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Augmentation of Chemotherapy-Induced Cytokine Production by Expression of the Platelet-Activating Factor Receptor in a Human Epithelial Carcinoma Cell Line

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In addition to their known cytotoxic effects, chemotherapeutic agents can trigger cytokine production in tumor cells. Moreover, many chemotherapeutic agents are potent pro-oxidative stressors. Although the lipid mediator platelet-activating factor (PAF) is synthesized in response to oxidative stress, and many epidermal carcinomas express PAF receptors (PAF-R) linked to cytokine production, it is not known whether PAF is involved in chemotherapeutic agent-induced cytokine production. These studies examined the role of the PAF system in chemotherapy-mediated cytokine production using a model system created by retroviral-mediated transduction of the PAF-R-negative human epidermal carcinoma cell line KB with the human PAF-R. The presence of the PAF-R in KB cells resulted in augmentation of the production of cytokines IL-8 and TNF-α induced by the chemotherapeutic agents etoposide and mitomycin C. These effects were specific for the PAF-R, as expression of the G protein-coupled receptor for fMLP did not affect chemotherapeutic agent-induced cytokine production. Moreover, ablation of the native PAF-R in the epithelial cell line HaCaT using an inducible antisense PAF-R strategy inhibited etoposide-induced cytokine production. Oxidative stress and the transcription factor NF-κB were found to be involved in this augmentative effect, because it was mimicked by the oxidant tert-butyl-hydroperoxide, which was blocked both by antioxidants and by inhibition of the NFκB pathway using a super-repressor 1κBBM mutant. These studies provide evidence for a novel pathway by which the epidermal PAF-R can augment chemotherapeutic-induced cytokine production through an NF-κB-dependent process.


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these studies and was found to be dependent on activation of NF-κB proteins, sequence-specific transcription factors induced in response to inflammatory and other stressful stimuli (reviewed in Ref. 21). The current findings describe a putative mechanism by which this GPCR can augment the cytokine-producing effects of chemotherapeutic agents in epithelial carcinomas.

Materials and Methods

Reagents

All chemicals were obtained from Sigma-Aldrich (St. Louis, MO) unless indicated otherwise.

Cell culture

The human epidermoid cell line KB was grown in DMEM (Life Technologies, Gaithersburg, MD) supplemented with 10% FBS (HyClone, Logan, UT). A KB PAF-R model system was established by transduction of PAF-R-negative KB cells with the MSCV2.1 retrovirus encoding the human leukocyte PAF-R as described previously (16). KB cells transduced with the PAF-R (KBP) or with control MSCV2.1 retrovirus (KBM) were characterized by Southern and Northern blot analyses and by radioligand binding and calcium mobilization studies to determine that the PAF-R was functional (16). Similarly, to create a KB cell line expressing the fMLP receptor (KFB), the fMLP-R cDNA was cloned into the MSCV2.1 retroviral vector. The presence of a functional fMLP-R in KFB cells was confirmed by Northern blotting to demonstrate fMLP-R mRNA and a positive intracellular calcium mobilization response to exogenous fMLP using the fluorescent dye fura-2-acetoxymethyl ester as previously described (22). All experiments were replicated with at least two separate KBM, KFB, and KBF clones.

Generation of HaCaT cells expressing an inducible PAF-R antisense

HaCaT as-PAF-R model system was established using the RevTet-on system (Clontech Laboratories, Palo Alto, CA) as previously described (22). PAF-R cDNA was cloned in a reversed orientation into the RevTet-On system consisting of both PAF-R-negative (KBM) and -positive (KBP) cells. As PAF may have both receptor-dependent and -independent effects (secondary to the formation of biologically active metabolites), our laboratory has previously created a model system by transduction of the PAF-R into a PAF-R-deficient epidermal cell line to study the role of the PAF-R in epithelial cell biology. The human epidermal carcinoma cell line KB does not express functional PAF-Rs, unlike normal human keratinocytes and the human keratinocyte-derivative carcinoma cell line HaCaT (15). A PAF-R-positive KB cell line, KBP, was created by transducing KB cells with a replication-deficient MSCV2.1 retrovirus containing the human PAF-R cDNA. KB cells were also transduced with the retrovirus backbone alone to establish a vector control cell line, KBM. Expression of the PAF-R protein was verified by binding studies using radiolabeled PAF-R antagonist WEB-2086 (16). Calcium mobilization studies demonstrated that the KB PAF-R was functionally active (16). Therefore, this in vitro epidermoid system consists of both PAF-R-negative (KBM) and -positive (KBP) cells.

