SYNTHESIS AND RELEASE OF PLATELET-ACTIVATING FACTOR IS INHIBITED BY PLASMA α_1-PROTEINASE INHIBITOR OR α_1-ANTICHYMOTRYPSIN AND IS STIMULATED BY PROTEINASES

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Platelet-activating factor (PAF)[1] is a mediator of inflammation and endotoxic shock identified as 1-*O*-alkyl-2-acetyl-*sn*-glyceryl-3-phosphorylcholine (1–3). PAF is produced by appropriately stimulated basophils (4), monocytes/macrophages (5, 6), polymorphonuclear neutrophils (6, 7), platelets (8), and endothelial cells (9, 10). A variety of stimuli induce these cells to synthesize PAF. For example, basophils produce PAF when sensitized by IgE (4), PMN, and macrophages upon phagocytosis (5, 6), and endothelial cells when incubated with thrombin (9, 10), angiotensin, or vasopressin (9).

Evidence has accumulated (see reference 11 for review) that endotoxin-induced inflammation is primarily mediated by IL-1 and TNF. We have recently shown that TNF stimulates rat peritoneal macrophages (RPM), human polymorphonuclear neutrophils (PMN), and vascular endothelial cells to synthesize and release PAF (12). This activity of TNF is regulated by cell-specific mechanisms, since PAF is rapidly but transiently synthesized by macrophages (12), whereas PAF synthesis peaks 4–6 h after addition of TNF in endothelial cells (12, 13). Production of PAF by these cells is stimulated also by IL-1 (14), with peak synthesis at 8–12 h (13). In endothelial cells, both TNF and IL-1 induce an acetyltransferase activity required for PAF synthesis (13, 14).

PAF shows a wide range of biological effects (see reference 15 for review): it induces aggregation of platelets (4), promotes chemotaxis of neutrophils and monocytes (16–18), increases vascular permeability, and alters the vascular tone (19). These activities of PAF show many similarities with some of the biological responses described for TNF (20). This observation led to the hypothesis that PAF may be an important secondary mediator of the inflammation induced by TNF (12).

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[1] *Abbreviations used in this paper:* AC, human plasma α_1-antichymotrypsin; BYS, baker's yeast spores; h, human; m, murine; PAF, platelet-activating factor; PI, human plasma α_1-proteinase inhibitor; PMN, human polymorphonuclear neutrophils; RPM, rat peritoneal macrophages; TPCK, L-1-tosylamide-2-phenylethyl chloromethyl ketone; TT, Tris-buffered Tyrode's solution.

In previous experiments, we noticed that the serine proteinase inhibitor L-1-tosylamide-2-phenylethylchloromethyl ketone (TPCK) drastically reduces PAF production induced by TNF in RPM (12) and endothelial cells (13). These cells are stimulated to synthesize and release measurable amounts of PAF only during incubations in serum-free medium, due to the presence of a serum acetylhydrolase that cleaves the 2-acetyl group from PAF (21). Therefore, it is not known from such experiments whether plasma proteinase inhibitors may interfere with PAF production similarly to TPCK. In the present investigation, we examined the effect of human plasma fractions on TNF-induced PAF synthesis and release. Preliminary experiments were carried out on PMN incubated with acid-treated serum to inactivate PAF acetylhydrolase (21), but PAF production and release was nevertheless drastically inhibited. This finding led us to analyze different plasma fractions for the presence of inhibitors of PAF synthesis, which were subsequently identified as proteinase inhibitors (antiproteinases).

