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Differential Expression of Platelet-Activating Factor Acetylhydrolase in Macrophages and Monocyte-Derived Dendritic Cells

Salma Al-Darmaki, Harvey A. Schenkein, John G. Tew, and Suzanne E. Barbour

Although macrophages (Mφ) and monocyte-derived dendritic cells (MDDC) come from a common precursor, they are distinct cell types. This report compares the two cell types with respect to the metabolism of platelet-activating factor (PAF), a biologically active lipid mediator. These experiments were prompted by our studies of localized juvenile periodontitis, a disease associated with high IgG2 production and a propensity of monocytes to differentiate into MDDC. As the IgG2 Ab response is dependent on PAF, and MDDC selectively induce IgG2 production, we predicted that PAF levels would be higher in MDDC than in Mφ. To test this hypothesis, human MDDC were prepared by treating adherent monocytes with IL-4 and GM-CSF, and Mφ were produced by culture in M-CSF. Both Mφ and MDDC synthesized PAF; however, MDDC accumulated significantly more of this lipid. We considered the possibility that PAF accumulation in MDDC might result from reduced turnover due to lower levels of PAF acetylhydrolase (PAFAH), the enzyme that catabolizes PAF. Although PAFAH increased when monocytes differentiated into either cell type, MDDC contained significantly less PAFAH than did Mφ and secreted almost no PAFAH activity. The reduced levels of PAFAH in MDDC could be attributed to lower levels of expression of the enzyme in MDDC and allowed these cells to produce PGE2 in response to exogenous PAF. In contrast, Mφ did not respond in this manner. Together, these data indicate that PAF metabolism may impinge on regulation of the immune response by regulating the accessory activity of MDDC. The Journal of Immunology, 2003, 170: 167–173.

Monocytes are a common precursor for two immune cells, macrophages (Mφ) and monocyte-derived dendritic cells (MDDC). Both monocytes and Mφ are components of the mononuclear phagocyte system. Mφ are important phagocytic cells that capture invading microbes and participate in eliminating them by either direct cytolyis or by presenting peptides to T cells and inducing specific immunity. Mφ also secrete cytokines and lipid mediators that regulate the immune response, including TNF-α, IL-1, IL-6, platelet-activating factor (PAF), and PGE2 (1–4). Like Mφ, immature MDDC are highly phagocytic cells, and Ag capture triggers MDDC maturation. Mature MDDC migrate to secondary lymphoid tissues, where they exhibit increased expression of the class II MHC proteins and co-stimulatory molecules associated with Ag presentation. Thus, like Mφ, mature MDDC are highly efficient APC. MDDC also regulate the immune response, but they differ from Mφ in that they can either enhance or suppress cell-mediated immunity in response to environmental cues (5, 6). Recent studies indicate that MDDC can regulate the immune response by secreting cytokines (such as IL-12), chemokines, and PGE2 (5, 7).

This report compares the changes in platelet-activating factor (PAF) metabolism that occur when monocytes differentiate into either Mφ or MDDC. Our interest in PAF metabolism in MDDC comes from studies of the immune response in localized juvenile periodontitis (LJP), a periodontal disease characterized by its circumpubertal onset and characteristic localized loss of supporting tissues around first molars and incisors (8). In a previous study we demonstrated that monocytes from LJP subjects have an increased propensity to differentiate into MDDC, while monocytes from healthy non-periodontitis (NP) subjects primarily differentiate into Mφ (9). Moreover, LJP subjects have elevated levels of IgG2 Ab in their sera, and LJP monocytes secrete cytokines that selectively promote the production of this subclass of Ab (10, 11). Most notably, the monocyte-derived lipid mediators, PAF and PGE2, were shown to induce IgG2 production, but had no effect on the production of IgG1 in a pokeweed mitogen-driven in vitro culture system. Furthermore, highly enriched MDDC cultures were shown to have a similar selective effect on IgG2 production (9). Together, these studies suggest that the outgrowth of MDDC in cultures of LJP monocytes may have important implications for the regulation of Ig subclass responses and that this regulation may be mediated through the levels of lipid mediators in the cultures.