Effects of chemotherapeutic agents on cytokine production in KB cells

The first studies assessed the ability of the chemotherapeutic agents etoposide and mitomycin C to induce the production of IL-8 in KB cells. As shown in Fig. 1, treatment of KB cells with the PAF-R agonist CPAF resulted in an accumulation of IL-8 mRNA

**FIGURE 1.** The effects of chemotherapeutic agents on IL-8 mRNA levels in KB cells. KBP and KMB cells were incubated with 100 nM CPAF, 1 μM PMA, or the chemotherapeutic agents etoposide (6 μg/ml) and mitomycin C (5 μg/ml). RNA was extracted at various times and subjected to Northern blot analysis using probes for IL-8 or GAPDH.
selectively in PAF-R-positive KBP, but not control KBM cells. However, PMA treatment resulted in similar levels of IL-8 mRNA in KBP (not shown) and KBM cells (Fig. 1). Treatment with the chemotherapeutic agents etoposide and mitomycin C resulted in increased levels of IL-8 mRNA, which was more apparent in KBP cells (Fig. 1). It should be noted that unlike the PAF-R ligand CPAF, which induced IL-8 mRNA by 1 h, chemotherapeutic agent-induced effects were not seen until several hours. Consistent with the Northern blotting data, etoposide and mitomycin C treatment of KBP cells resulted in an increased release of IL-8 protein compared with KBM cells (Fig. 2). PMA-induced IL-8 protein release was similar in KBM and KBP cells (Fig. 2). As shown in Fig. 3, TNF-α production was also selectively enhanced in KBP cells treated with etoposide. Similarly, mitomycin C induced more TNF-α protein release in KBP over KBM cells (data not shown). These studies indicate that expression of the PAF-R in a carcinoid cell line results in enhanced cytokine production in response to etoposide and mitomycin C.

To assess whether the PAF-R-mediated enhanced cytokine production in response to chemotherapeutic agents is a general characteristic of GPCR, KB cells were transduced with the fMLP-R. This GPCR was chosen as a control because epithelial cells, unlike bacteria, do not produce the peptide ligand fMLP. Calcium mobilization assays with fura-2-loaded KBF cells resulted in intracellular calcium responses to 100 nM fMLP confirmed that the fMLP-R was functional (data not shown). As shown in Fig. 4, treatment of KBF cells with fMLP resulted in increased release of IL-8. Consistent with the idea that chemotherapeutic agents are not activating GPCRs in a ligand-independent fashion, transducing KB cells with the fMLP-R did not affect responses to etoposide (Fig. 4).

Effects of the endogenous PAF-R on etoposide-induced IL-8 production

The next studies were designed to assess whether the levels of PAF-R expression found on the epithelial cell line HaCaT (15) could modulate chemotherapeutic agent-induced cytokine production. To address this question, HaCaT keratinocytes that express native PAF-Rs were transduced with a retrovirus from which an antisense RNA corresponding to the PAF-R mRNA could be induced (HaCaTpRevTRE-as-PAF-R). This model system has been previously characterized, and measurement of intracellular calcium flux or IL-8 production in response to CPAF was used to confirm that treatment of HaCaTpRevTRE-as-PAF-R cells with doxycycline resulted in ablation of endogenous PAF-R expression (22). As shown in Fig. 5, doxycycline (10 mg/ml) treatment of HaCaTpRevTRE-as-PAF-R cells inhibited CPAF-induced, but not PMA-induced, IL-8 production in HaCaTpRevTRE-as-PAF-R cells. It should be noted that doxycycline pretreatment did not affect Ca²⁺ mobilization responses (not shown) or IL-8 production by the nonmetabolizable PAF-R agonist CPAF in control HaCaTpRevTRE cells (Fig. 5).

Ablation of the endogenous PAF-R in HaCaT cells resulted in diminished IL-8 production due to the chemotherapeutic agent etoposide. It should be noted that the modest decrease in etoposide-induced IL-8 production in HaCaTpRevTRE-as-PAF-R cells compared with HaCaTpRevTRE (Fig. 5) was less than the differences between KBP and KBM cells (Fig. 2). This fits with the decreased magnitude of IL-8 production induced by CPAF in HaCaT vs KBP cells and is probably due to the decreased levels of PAF-R expression found in HaCaT compared with KBP cells (16). These studies indicate that endogenous levels of PAF-R expression in carcinomas are adequate to modulate chemotherapeutic agent-induced cytokine production.
Role of oxidative stress on PAF-R augmentation of chemotherapeutic agent-induced cytokine production

