Augmentation of Ultraviolet B Radiation-induced Tumor Necrosis Factor Production by the Epidermal Platelet-activating Factor Receptor*©

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Ultraviolet B radiation (UVB) has been shown to damage human keratinocytes in part by inducing oxidative stress and cytokine production. Indeed, UVB-induced production of the pro-inflammatory and cytotoxic cytokine tumor necrosis factor α (TNF-α) has been implicated in the epidermal damage seen in response to acute solar radiation. Though the lipid mediator platelet-activating factor (PAF) is synthesized in response to oxidative stress, and keratinocytes express PAF receptors linked to cytokine biosynthesis, it is not known whether PAF is involved in UVB-induced epidermal cell cytokine production. These studies examined the role of the PAF system in UVB-induced epidermal cell TNF-α biosynthesis using a novel model system created by retroviral-mediated transduction of the PAF receptor-negative human epidermal cell line KB with the human PAF receptor (PAF-R). Treatment of PAF-R-expressing KB cells with the metabolically stable PAF-R agonist car bamoyl-PAF resulted in increased TNF-α mRNA and protein, indicating that activation of the epidermal PAF-R was linked to TNF-α production. UVB irradiation of PAF-R-expressing KB cells resulted in significant increases in both TNF-α mRNA and protein in comparison to UVB-treated control KB cells. However, UVB treatment up-regulated cyclooxygenase-2 mRNA levels to the same extent in both PAF-R-expressing and control KB cells. Pretreatment with the antioxidant vitamin E or the PAF-R antagonists WEB 2086 and A-85783 inhibited UVB-induced TNF-α production in the PAF-R-positive but not control KB cells. These studies suggest that the epidermal PAF-R may be a pharmacological target for UVB in skin.

Ultraviolet B radiation (280–320 nm; UVB) can have profound effects upon human keratinocytes. Acute short term UVB absorption by keratinocytes results in oxidative stress and DNA damage (1–3). UVB can also induce cytokine production in keratinocytes including interleukin (IL)-1, IL-6, IL-8, IL-10, and tumor necrosis factor α (TNF-α) (4–6). Originally described as a cytotoxic factor for proliferating tumor cells, TNF-α has a wide range of pro-inflammatory and cytotoxic effects (reviewed in Ref. 7). Ultraviolet radiation-induced TNF-α production in keratinocytes has been implicated in UVB-induced inflammation and epidermal cell apoptosis (8).

In addition to its ability to induce the production of protein cytokines, ultraviolet radiation can induce the production of lipid mediators such as prostanoids and platelet-activating factor (1-alkyl-2-acetyl-glycerol-3-phosphocholine (PAF)) in epidermal cells (9–12). Derived from glycerophospholipids, PAF is a potent activator of many cell types including platelets, monocytes, polymorphonuclear leukocytes, mast cells, and vascular endothelium cells (reviewed in Ref. 13). PAF also has trophic effects on diverse cell types (14, 15). Although this glycerophospholipid mediator can be metabolized to potentially biologically active neutral lipid or phosphatidic acid species (16, 17), the majority of PAF effects are thought to be mediated through a single G protein-linked transmembrane receptor (PAF-R) (reviewed in Ref. 18). PAF is the best characterized ligand for the PAF-R; yet other natural products can bind to and signal through this receptor. These other ligands include oxidized phospholipids derived from low density lipoproteins (19, 20), lipopolysaccharide and protein A (21), lipoteichoic acid moieties on Streptococcus species (22), and 1-acyl-2-acetylglycerophosphocholines (23, 24). This diversity of ligands recognized by the PAF-R could potentially allow involvement of this system in a wide range of pathological conditions including oxidative damage and bacterial infection.

Recent studies suggest that the PAF system is involved in keratinocyte function and skin inflammation. Indeed, PAF is found in association with inflammatory skin diseases (24, 25), intradermal injections of PAF induce inflammation (24, 26), and human keratinocytes both synthesize PAF and 1-acyl PAF species as well as express functional PAF-Rs (27–29). Activation of the epidermal PAF-R leads to the production of PAF, prostaglandins, IL-6, IL-8, and the inducible form of cyclooxygenase (COX-2) (30).