Although it is well established that LJP monocytes secrete more PGE2 than NP monocytes (12), PAF metabolism has not yet been compared in these two populations. A previous study demonstrated that PAF levels change dramatically when monocytes differentiate into Mφ (3). Although both Mφ and monocytes can synthesize PAF, monocytes accumulate considerably more of this lipid mediator. This difference can be attributed to the expression of PAF acetylhydrolase (PAFAH), a catalytic phospholipase A2 that selectively hydrolyzes PAF. The activity of this enzyme increases nearly 300-fold when monocytes differentiate into Mφ (3). Given

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the apparent connections between MDDC, PAF, and the production of IgG2 in our model system, we hypothesized that the expression of this enzyme might be considerably lower in MDDC than in mature Mϕ. To investigate this possibility, we compared PAF metabolism in highly enriched cultures of MDDC and Mϕ. Our data indicate that MDDC do indeed accumulate more PAF than do Mϕ and that this accumulation is associated with a reduction in the expression of PAF acetylhydrolase (PAFAH). Moreover, while MDDC synthesize PGE₃ in response to nanomolar concentrations of PAF, Mϕ make no comparable response, presumably because the exogenous PAF is readily catabolized by Mϕ-derivd PAFAH. Together, these data indicate that the differentiation pathway of monocytes has important implications for the levels of PAF and other lipid mediators that are available to regulate the immune response.

**Materials and Methods**

**Human subjects**

Human studies were performed in compliance with all relevant federal guidelines and the institutional policies of Virginia Commonwealth University. Buffy coat preparations were obtained from Virginia Blood Services (Richmond, VA) and were used within 24 h of blood draw. In some experiments subjects for study were located through the Clinical Research Center for Periodontal Disease, School of Dentistry, Virginia Commonwealth University (Richmond, VA). Patients with LJP were 35 years old or less and had localized patterns of severe periodontal destruction limited to the first molar or incisor teeth and up to two additional teeth. The NP control subjects were age- and race-matched and had no evidence of attachment loss, except for recession on the buccal surface of anterior teeth at no more than one site and no pockets > 3 mm. All NP and LJP subjects were nonsmokers.

**Isolation of leukocytes from peripheral blood (PBL)**

Thirty milliliters of heparinized or citrated human peripheral blood was layered over 15 ml of Lymphocyte Separation Media (ICN Pharmaceuticals, Aurora, IL) and centrifuged at 400 × g for 30 min. Buffy coat preparations were obtained from Virginia Blood Services by centrifugation and release. The phenotypes of the cell cultures were confirmed through FACS analyses of CD14low, class II MHC high, and Mϕ-derived PAFAH. Together, these data indicate that the differentiation pathway of monocytes has important implications for the levels of PAF and other lipid mediators that are available to regulate the immune response.

**Real-time PCR**

Real-time PCR was performed by the Nucleic Acid Research Facility at Virginia Commonwealth University. RNA was prepared from Mϕ or MDDC with the TRIzol RNA isolation reagent (Invitrogen/Life Technologies). The primers for the real-time PCR were 5'-CCACGGACACCGTCTC-3' and 5'-CATGTGCTTTTCTGCCTCTG-3', complementary to bp 84–100 and 179–200, respectively. The probe was 5'-CTCCGCCGCGCCGTGAGAAGCT-3' (complementary to bp 110–137). The probes and primers were designed using the Primer Express 1.5 version (Applied Biosystems, Foster City, CA). The TaqMan probe (Applied Biosystems) was labeled in the 5' end with FAM (fluorescein) and in the 3' end with TAMRA.

The experiments were performed with the ABI PRISM 7700 Sequence Detection System (PE Applied Biosystems, Foster City, CA) using the TaqMan One Step PCR Master Mix Reagents Kit (PE Applied Biosystems). The PGE₂ assay was performed with the ECL Western blot detection system (both from Amersham Pharmacia Biotech, Arlington Heights, IL). Western blotting was performed with 10 μg of total protein using 5% SDS-PAGE, and the proteins were transferred onto nitrocellulose using standard methods (16). PAFAH protein was detected with an Ab directed against the C terminus of plasma PAFAH (Cayman Chemical, Ann Arbor, MI). An Ab against Raf-1 (Santa Cruz Biotechnologies, Santa Cruz, CA) was used as a protein loading control. Protein A-HP and the ECL Western blotting detection system (both from Amersham Pharmacia Biotech, Arlington Heights, IL) were used. In a peptide blocking experiment with PAFAH Ab was incubated with 10 μg/ml of PAFAH blocking peptide (Cayman Chemical) for 1 h at room temperature before addition to the blot. This treatment eliminated the 45-kDa PAFAH band (data not shown). In addition to the 45-kDa PAFAH band, anti-PAFAH recognized the blot. This treatment eliminated the 45-kDa PAFAH band (data not shown).