Our previous studies using a fluorescent H$_2$O$_2$-sensitive dye demonstrated that etoposide and mitomycin C treatment of KB carcinoma results in increased levels of intracellular H$_2$O$_2$ (22). The next studies used an antioxidant to confirm the involvement of oxidative stress in the ability of chemotherapeutic agents to activate the epidermal PAF-R and thus modulate IL-8 production. Pretreatment of KB cells with the antioxidant trolox blunted etoposide-mediated IL-8 release in KBP, but not KBM, cells and tended to negate the effect of the PAF-R in KBP cells (Fig. 6). However, trolox did not affect CPAF-induced IL-8 release in KBP cells. Similarly, pretreatment of KB cells with 10 mM of the antioxidant reservatrol resulted in effects similar to those of trolox (not shown). Consistent with the idea that oxidative stress could induce cytokines via activation of the epidermal PAF-R, exposure of KB cells to the potent pro-oxidative stressor tert-butyl-hydroperoxide resulted in cytokine production only in KBP cells (Fig. 7). These effects of tert-butyl-hydroperoxide were inhibited by preincubation with the antioxidants trolox (Fig. 7) and reservatrol (not shown).

Involvement of the NF-κB system in PAF-R-mediated augmentation of chemotherapeutic agent-induced cytokine production

The following studies were designed to assess the mechanism by which the PAF-R could augment chemotherapeutic agent-induced cytokine production. Indeed, activation of the epidermal PAF-R is linked to numerous signal transduction systems that could be responsible for IL-8 production. It should be noted that, like a diverse group of stimuli including pro-oxidative stressors, the epidermal PAF-R induces the transcription factor NF-κB, and activation of this same pathway has also been reported to induce IL-8 production (24).

To test whether PAF-R-mediated activation of the NF-κB pathway was involved in the augmentation of chemotherapy-mediated cytokine production.
IL-8 production, we examined whether a dominant negative inhibitor of NF-κB could affect PAF-R-mediated augmentation of cytokine production in response to these chemotherapeutic agents. To that end, KBP cells were transduced with a super-repressor IκB protein (KBP-MIEG-IκBM) or vector control (KBP-MIEG). We have previously shown that increased IκBo degradation and increased NF-κB binding activity induced by PAF-R agonist CPAF or IL-1β were blocked in these cells transduced with this mutant nondegradable IκB protein (22, 23). As shown in Fig. 8, blockade of the NF-κB pathway inhibited the IL-8 production induced by the PAF-R agonist CPAF as well as that induced by etoposide and mitomycin C. However, IL-8 production induced by the protein kinase C agonist PMA was essentially equal in KBP-MIEG and KBP-IκBM cells. These findings suggest that PMA-mediated IL-8 production in KB cells is independent of the NF-κB pathway. It should also be noted that our previous studies have indicated that PMA-induced IL-6 and IL-8 production is similar in KBM and KBP cells, indicating that PMA effects are independent of the PAF-R (16). These findings fit with previous reports that phorbol esters do not induce PAF biosynthesis (25). The present studies indicate that activation of the NF-κB pathway is responsible for PAF-R augmentation of chemotherapeutic agent-induced cytokine production (Fig. 8).

Discussion
These studies provide evidence that in epithelial carcinoma cells, the PAF-R can augment cytokine production induced by chemotherapeutic agents via an NF-κB-dependent process. The augmentation of chemotherapeutic agent-induced cytokine production was mediated through the PAF-R, as it was only seen in PAF-R-expressing KBP carcinoma cells. Our results describe a novel function for a GPCR, namely, the induction of an NF-κB-dependent pathway by which the PAF-R can augment the cytokine production of chemotherapeutic agents.

Recent studies have shown that oxidative stress can trigger the production of lipids with PAF-R agonist activity. For example, chemical oxidation of low density lipoproteins results in the production of a PAF-R activity that has been shown to consist of fragmented alkyl GPC including 1-hexadecyl-2-butanoyl-GPC and 1-hexadecyl-2-butenoyl-GPC, along with trace levels of authentic PAF (18). Of significance, systemic exposure to the strong oxidative stress of tobacco smoke has been shown to induce the production of these PAF-R agonists in hamsters in vivo (26). Other pro-oxidative stressors, such as UV-B, can also stimulate the PAF-R through production of PAF and PAF-like lipids (13). Several lines of evidence suggest that the chemotherapeutic agents etoposide and mitomycin C are activating the PAF-R through the production of PAF/PAF-like species. First, these chemotherapeutic agents are known pro-oxidative stressors (22, 27, 28). Second, pretreatment with antioxidants blocked the augmentation of chemotherapy-mediated cytokine production found in PAF-R-expressing KBP cells. The finding that expression of the GPCR for fMLP in KB cells (KBF) did not affect chemotherapy-induced IL-8 production suggests that these agents were not nonspecifically activating the GPCR in a ligand-independent fashion.