It is not presently known whether the PAF system participates in UVB-induced cytokine production. However, ultraviolet radiation has been reported to be a stimulus for PAF biosynthesis in corneal epithelial cells (11). In addition, cytokines and the oxidative stress generated in response to UVB irradiation in epidermal cells can cause PAF production in other cell types (31–33).

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‡ The abbreviations used are: IL, interleukin; UVB, ultraviolet B radiation; PAF, platelet-activating factor; PAF-R, PAF receptor; KBP, CPAF; 1-hexadecyl-2-acetylglycerol-3-phosphocholine; TNF-α, tumor necrosis factor α; [Ca2+]i, intracellular calcium concentration; COX-2, cyclooxygenase type 2.
The objective of these studies was to assess whether PAF-R activation can modulate UVB-induced TNF-α production. Using a model system our laboratory has developed by retroviral-mediated gene transduction to express the human PAF-R in the PAF-R-negative human epidermoid cell line KB (24, 30), we present evidence indicating that the PAF-R can modulate UVB-induced TNF-α production, in part by the production of a soluble PAF-R agonistic activity that is structurally not PAF nor 1-acyl PAF.

MATERIALS AND METHODS

Reagents—Routine chemicals, PAF, 1-hexadecyl-2-N-acetyl-3-glycerophosphocholine (CPAF), α-tocopherol (vitamin E), and fatty acid-free bovine serum albumin were obtained from Sigma. Growth media and supplements were purchased from Life Technologies, Inc., and fetal bovine serum was from Intergen (Purchase, NY). The PAF-R antagonists were kindly provided as follows: WEB 2086 from Boehringer Ingelheim (Ridgefield, CT), CV-6209 from Takeda Chemical, Ltd., and MK-886 from Dr. James Summers, Abbot Pharmaceuticals (Abbott Park, IL).

KB PAF-R Model System—The epithelial cell line KB was cultured as described previously (28, 29). KB cells were transduced with the MSCV2.1 retrovirus containing the human leukocyte PAF-R cDNA as described previously (24, 30). KB cell clones transduced with PAF-R (KBP) or with control MSCV2.1 retrovirus (KBM) were characterized by Southern and Northern blot analysis and by binding and calcium mobilization studies to demonstrate that the KB PAF-R was functional (24, 30). All experiments were replicated with at least two different KB or KBM clones.

UVB Irradiation—Epidermal cells were irradiated as described previously (12). For studies examining PAF-R agonistic activity, 10-cm dishes containing KBM cells (90% confluent) were washed three times with HBSS, and 1 ml of prewarmed (37°C) HBSS with 0.25% bovine serum albumin was added and then UVB-irradiated (FS20 Westinghouse Electric Corp., Pittsburgh, PA). Immediately after irradiation, the reaction was quenched with ice-cold methanol, and lipids were extracted (34). The chloroform-containing fraction was dried under a stream of nitrogen gas and brought up in absolute ethanol for calcium mobilization studies or select ion monitoring gas chromatography/mass spectrometry as described previously (12).

TNF-α Measurements—Total RNA from KBM cells was extracted, and 10 μg of RNA was subjected to Northern blot analysis exactly as described previously (30). Human glyceraldehyde-3-phosphate dehydrogenase and TNF-α cDNA probes were obtained from American Type Culture Collection (Rockville, MD); human COX-1 and COX-2 cDNA were kind gifts from Dr. Jana Stankova (University of Sherbrooke, Culture Collection (Rockville, MD); human COX-1 and COX-2 clones were used for their mRNA levels in KBM, but not KBM cells, induced transient intracellular calcium mobilization responses in KBP cells (Fig. 2). CPAF treatment of KBM cells did not result in a significant TNF-α protein secretion. However, treatment of KBM cells with 10 μM PMA did result in increased TNF-α release (data not shown), indicating that the PAF-R is not necessary for the production/release of this cytokine in these cells.

