systemic i.p. administration of PTX blocked the UVB light–induced production of TNF-α within the epidermis. The inhibitory effect of PTX on epidermal proliferation and cutaneous TNF-α protein levels may occur as a direct effect of PTX or may result from the inhibition of other factors that are released systemically following UVB exposure. Schwarz et al. [60] demonstrated that PTX suppresses UVB-induced interleukin-1 and TNF-α cytokine release by keratinocytes in vitro. However, the addition of PTX to unirradiated cells increases constitutive cytokine secretion. These results support our observation of a decrease in UVB light–induced cutaneous TNF-α gene expression at 24 h but a slight increase in TNF-α gene expression in nonirradiated skin isolated 24 h and 1 wk after i.p. injection with PTX. These results provide further evidence in support of the importance

Figure 6. Immunochemical detection of TNF-α protein in cutaneous tissue isolated from Skh/hr hairless mice. TNF-α–immunoreactive protein was present at low levels in the epidermis of skin isolated from mice injected with PBS (A). Similar levels of epidermal TNF-α were detected in tissue isolated from mice injected with 50 μg/g PTX (data not shown). There was a significant increase in TNF-α–immunoreactive protein detected within the epidermis 24 h after exposure to UVB light (B). The inhibition of UVB-induced TNF-α–immunoreactive protein within the epidermis by i.p. PTX treatment was evident by the lightly stained epidermis (C). The low level of isotypic antibody binding within the epidermis 24 h after exposure to UVB light demonstrated the specificity of the immunoreactive TNF-α localization (D). (Magnification, 20×)

of this cytokine in the mediation of the cutaneous effects of UVB light exposure and suggest that PTX is an effective anti-inflammatory agent. The ability of PTX to decrease *TNF-α* transcription, and subsequently decrease protein production [61–65], suggests that PTX may be a promising agent in the development of strategies to modulate TNF-α–mediated response to deleterious agents such as UVB light.

At the site of tissue injury, the production of inflammatory cytokines may benefit the host by promoting both the accumulation and activation of neutrophils. However, prolonged or excessive activation of neutrophils can initiate microvascular injury, resulting in increased vasopermeability, hemorrhage, reperfusion injury, and thrombosis [66]. Neutrophil infiltration into the dermis is a characteristic component of the host response to a topical inflammatory stimulus. MPO has been used as a marker to quantitate the extent to which leukocytes that have infiltrated into the dermis produce reactive oxygen intermediates in response to topical application of tumor promoters [67–69]. Our study demonstrated that while systemic i.p. injection of PTX had no effect on the number of neutrophils infiltrating the dermal microenvironment 24 h after UVB exposure as detected by quantitation of Gr-1–positive cells within the dermis, it did significantly decrease neutrophil MPO activity. These results are supported by a previous study showing that micromolar concentrations of PTX decrease native and recombinant TNF-α–primed, formyl Met-Leu-Phe–stimulated neutrophil superoxide production and MPO release [70]. PTX has been shown to inhibit the oxidative burst by neutrophils in response to formyl Met-Leu-Phe by binding to the neutrophil surface [71]. The effects of PTX on neutrophils in combination with the PTX-induced inhibition of *TNF-α* gene expression and protein production could be beneficial in conditions in which high TNF-α production may induce excessive neutrophil activation, leading to vascular damage and tissue injury.

In summary, we characterized the role of TNF-α in the normal cutaneous environment and documented the significant acute alterations induced by exposure to UVB light, including increases in epidermal proliferation, *TNF-α* gene synthesis and protein production, and neutrophil activation. Furthermore, these studies indicated that PTX is an effective pharmacologic probe useful for dissecting the importance of individual cytokines in the regulation of epidermal proliferation and in leukocyte activation after exposure to UVB light. Taken together with the results of previous studies, our results suggest that the pharmacologic effects of PTX may have important implications in clinical disease states in which the local and systemic modulation of TNF-α may be essential for the control of specific inflammatory processes.

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