Materials and Methods

Materials. Human and murine rTNF (hTNF and mTNF) were respectively gifts of Dr. Tatsuro Nishihara of the Suntory Institute for Biomedical Research, Osaka, Japan, and of Dr. Walter Fiers, University of Ghent, Ghent, Belgium. Human rIL-1α was a gift of Immunex Corp. (Seattle, WA). Human plasma α_1-proteinase inhibitor (PI) and α_1-antichymotrypsin (AC), rabbit anti-PI IgG, leukocyte myeloperoxidase, human neutrophil elastase and cathepsin G, and human erythrocyte catalase were purchased from Athens Research and Technology Inc. (Athens, GA). The chloromethyl ketones proteinase inhibitors MeO-Suc-Ala_2-Pro-ValCH_2Cl and Z-Gly-Leu-Phe-CH_2Cl were from Enzyme Systems, Inc. (Livermore, CA). Baker's yeast spores (BYS) were prepared by boiling baker's yeast for 30 min in isotonic saline. Spores were collected by 10-min centrifugation at 1,500 *g* and washed twice with saline. To prepare opsonized spores (BYS-C3b), 20 mg of spores were incubated 30 min at 37°C with 1 ml of fresh human serum, centrifuged and washed twice with Tris-buffered Tyrode's solution (TT) without calcium and magnesium, pH 6.5, supplemented with 0.25% delipidated BSA (TT-BSA). [^{3}H]PAF was synthesized by reacting 10 mM 1-octadecyl-2-lyso-sn-3-phosphocholine (Bachem Feinkemikalien, Bubendorf, Switzerland) in methanol with 0.1 Ci of [^{3}H]acetic anhydride (6 Ci/mmol; Amersham Int., Amersham, UK) in the presence of 32.7 mM dimethylaminopyrimidine (22). The specific activity of [^{3}H]PAF was 90 mCi/mol.

Plasma Fractionation. Human plasma freshly collected in 5 mM EDTA was fractionated on a 1 × 100 cm Sephacryl-200 superfine column (Pharmacia Fine Chemicals, Piscataway, NJ), equilibrated with 0.01 M phosphate buffer, pH 7.4, and eluted with this buffer at 4°C through a recording spectrophotometer (LKB Instruments, Inc., Gaithersburg, MD). Eluted fractions were immediately assayed for PAF acetylhydrolase activity, pooled, frozen at −80°C, and lyophilized. These fractions were reconsituted with distilled water before incubation with PMN or RPM. A corresponding volume of elution buffer was used as control.

PAF Acetylhydrolase Assay. The PAF acetylhydrolase activity was measured according to Blank et al. (22). Briefly, 0.5 ml of Sephacryl-200 column fractions were incubated for 10 min at 37°C with 4 μM [^{3}H]PAF (0.5 nCi) in polypropylene tubes. The reactions were stopped by adding 0.5 ml of 10% $NaHCO_3$ and 1 ml of chloroform, shaking, and separating the organic phase by centrifugation. The upper water phase was extracted three times with 1-ml aliquots of chloroform, and the [^{3}H]acetate in the water phase was measured by scintillation counting.

Cell Preparation and Treatment. Human PMN were prepared as described (9). Briefly, blood from healthy donors was drawn into plastic tubes containing 5 mM EDTA and centrifuged for 20 min at 700 *g*. Plasma and buffy coat were removed, and pelleted cells were resuspended in 2 volumes of 2.5% gelatin (Difco Laboratories, Inc., Detroit, MI) in saline. The bulk of the erythrocytes was removed by low speed centrifugation, and the remaining cells were pelleted

by centrifugation for 20 min at 700 *g*. These cells were subjected to osmotic shock to eliminate contaminating erythrocytes and were washed twice with TT-BSA without calcium and magnesium. Smears of cells stained with May-Gruenwald-Giemsa showed 85–95% PMN. The cells were resuspended at 5 × 10^6/ml in TT-BSA with calcium and magnesium, and incubated at 37°C with the additions indicated in the text. Rat peritoneal macrophages and human vascular endothelial cells were prepared and cultured as described (12).

Assay of Cell-associated and Released PAF. In standard assays, 2.5 × 10^6 PMN or 10^6 RPM were incubated in 0.5 ml reactions with TNF, 20 μl of BYS-C3b suspension (~10 spores/cell) or other additions indicated in the text. Endothelial cells were grown in multiwell plates (~5 × 10^5 cells/35-mm well) and treated in 1 ml of Iscove's medium containing 0.25% BSA. Preincubation with proteinase inhibitors was for 20 min at 22°C. PAF released into the medium and cell associated was isolated, characterized, and measured as described (12).

Results

Inhibition of PAF Synthesis by Plasma Fractions. In preliminary experiments, human PMN were prepared by different procedures and stimulated to produce PAF in incubations with TNF. Only PMN prepared by differential centrifugation in buffer containing 2.5% gelatin and used within 2 h responded reproducibly to TNF (data not shown). These experiments were carried out in serum-free medium (12) because of the presence of an enzymatic activity in serum that rapidly hydrolyses PAF (21, 22). This PAF acetylhydrolase can be inactivated by treating serum with 0.1 N HCl at pH 3 (22). However, when serum treated in this way was added to incubations of PMN and hTNF, an almost complete suppression of PAF production was still observed (data not shown). This finding suggested that other inhibitors of PAF synthesis are present in serum.