The mechanism by which chemotherapeutic agents can activate the PAF-R is most likely via production of PAF-R agonistic activity, rather than acting directly as PAF-R agonists. Consistent with this, etoposide and mitomycin C are unable to trigger an intracellular Ca2+ mobilization response in fura-2-loaded KBP cells (data not shown). This is in contrast to a PAF-R agonist such as CPAF, which induces an almost immediate Ca2+ flux (16). The lag time in IL-8 mRNA accumulation in response to etoposide vs the more rapid response seen with CPAF in KBP cells (Fig. 1) also fits with the idea that chemotherapeutic agents are inducing the biosynthesis of a PAF-R agonistic activity rather than acting as direct PAF-R agonists. Thus, the data presented in these studies support the hypothesis that these chemotherapeutic agents have the ability to induce the production of PAF/PAF-like species. Ongoing studies are attempting to define the structural identity of chemotherapeutically activated PAF-R agonistic activity.

The current studies also begin to define mechanistically how PAF-R activation can augment cytokine production by chemotherapeutic agents. GPCR, including the PAF-R, have been shown to activate the NF-κB system (22, 23). Consistent with the involvement of NF-κB in the augmentation of chemotherapeutic-induced cytokine production, we have recently reported that etoposide and mitomycin C treatment of KBP cells resulted in much greater levels of NF-κB binding activity than those in KBM cells (22). Blocking NF-κB activation with a super-repressor IκB mutant decreased the augmentative effect of PAF-R expression on chemotherapy-induced IL-8 production (Fig. 7).

Although expression of the fMLP-R in KB cells did not affect chemotherapeutic-induced IL-8 production, this receptor was found to have in common with the PAF-R the ability to activate the NF-κB pathway. Indeed, fMLP treatment of KBF cells resulted in enhanced NF-κB binding by gel shift assays (22). Transfection of KBF cells with an NF-κB-luciferase reporter plasmid to measure NF-κB activity also revealed increased levels in KBF cells in response to fMLP (22). The ability of the fMLP-R to stimulate the NF-κB system, yet not affect chemotherapeutic-induced responses of NF-κB activation or cytokine production, supports the contention that these pro-oxidative chemotherapeutic agents activate the epidermal PAF-R via production of endogenous ligands.

The present studies using antioxidants also suggest that chemotherapeutic agent-induced cytokine production does not appear to depend upon the pro-oxidative effects of the agents. Indeed, incubation of KBM cells with trolox did not have a significant effect on etoposide-induced IL-8 production (Fig. 6), yet ablated that induced by tert-butyl-hydroperoxide (Fig. 7). That and the significant difference in IL-8 production between the potent oxidant tert-butyl-hydroperoxide vs the weaker oxidant etoposide fit with a less important role of oxidative stress in chemotherapeutic-mediated cytokine production. However, the presence of the PAF-R allows the cells to respond to chemotherapeutic agent-generated oxidative stress to augment cytokine production.

The significance of chemotherapeutic-induced cytokine production is unclear. However, the ability of populations to degrade PAF and PAF-like lipids differs greatly, with both acquired and inherited deficiencies of PAF acetylhidrolases described (29). Thus, it is possible that pro-oxidative chemotherapeutic agent-induced PAF activity might be handled differently in different populations, which could result in either increased or possibly decreased tumor cell killing or increased chemotherapeutic-induced side effects.

In summary, the present studies demonstrate that chemotherapeutic agents such as etoposide and mitomycin C can induce the production of the cytokines IL-8 and TNF-α in a human epithelial carcinoid cell line. Expression of the epidermal PAF-R results in an augmentation of chemotherapeutic agent-mediated cytokine production via a pro-oxidative process involving the production of PAF-R agonists. We describe a novel pathway by which these pro-oxidative stressors induce PAF/PAF-like species, which then activate the NF-κB pathway through the PAF-R. The biological significance of this pathway is not clear, but could be the impetus for further studies to define whether the presence of the PAF-R could modulate chemotherapeutic responses in vivo. Finding that this novel pathway is an important determinant in the effectiveness and/or side effect profile of certain chemotherapeutic agents would...
be useful for planning chemotherapeutic strategies in PAF-R-expressing tumor cells.

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References

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