**Preparation of conditioned medium**

Mϕ and MDDC were incubated overnight in serum-free RPMI medium at 37°C in 5% CO₂ and 100% humidity. The cell-free medium was harvested and centrifuged to remove floating cells, and supernatant fluids were collected and stored at -80°C until used.

To measure PAF accumulation, a metabolic labeling assay was performed as previously described (14). Adherent Mϕ or MDDC were incubated 1 ml of HBSS (Cellgro, PeproTech, Rocky Hill, NJ) containing 25 μCi of [³H]acetic acid (NEN, Boston, MA; 2.5 Ci/mmol) for 50 min at 37°C. The cells were harvested and washed twice with HBSS to remove excess radiolabel. Cellular lipids were extracted and [³H]PAF was separated on TLC plates using a previously described method (14). PAF was identified based on comigration with authentic PAF. The PAF band was scraped from the TLC plate, and radiolabeled PAF was quantified by scintillation counting and normalized to cell protein. This assay measures total PAF accumulation and does not distinguish between fatty-acyl PAF and fatty-acyl PAF.

**PAFH assay**

PAFH activity was measured using a previously described method (15) with minor modifications. The substrate consisted of 50 μM 1-alkyl-2-acetyl-sn-glycero-3-phosphocholine (PAF; obtained from Avanti Polar Lipids, Alabaster, AL) with 0.05 μCi of hexadecyl-2-acetyl-sn-glyceryl-3-phosphocholine, 1-O-[acyl-(N)-H] (NEN/PerkinElmer; 13.5 Ci/mmol) added as a tracer. Substrate was incubated together with enzyme source (cell homogenate or conditioned medium) in a total volume of 500 μl. The reactions were incubated at 37°C for 30 min and were terminated by the addition of 500 μl of 14% TCA. Samples were incubated at 4°C for 10 min and then centrifuged at 1000 × g for 15–20 min at 4°C. Four hundred microliters of the supernatant containing released [³H]acatec was mixed with 5 ml of BioSafe II scintillation cocktail (RPI, Mount Prospect, IL), and radiolabeled acatec was quantified by scintillation counting. The reactions were performed in triplicate, and a reaction without enzyme was used as a control for nonenzymatic hydrolysis. All assays were performed under conditions in which substrate hydrolysis was linear with respect to both enzyme input and time.

**Western blot**

Proteins in Mϕ, MDDC, and mature MDDC cell homogenates were separated by SDS-PAGE, and the proteins were transferred onto nitrocellulose using standard methods (16). PAFAH protein was detected with an Ab directed against the C terminus of plasma PAFAH (Cayman Chemical, Ann Arbor, MI). An Ab against Raf-1 (Santa Cruz Biotechnologies, Santa Cruz, CA) was used as a protein loading control. Protein A-HP and the ECL Western blotting detection system (both from Amersham Pharmacia Biotech, Arlington Heights, IL) were used. In a peptide blocking experiment with PAFAH Ab was incubated with 10 μg/ml of PAFAH blocking peptide (Cayman Chemical) for 1 h at room temperature before addition to the blot. This treatment eliminated the 45-kDa PAFAH band (data not shown). In addition to the 45-kDa PAFAH band, anti-PAFAH recognized the absence of a protein of ~50 kDa, which was eliminated by the peptide. This band may be the glycosylated form of PAFAH that has recently been described (17).
MDDC were metabolically labeled with [3H]acetate, cell-associated lipids were extracted, and accumulated alkyl- and/or acyl-PAF was quantified by scintillation counting (as described in Materials and Methods). Both Mφ and MDDC synthesized PAF (Fig. 1). However, MDDC accumulated ∼3 times more PAF than did Mφ (p = 0.039).