To identify these inhibitors, huma plasma was fractionated by gel filtration chromatography (Fig. 1). The fractions eluted were tested on PMN in a standard assay for hTNF-mediated induction of PAF production. Two peaks of inhibitory activity were clearly separated; PAF acetylhydrolase was detected only in the first peak (fractions 1–3). Addition of these fractions presumably resulted in hydrolysis of PAF released by PMN in response to hTNF. The second peak of inhibitory activity (fractions 7–9)

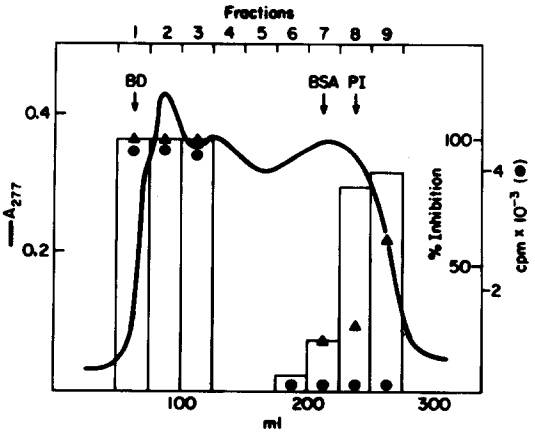

FIGURE 1. Fractionation of human plasma by chromatography on Sephacryl-200 and assay for inhibitors of TNF-induced PAF synthesis. The plasma fractionation and other assays are described in Materials and Methods. The histogram shows the percent inhibition of PAF release by 50 μl of plasma fractions preincubated for 30 min at 22°C with 2.5 × 10^6 PMN before stimulation with 10 ng hTNF for 10 min at 37°C. The PAF-acetylhydrolase activity of plasma fractions (●) is expressed as cpm of [^{3}H]acetate recovered in the inorganic phase. The percent inhibition of PAF synthesis in assays carried out in the presence of anti-PI IgG is also indicated (▲). These cells were preincubated for 20 min at 22°C with 0.1 mg of IgG before addition of 10 ng hTNF. The elution volume of blue dextran (*BD*) and of other marker proteins is indicated by the arrows.

was eluted after BSA (Fig. 1). In previous experiments, the protease inhibitor TPCK blocked the synthesis of PAF induced by TNF in RPM (12) and vascular endothelial cells (13). Thus, it seemed possible that plasma proteinase inhibitors could have a similar effect. Furthermore, the second peak of inhibitory activity coeluted with PI (Fig. 1). To identify this inhibitory activity with PI, we added anti-PI-neutralizing IgG to column fractions, which were incubated with PMN before stimulation with hTNF. The inhibition of PAF release was greatly reduced when fraction 8 was treated in this way, whereas, anti-PI IgG had less effect on the activity of fraction 9. The identification of the inhibitor in fraction 8 as PI was further confirmed by generating oxidants with myeloperoxidase (Table I). Biological oxidants inactivate PI (23) and, accordingly, suppressed the inhibitory activity of fraction 8 to a large extent in assays with both PMN and RPM stimulated by hTNF and mTNF, respectively. The same effect was observed when purified PI was used in control experiments (Table I). These findings show that PI inhibits PAF release induced by TNF and raise the possibility that other plasma antiproteinases may have a similar activity.

Synthesis of PAF can be induced in PMN and RPM by phagocytosis of opsonized yeast spores (24). Thus, it was of interest to examine whether fraction 8 or PI inhibited PAF production stimulated by phagocytosis of BYS-C3b. Partial inhibition of PAF release (Table I) and of cell-associated PAF synthesis (data not shown) was observed in assays with both fraction 8 and PI. However, this inhibitory activity was greatly increased when catalase, which decomposes hydrogen peroxides, was added to the assays (Table I). Presumably, during phagocytosis, PMN and RPM release peroxides that inactivate PI.