The Effects of UVB on the Production of PAF-like Species in KB Cells—Ultraviolet radiation has been shown to stimulate PAF biosynthesis in epidermal cells (11). Our previous studies used gas chromatography/mass spectrometry to demonstrate that UVB treatment of KB cells resulted in the biosynthesis of PAF and 1-acyl PAF in KBM but not KBM cells (12). Inasmuch as preincubation with PAF-R antagonists and antioxidants inhibited UVB-induced KBF PAF biosynthesis, we hypothesized that this pro-oxidative stressor triggered the production of oxidatively modified phospholipids, which then acted upon the PAF-R-positive KBP (but not KBM cells) to stimulate PAF biosynthesis. To test this hypothesis, lipids were extracted from KBM cells after UVB irradiation and tested for PAF-R agonistic activity by bioassay. Lipid extracts from UVB-irradiated KBM, but not untreated KBM cells, induced transient intracellular calcium mobilization responses in KBM cells (Fig. 3). The peak changes in [Ca²⁺]i, from the lipid extracts were compared with a standard curve derived from authentic C-16 PAF (1-hexadecyl-2-N-acetyl-3-glycerophosphocholine) treatment of KBM cells. In 6 separate experiments, lipid extracts from KBM cells irradiated with 1000 J/m² contained 782 ± 105 fmol/10⁶ cells (mean ± S.D.) C-16 PAF equivalent biological activity. The Ca²⁺ flux generated by lipid extracts derived from irradiated KBM cells was inhibited by pretreatment of indo-1-loaded KBM cells with the structurally dissimilar PAF-R antagonists CV-6209 (10 μM) and WEB 2086 (1 μM) (data not shown). In addition, treatment of indo-1-loaded KBM cells with was created by transducing KB cells with the replication-defective MSCV2.1 retrovirus containing the entire human PAF-R cDNA (24, 30). By comparing the effects of stimuli on both PAF-R-positive (KBP) and -negative (transduced with empty MSCV 2.1 retrovirus; KBM) KB cells, the role of the PAF-R on cytokine production could be readily assessed.

The Effects of CPAF on TNF-α Production in KB Cells—Our first studies assessed the ability of PAF-R activation to stimulate TNF-α production using the KBP-PAF-R model system. KBM and KBM cells were treated with 100 nM metabolically stable PAF-R agonist CPAF for various times, and TNF-α mRNA or released protein was measured. As shown in Fig. 1, incubation of KBM, but not KBM cells, with CPAF resulted in an increased accumulation of TNF-α mRNA. Increased cytokine mRNA was first seen by 30 min, was maximal at 1–2 h, and returned to base line by 6 h. Consistent with the Northern blotting data, CPAF treatment of KBM cells resulted in an increase in immunoreactive TNF-α protein secretion as shown in Fig. 2. CPAF treatment of KBM cells did not result in a significant TNF-α protein secretion. However, treatment of KBM cells with 10 nM PMA did result in increased TNF-α release (data not shown), indicating that the PAF-R is not necessary for the production/release of this cytokine in these cells.

RESULTS

The KB PAF-R Model System—Since PAF may have both receptor-dependent and -independent effects (secondary to the formation of biologically active metabolites), a model system was developed to study the role of the PAF-R in epidermal cell function. This system utilizes the human epidermal cell line KB, which, unlike normal human keratinocytes, does not express functional PAF-Rs (28, 29). A PAF-R-positive KB cell line

FIG. 1. The effect of CPAF treatment on TNF-α mRNA levels in KB cells. KBP or KBM cells were incubated with 100 nM CPAF, and RNA was extracted at various times and subjected to Northern blot analysis using probes for TNF-α or glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The results shown are typical for at least two separate KBM or KBM clones.
lipid extracts from UVB-treated KBM cells did not result in an increase in [Ca\(^{2+}\)]. Three of the six UVB-irradiated KBM extracts were subjected to select ion gas chromatography/mass spectrometry with deuterated PAF and 1-acyl PAF internal standards (29). In these specimens, less than 100 fmol/10^6 cells of either PAF or 1-palmitoyl acyl PAF species were found, consistent with our previous report that UVB does not stimulate the production of PAF or 1-acyl PAF species in PAF-R-negative KB cells (12). Altogether, these findings indicate that UVB can stimulate the formation of a PAF-R agonistic activity, which our previous mass spectrometry studies indicate is structurally not PAF nor acyl-PAF.