PAFAH activity is higher in Mφ than MDDC

The accumulation of PAF in MDDC could be the result of increased synthesis of the lipid mediator or might be related to a reduction in its catabolism by PAFAH, a phospholipase A2, that selectively hydrolyzes PAF. To address the question of PAFAH, we quantified the PAFAH activity secreted by Mφ and MDDC. As is shown in Fig. 2, Mφ secreted significantly more PAFAH than did immature MDDC (17-fold difference; p < 0.0001). A previous study demonstrated that PAFAH expression increases dramatically when monocytes differentiate into Mφ (3). However, the expression of PAFAH during MDDC differentiation has not been described. Based on the results shown in Fig. 2, we predicted that the increase in PAFAH expression would be less pronounced in differentiating MDDC than in Mφ. To address this, PAFAH activity was measured in homogenates of monocytes, Mφ, MDDC, and mature MDDC. A representative experiment is shown in Fig. 3. PAFAH activity increases when monocytes differentiate into either Mφ or MDDC. However, PAFAH activity is ∼4-fold higher in homogenates of Mφ than in MDDC (p < 0.00001). Both cell-associated and secreted PAFAH activities were similar in MDDC and mature MDDC. In addition to Mφ, MDDC express other forms of PLA2 that preferentially hydrolyze phospholipids with longer sn-2 acyl chains. (18). Thus, we cannot rule out the possibility that other PLA2s contribute to the hydrolysis of PAF in MDDC and Mφ cell homogenates. In fact, this may explain why the ratio of catabolic activity in Mφ vs MDDC is lower in the cell homogenates (Fig. 3) than when secreted activity is measured (Fig. 2). Nevertheless, these experiments strongly suggest that enhanced accumulation of PAF in MDDC can be explained by a reduction in PAF catabolism and that this can be at least partially attributed to a reduction in its catabolism by PAFAH.

PAFAH expression is higher in Mφ than MDDC

To determine whether differences in PAFAH activity between Mφ and MDDC could be attributed to differences in expression, we quantified PAFAH protein and mRNA in Mφ and MDDC. PAFAH mRNA expression was quantified by real-time PCR and was normalized to the expression of β-actin. As is shown in Fig. 4, PAFAH mRNA was at least 5 times higher in Mφ than MDDC or mature MDDC (p = 0.003 and 0.0004, respectively). PAFAH protein expression was quantified by Western blot analysis with a polyclonal Ab directed against the C terminus of plasma PAFAH. As shown in the inset to Fig. 4, PAFAH protein expression was higher in Mφ than MDDC or mature MDDC. Thus, the expression of PAFAH protein and mRNA in MDDC and Mφ was consistent with differences in PAFAH activity in these cell types.

MDDC respond to PAF stimulation

As MDDC contained less PAFAH activity than Mφ, we predicted that PAF turnover would be low in these cells and that they would respond more readily to low concentrations of exogenous PAF than would Mφ. To test this hypothesis, Mφ and MDDC were treated with various concentrations of PAF, and PGE2 production

FIGURE 1. PAF Accumulation in Mφ and MDDC. Mφ and MDDC were metabolically labeled with [3H]acetate as described in Materials and Methods. Lipids were extracted, PAF was isolated by TLC, and associated radiolabel was quantified by scintillation counting. [3H]PAF was normalized for recovery of cellular protein, and the data are presented as disintegrations per minute per microgram of protein. The data shown are compiled from four independent experiments. Error bars indicate the SEM. MDDC accumulated significantly more PAF than did Mφ (p = 0.036, by unpaired t test).