Inhibition of PAF Synthesis by Plasma Proteinase Inhibitors. The dose response to PI inhibition was measured in standard TNF induction assays of PAF production by neutrophils and macrophages. Complete inhibition of PAF release was observed with 20 μg/ml of PI, and significant inhibition with 0.2 μg/ml (Fig. 2 *A*). The dose response to PI inhibition was similar for RPM (Fig. 2 *B*) and for cell-associated PAF

TABLE I

Effect of Oxidants on the Inhibition of PAF Production

Additions	Neutrophils		Macrophages	
	Plasma no. 8	PI	Plasma no. 8	PI
	%	%	%	%
TNF	0.4 ± 0.7 (6)	0.9 ± 0.8 (13)	0.4 ± 0.7 (4)	0.3 ± 0.5 (3)
TNF + myeloperoxidase	3.8 ± 1.1 (56)	7.9 ± 0.6 (116)	6.0 ± 0.7 (66)	6.8 ± 1.8 (75)
BYS-C3b	4.9 ± 0.5 (42)	6.3 ± 1.5 (54)	1.8 ± 0.7 (20)	3.1 ± 1.0 (35)
BYS-C3b + catalase	0.9 ± 0.7 (8)	1.7 ± 1.8 (15)	ND	0.2 ± 0.3 (2)

For each experiment, 2.5×10^6 PMN or 10^6 RPM were assayed in triplicate for PAF release induced by 10 ng/ml hTNF or mTNF, respectively, and by BYS-C3b (see Materials and Methods). The cells were preincubated 30 min with plasma fraction #8 (described in Fig. 1) or 10 μg PI ± 0.03 U/ml of myeloperoxidase and 0.05 U/ml of glucose oxidase, before addition of TNF. Alternatively, the cells were preincubated 20 min ± 1,500 U of catalase and then stimulated with 10 μl of BYS-C3b suspension. The medium was harvested after 10 and 60 min from PMN and RPM, respectively. The mean ng of PAF released in the medium ± SD are indicated. When treated with TNF alone, the PMN and RPM released 6.8 ± 0.7 and 9.1 ± 1.1 ng of PAF, respectively. When treated with BYS-C3b alone, the PMN released 11.6 ± 2.1 ng of PAF and the RPM 8.9 ± 3.2 ng. These values were taken as 100% to calculate the percent PAF released relative to these controls.

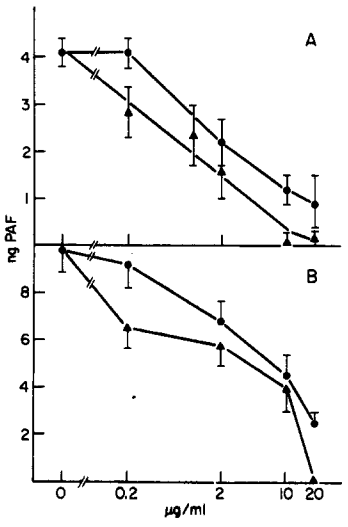

FIGURE 2. Dose response of the inhibition of PAF release by PI (●) and AC (▲) in neutrophils (*A*) and macrophages (*B*). The amounts of PI or AC indicated in the abscissa were added to incubations containing either 2.5 × 10^6 PMN or 10^6 RPM for 30 min at 22°C before addition of 10 ng hTNF or mTNF, respectively. The PMN were then incubated for 10 min at 37°C, whereas the RPM were incubated for 30 min before removing the medium to measure PAF release.

in both cell types (data not shown). Since the experiments with human plasma fractions (Fig. 1) suggested that other proteins could be inhibitory for TNF induction of PAF synthesis, we assayed the human plasma proteinase inhibitor AC in parallel experiments. This antiproteinase was slightly less effective than PI in decreasing PAF release by PMN or RPM (Fig. 2). Essentially identical data were obtained for cell-associated PAF (data not shown).

Pretreatment with plasma proteinase inhibitors before TNF addition did not result in inhibition of PAF synthesis. When PMN were preincubated for 30 min with 20 μg/ml of either PI or AC and then washed, the amount of PAF released 10 min after addition of 20 ng/ml hTNF was comparable with that released from control cells preincubated without antiproteinases (data not shown). This finding suggests that these proteins do not interact with a proteinase present in untreated PMN, but presumably inhibit an enzyme released by these cells or activated on their surface after hTNF addition. This hypothesis may explain the lack of effect of the preincubation with PI, since this antiproteinase reacts slowly with zymogens by forming stable complexes (see reference 25 for review).