**The Effects of UVB on TNF-α Production in KB Cells**— Keratinocytes have been shown to synthesize TNF-α in response to UVB (4, 6). Given the findings that PAF-R activation is a potent stimulus for TNF-α production in epidermal cells and UVB can induce a PAF-R agonistic activity, the next experiments sought to define whether the presence of the PAF-R can modulate UVB-induced TNF-α biosynthesis. KBM and KBP cells were treated with 400 J/m\(^2\) UVB, and TNF-α mRNA or released protein was measured at various times after UVB irradiation. As shown in Fig. 4, UVB irradiation of KBP cells resulted in a significant increase in the accumulation of TNF-α mRNA in comparison with similarly treated KBM cells. The time course of TNF-α mRNA accumulation in UVB-treated KBP cells appeared to be somewhat similar although of slightly longer duration than that seen in CPAF-treated KBM cells. Unlike UVB-induced accumulation of TNF-α mRNA, COX-2 mRNA levels appeared essentially equal in KBP versus KBM cells (Fig. 4). Both KBM and KBP cells contained similar amounts of COX-1 mRNA levels, which were not affected by CPAF (30) or UVB (not shown) treatment. Consistent with the Northern blotting data, UVB treatment of KBP cells resulted in increased TNF-α protein secretion in KBP over KBM cells (Fig. 5). As has been previously reported for wild-type KB (4), UVB treatment of KBM cells did result in increased levels of TNF-α protein release, albeit at much lower amounts than in KBP cells.

The ability of pretreatment with a PAF-R antagonist or antioxidant to inhibit UVB-stimulated TNF-α release in KBP cells was next assessed. As shown in Fig. 6A, pretreatment of KBP cells with the antioxidant vitamin E inhibited subsequent TNF-α production in response to UVB stimulation. Similarly, other antioxidants 1,1,3,3-tetramethylthiourea (1 mM) and N-acetyl cysteine (10 mM) inhibited UVB-induced TNF-α production in KBP cells (data not shown). However, vitamin E (or other antioxidants) did not affect TNF-α release in response to CPAF (Fig. 6B). In addition, preincubation of KBP cells with 10 μM PAF-R antagonists WEB 2086 (not shown) inhibited both UVB- and CPAF-induced TNF-α production (Fig. 6A and B). However, vitamin E or these PAF-R antagonists did not affect TNF-α production in response to UVB in KBM cells (Fig. 6C). Incubation of KBM cells with antioxidants or PAF-R antagonists did not affect base-line TNF-α release (data not shown). Altogether, these findings support the hypothesis that UVB can stimulate the formation of a soluble PAF-R agonistic activity, which then can act upon a PAF-R-expressing cell to augment TNF-α production.

**DISCUSSION**

These studies provide evidence that the epidermal PAF-R may be involved in UVB-induced cytokine production. The study of PAB/PAF-R is limited by the rapid metabolism of PAF and the fact that PAF metabolites can exert biological activity independent of the PAF-R (16, 17). The model system used in these studies was developed to overcome some of the current limitations in the study of PAF/PAF-R and to account for the diverse ligands recognized by the PAF-R (19–24). In particular, this model system can account for non-PAF PAF-R agonists such as sn-2 short chain phosphocholines, which have been shown to be produced in response to lipid peroxida (reviewed in Refs. 35 and 36).
Activation of the KB PAF-R resulted in an increased accumulation of TNF-α mRNA and protein. Similarly, PAF treatment of monocytes and endothelial cells also has been reported to increase TNF-α biosynthesis (37, 38). Inasmuch as TNF-α is a known stimulus for PAF biosynthesis in these same cell types (31), this lipid mediator and cytokine could be involved in pathophysiological effects ascribed to each other through positive feedback mechanisms. Characterization of this complex relationship between PAF and TNF-α could have therapeutic implications given the availability of PAF-R antagonists.