FIGURE 2. Mφ secrete more PAFAH than do MDDC. PAFAH activity was measured in conditioned medium from Mφ, MDDC, and mature MDDC. The data are reported as the percentage of substrate hydrolyzed in the PAFAH assay. A representative experiment is shown (experiment was repeated four times. Error bars indicate the SD of four determinations. Mφ secreted significantly more PAFAH than MDDC (p < 0.0001, by unpaired t test).
was measured as explained in Materials and Methods. As illustrated in Fig. 5A, exogenous PAF induced a concentration-dependent increase in PGE₂ production in MDDC. In contrast, the addition of exogenous PAF to M/H9278 had no comparable effect. We hypothesized that this failure to respond could be attributed to the presence of high levels of PAFAH in M/H9278 cell cultures, as the enzyme catabolizes PAF and thereby limits the ability of the cells to respond to this lipid mediator. To test this possibility, M/H9278 and MDDC were treated with cPAF (a PAFAH-resistant PAF analog), and PGE₂ production was measured. As shown in Fig. 5B, both M/H9278 and MDDC produced PGE₂ in response to cPAF stimulation in a dose-dependent manner. In fact, the response of M/H9278 to low concentrations of cPAF was more robust than that of MDDC, although both cell types produced similar amounts of PGE₂ in response to 100 nM cPAF. The effect of exogenous PAF on mature MDDC was also determined. Although mature resting MDDC produced larger quantities of PGE₂ than did immature MDDC (data not shown), exogenous PAF did not stimulate increased PGE₂ production (fold induction, 1.0 ± 0.2 (mean ± SE)). This is most likely due to the loss of the PAF receptor during MDDC maturation (19), which renders mature MDDC unresponsive to PAF treatment.

**PAFAH is lower in LJP monocytes than NP monocytes**

As noted above, our studies were prompted by the observation that LJP monocytes exhibit a propensity to differentiate into MDDC when cultured for 4 days in human serum (9). The presence of MDDC in LJP monocyte cultures is believed to augment IgG2 production, possibly by promoting increased PAF accumulation. As MDDC contained less PAFAH than did M/H9278, we reasoned that PAFAH levels would be lower in cultures of LJP monocytes than in cultures of NP monocytes. To test this hypothesis, LJP and NP monocytes were cultured for 4 days in human serum, and PAFAH activity was measured in cell homogenates. As shown in Fig. 6, homogenates of LJP monocyte cultures do indeed contain less PAFAH activity than NP monocytes (37% decline; p < 0.05). This observation is consistent with our hypothesis that the differentiation of LJP monocytes to MDDC may induce IgG2 production by regulating PAF levels.

**Discussion**

The results of this study have unveiled differences in PAF metabolism in M/H9278 and MDDC, two immune cell types that differentiate from a common monocyte precursor. As is illustrated in Fig. 1, MDDC accumulate 3-fold more PAF than M/H9278. The availability of PAF is determined both by the rate of its synthesis and its degradation by PAFAH, a calcium-independent phospholipase A₂ that selectively hydrolyzes the sn-2 acyl chain of PAF to generate biologically inactive lyso-PAF (18). As shown in Fig. 3, PAFAH activity increases when monocytes differentiate into either M/H9278 or MDDC. However, M/H9278 contain ~4 times more PAFAH activity than MDDC, and this may allow MDDC to accumulate more PAF.
More remarkably, Mϕ secrete ~17-fold more PAFAH activity than do MDDC (Fig. 2), and this may explain why MDDC respond to exogenous PAF while Mϕ fail to do so (Fig. 5). As shown in Fig. 4, both PAFAH mRNA and protein expression are lower in MDDC than in Mϕ. These data are consistent with the difference in PAFAH activity in the two cell types and indicate that the reduction in PAFAH activity in MDDC is a result of lower expression of the enzyme.

The expression of PAFAH is known to be susceptible to modulation by both host-derived cytokines and pro-inflammatory products derived from bacteria (20). For example, both IFN-γ (a host-derived, pro-inflammatory cytokine) and LPS (a component of Gram-negative bacteria) have been shown to suppress the transcriptional activity of the PAFAH promoter. Interestingly, mouse DC have been shown to produce IFN-γ in response to IL-12 (21), suggesting that these cells may actively antagonize the synthesis of PAFAH. In contrast, transcriptional activity is induced by PAF itself, suggesting the existence of a feedback mechanism that maintains the levels of this biologically active lipid (20). Other monocyte/Mϕ-derived cytokines, including IL-1α, TNF-α, and IL-6 and the anti-inflammatory cytokine IL-4 have no effect on transcription of the PAFAH gene (20). It is possible that the expression of PAFAH is modulated by the cytokines that are used to induce monocyte differentiation to Mϕ (M-CSF) and MDDC (GM-CSF). To this end, these cytokines are reported to have modest effects on PAFAH secretion by mature monocyte-derived Mϕ (20). However, their effects on PAFAH expression during monocyte differentiation are not yet known. Based on our observations, we predict that during differentiation, the expression of PAFAH may be induced by both M-CSF and IL-4/GM-CSF, but that M-CSF is the more potent inducer of expression. Verification of this hypothesis awaits further studies in our laboratory.