These findings show that two different plasma proteinase inhibitors interfere with the induction of PAF synthesis by TNF. It was therefore of interest to compare the effect of these antiproteinases on PAF production induced by other stimuli, such as phagocytosis of BYS-C3b or treatment with the calcium ionophore A23187 (Table II). Both PI and AC drastically inhibited PAF production induced by TNF, but had no effect on cells treated with A23187. PI was somewhat inhibitory for PMN, but clearly inhibitory for RPM incubated with BYS-C3b. The interpretation of these data has to take into account the finding that oxidants produced during phagocytosis of BYS-C3b inactivate PI, as shown by the experiment where the addition of catalase increased the inhibitory activity of PI (Table I). AC had no effect on PAF production by PMN, whereas, it significantly inhibited PAF production by RPM (Table II). At this time, we cannot distinguish between a lack of effect of AC on

TABLE II

Effect of Proteinase Inhibitors on PAF Synthesis and Release

Treatment	Neutrophils		Macrophages	
	Released	Cell associated	Released	Cell associated
None	0.4 ± 0.6	0.4 ± 0.8	0.8 ± 1.2	1.7 ± 1.2
TNF	7.6 ± 3.6	8.3 ± 3.2	12.4 ± 2.6	12.1 ± 2.9
TNF + PI	1.3 ± 0.6	0.5 ± 0.5	0.5 ± 0.8	0.9 ± 0.9
TNF + AC	0.9 ± 0.9	2.5 ± 4.2	2.6 ± 1.1	3.2 ± 1.5
BYS-C3b	11.2 ± 2.7	12.1 ± 3.0	15.4 ± 2.2	16.2 ± 3.2
BYS-C3b + PI	8.1 ± 2.9	8.9 ± 3.0	3.3 ± 1.6	7.4 ± 1.2
BYS-C3b + AC	13.5 ± 0.7	11.5 ± 0.6	4.9 ± 1.5	8.7 ± 2.4
A23187	11.3 ± 2.5	12.0 ± 2.0	22.5 ± 2.2	25.0 ± 3.0
A23187 + PI	14.5 ± 0.7	12.0 ± 3.5	20.3 ± 1.5	23.9 ± 4.4
A23187 + AC	14.3 ± 0.6	11.5 ± 0.6	22.0 ± 2.0	24.3 ± 4.5

For each assay, 2.5×10^6 PMN or 10^6 RPM were preincubated for 30 min with 20 μg/ml of proteinase inhibitors or kept as untreated controls. The cells were then treated for 10 min with 20 ng/ml hTNF or mTNF, respectively, for 20 min with 20 μl of BYS-C3b suspension, or for 30 min with 1 μg/ml of A23187. PAF released into the culture medium or cell associated was measured as described in Materials and Methods. Each experiment was carried out in quadruplicate and the data shown are mean ng of PAF ± SD.

PMN stimulated by phagocytosis, or an inactivation of this antiproteinase during the incubation of PMN with BYS-C3b. However, the effect of AC on RPM suggests that these cells differ from PMN in their sensitivity to different plasma proteinase inhibitors.

In subsequent experiments, we examined whether PI and AC inhibit the production of PAF induced by hTNF in human vascular endothelial cells. The response of these cells to hTNF is much slower than that of RPM to mTNF (12, 13). Therefore, PAF production was measured after 6 h of incubation with hTNF (Table III). Both PI and AC inhibited the production of cell-associated PAF and its release, but PI was more inhibitory than AC. This result indicates that plasma antiproteinases

TABLE III

The Effect of Serum Proteinase Inhibitors on the Stimulation of PAF Production by hTNF in Human Vascular Endothelial Cells

Additions	μg/ml	PAF	
		Cell associated	Released
		ng	
None	-	0.6 ± 0.2	0
hTNF	-	6.1 ± 2.0	3.1 ± 0.6
hTNF + PI	5	3.8 ± 1.1	0.8 ± 0.2
hTNF + PI	10	1.6 ± 0.4	0.7 ± 0.3
hTNF + AC	5	4.7 ± 1.3	1.8 ± 0.5
hTNF + AC	10	2.3 ± 0.7	1.1 ± 0.7

For each assay, 2.5×10^5 cells were pretreated for 30 min with the proteinase inhibitor indicated and then incubated for 6 h with 17 ng/ml hTNF. The cell-associated and released PAF were measured as described in Materials and Methods.

inhibit PAF production by vascular endothelial cells that were previously shown to respond to hTNF in serum-free medium (12, 13).