Our previous studies have demonstrated that expression of the PAF-R leads to enhanced apoptosis in response to UVB treatment (12). This augmentation of UVB-induced apoptosis seen in KBP over KBM cells was inhibited by pretreatment with antioxidants and PAF-R antagonists. The present experiments show that UVB treatment can generate a biologically active PAF-like activity in KB cells. However, mass spectrometry studies indicate these lipid extracts do not contain PAF or 1-acyl PAF species (12). These findings are compatible with the hypothesis that the pro-oxidative stressor UVB can generate oxidized phospholipids with PAF-like activity. Recent evidence from several laboratories indicate that free radical-induced oxidation of unsaturated fatty acids in the sn-2 position of glycerophosphocholines results in non-PAF PAF-R agonists (39, 40). This activity has been demonstrated in association with oxidized low density lipoprotein and after exposure of rodents to the known oxidant cigarette smoke (41). The production of this potent biological activity would be expected to be dependent upon the amount of free radical-induced damage and the redox state of the cell, unlike the tightly regulated enzymatic biosynthesis of native PAF. Although this non-PAF PAF-R activity generated in response to oxidative stress has been reproduced in several laboratories in various model systems, the exact structures of the active metabolites are not known at this time.

Consistent with the ability of UVB to generate PAF-R agonistic activity, UVB irradiation resulted in increased TNF-α mRNA accumulation and protein release in KBP over KBM cells. That UVB appeared to increase COX-2 mRNA levels independent of PAF-R expression suggests different mechanisms.
nisms for the induction of these two UVB-induced proteins. The significance of COX-2 in epidermal function is not clear. However, high amounts of COX-2 protein have been reported in carcinomas (42). The ability of the known tumor initiator and promoter UVB to up-regulate this protein in epidermal cells in vitro as well as in vivo has suggested possible involvement of COX-2 in epidermal carcinogenesis (43).

In addition to the ability of UVB to generate PAF-R agonistic activity, several other possible mechanisms exist that could explain in part the ability of the PAF-R to augment UVB-induced TNF-α production. One possibility is that UVB could have inhibitory effects on PAF-acetylhydrolase enzymes. Indeed, oxidative stress has been reported to inactivate these enzymes that serve to degrade PAF and short-chained sn-2 glycerophosphocholines (44). A second possibility is that UVB is activating the PAF-R directly. Although UV radiation has been reported to activate growth factor or TNF type 1 receptors (45, 46), it is not known whether UV can activate a G protein membrane receptor. The ability of preincubation of KBP cells with antioxidants or the PAF-R antagonists WEB 2086 and A-82587 to inhibit this UV-induced augmentation of TNF-α production in KBP cells was not expected to differentiate PAF-R activation due to the production of a soluble PAF-R activity versus direct activation. However, the finding of a soluble PAF-R activity in UVB-irradiated KB cells suggests that this activity is responsible for a component of UVB-induced PAF-R activation. We hypothesize oxidized lipids with PAF-R activity as the source of this PAF-R agonistic activity in KB cells. Future studies will attempt to structurally characterize this PAF-like activity as well as to define whether UVB can activate G protein receptors directly.

Although human keratinocytes synthesize PAF (27, 29) and express functional PAF-Rs (28), the role of the PAF-R in epidermal cell function is not clear. These studies suggest that one of the functions of the PAF-R in epidermal keratinocytes could be to augment production of the known proinflammatory and cytotoxic cytokine TNF-α in response to UVB radiation. Although UVB can stimulate more TNF-α production in KB over KBM cells, ongoing studies indicate that the augmentation of UVB-induced cytokine seen in KB over KBM cells (12) is not affected by pretreatment with the protein synthesis inhibitor cycloheximide (0.1 mg/ml) or RNA synthesis inhibitor actinomycin D (0.1 mg/ml). The lack of effects by these inhibitors at dosages that inhibit keratin protein/RNA synthesis suggests that endogenous TNF-α production does not play an important role in PAF-R augmentation of UVB-induced apoptosis in these cells. However, increased TNF-α production by epidermal cells could certainly modulate cutaneous inflammation given the potent proinflammatory effects of this cytokine (7).

The ability of the PAF-R to recognize oxidatively modified glycerophosphocholines produced in response to noxious stimuli and produce pro-inflammatory cytokines such as IL-6, IL-8, and TNF-α fits with the notion that the epidermal PAF-R may act as an endogenous “damage sensor.” Inasmuch as certain populations are potentially more susceptible to PAF effects due to inherited or acquired PAF-acetylhydrolase deficiencies (reviewed in Ref. 47), these findings may have clinical implications. A better understanding of the functions of the PAF system in keratinocyte biology and cutaneous inflammation may lead to therapeutic interventions designed around this lipid mediator.

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