Although it is well established that primed macrophages both produce and respond to PAF and other pro-inflammatory lipids (4, 3, 22), only a few previous studies have investigated the ability of MDDC to produce and respond to lipid mediators. Like macrophages, immature MDDC respond to PAF with increases in intracellular calcium and polymerization of the actin cytoskeleton (19). Interestingly, these responses do not occur in mature MDDC due to the loss of PAF receptor expression upon maturation (19). This is consistent with our observation that PAF elicits PGE$_2$ production by immature MDDC, but not by mature MDDC (data not shown).
have recently confirmed in Mφ and the production of IgG2 Abs. Our studies of PAF metabolism have important implications for both the inflammatory response in patients with LJP, a disorder that is associated with abnormalities in the myeloid compartment (10, 11). Our data are also in line with a growing body of evidence suggesting that MDDC are a potential source of eicosanoids. Unlike Mφ that only express the inducible form of cyclooxygenase (COX2) after activation, resting MDDC have been shown to constitutively express both COX1 and COX2 and to produce prostanoids, PGE2, in particular, although in lower amounts than macrophages (7). This PG, in turn, has dramatic effects on the biology of MDDC. PGE2 and other COX-derived products may induce the maturation of MDDC and thereby augment their ability to stimulate all T cells (7). Most studies suggest that PGE2 treatment of MDDC shifts these cells from inducing a pro-inflammatory Th1 response to a Th2 response (23–25). However, another study has demonstrated that nanomolar concentrations of PGE2 can induce IFN-γ production by Ag-stimulated murine Th1 cells (26), and we have recently confirmed this in a human system. Thus, it is possible that the PAF-stimulated MDDC could induce a Th1 response through the low levels of PGE2 that are produced by these cells. Clearly, additional experiments are necessary to delineate the roles of PAF and PGE2 in MDDC biology and to determine the relevance of these to the immune response.

Although Mφ and MDDC originate from a common monocyte precursor, they have distinct, but overlapping, functions. Our studies indicate that PAF metabolism is another aspect that is somewhat different in MDDC and Mφ biology. This difference may have important implications for both the inflammatory response and the production of IgG2 Abs. Our studies of PAF metabolism in Mφ and MDDC were prompted by our investigations of the immune response in patients with LJP, a disorder that is associated with a number of abnormalities in the myeloid compartment (10, 12, 27–29), including a propensity of monocytes to differentiate into MDDC (9). LJP subjects also exhibit high levels of circulating IgG2, and this subclass of Ab is thought to lessen the severity of their disease (30, 31). We have also shown that the IgG2 Ab response is selectively induced by PAF and enriched cultures of MDDC (9, 10). The present study extends our knowledge of the regulation of the IgG2 Ab response in periodontal disease and provides us with another clue regarding the relationship of monocytes to this response. Although PAFAH expression increases when monocytes differentiate into either Mφ or MDDC, expression is at least 3-fold lower in MDDC (Fig. 4), and these cells secrete very little of the catabolic enzyme (Fig. 3). Hence, in periodically healthy (NP) subjects in whom monocytes differentiate primarily into macrophages, large amounts of PAFAH are secreted, thereby limiting the availability of PAF and the production of IgG2. In contrast, the propensity of LJP monocytes to differentiate into MDDC results in lower levels of PAFAH (Fig. 6), and this allows PAF to accumulate and promote the production of a protective IgG2 Ab response. We are currently performing experiments to further delineate the role of PAF in the IgG2 Ab response and to identify the mechanisms that skew monocyte differentiation toward the MDDC phenotype in LJP subjects.

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