Production of PAF can be induced in PMN and vascular endothelial cells also by treatment with IL-1, a cytokine that shares several activities with TNF (25). It was thus of interest to establish whether the plasma proteinase inhibitors interfere with the release of PAF or with the production of cell-associated PAF induced by IL-1. Both PI and AC significantly inhibited the production of PAF in PMN and endothelial cells treated with IL-1 (Table IV). This finding suggests that antiproteinases interfere with some common step in the induction of PAF synthesis stimulated by TNF and IL-1.

Effect of Proteinases on PAF Synthesis. The inhibition of PAF production by PI and AC may be explained by the inhibition of specific proteinase(s) in TNF-treated cells. To show that a proteinase can trigger PAF synthesis, PMN and RPM were treated with human neutrophil elastase. This proteinase induced PAF production and release in both cell types (Fig. 3). The time course of PAF production and the dose response to different concentrations of elastase were determined for both PMN and RPM. The maximum level of cell-associated PAF was observed in PMN after 2 min (the first time point sampled), whereas, PAF release peaked 5 min after the addition of elastase (Fig. 3 *A*). Both cell-associated and released PAF subsequently decreased to undetectable levels after 60 min. PMN were stimulated to produce PAF by elastase concentrations as low as 10 ng/ml, but significant PAF release was observed with greater elastase concentrations (2 μg/ml; Fig. 3 *B*). The RPM were stimulated to produce maximal amounts of PAF 10 min after addition of elastase (Fig. 3 *C*). About equal amounts of cell-associated and released PAF were detected at this time; similar PAF levels were present at 30 min, but the cell-associated PAF decreased to undetectable levels afterwards (Fig. 3 *C*). Greater amounts of elastase were required to induce PAF production and release in RPM than in PMN (Fig. 3 *D*).

Vascular endothelial cells were similarly stimulated to produce PAF by the addition of elastase (Fig. 4). Maximum levels of cell-associated and released PAF were present after 30 min; the amount of PAF decreased afterwards, and it returned to

TABLE IV

The Effect of Serum Proteinase Inhibitors on Synthesis of PAF by Neutrophils and Vascular Endothelial Cells Stimulated with IL-1

Cells	Additions	PAF	
		Released	Cell associated
		ng	
Neutrophils	IL-1	4.0 ± 0.1	4.9 ± 0.9
	IL-1 + PI	1.5 ± 0.7	1.8 ± 0.8
	IL-1 + AC	2.5 ± 0.3	2.9 ± 1.1
Endothelial	IL-1	1.7 ± 0.2	4.6 ± 0.7
	IL-1 + PI	0.5 ± 0.4	1.1 ± 0.8
	IL-1 + AC	0.8 ± 0.1	3.3 – 0.6

For each assay, 2.5×10^6 PMN or 2.5×10^5 endothelial cells were preincubated 30 min at 22°C in 0.5 ml of medium containing 10 μg/ml of proteinase inhibitors; the cells were then incubated for 30 min (PMN) or 6 h (endothelial cells) with 0.1 nM IL-1. The PAF released in the medium or cell associated was measured as described in Materials and Methods.

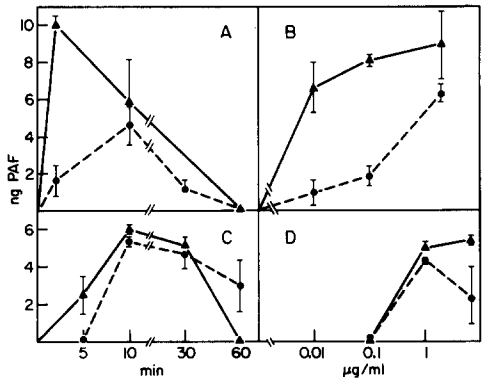

FIGURE 3. The effect of elastase on production of cell-associated PAF (▲) and release of PAF (●) in neutrophils (*A* and *B*) or macrophages (*C* and *D*). Standard assays were incubated for the time indicated with 1 μg/ml of elastase (*A* and *C*) or for 10 min with the concentration of elastase indicated in the abscissa (*B* and *D*).

a basal level after 3 h (Fig. 4 *A*). Therefore, the addition of elastase stimulated PAF production much faster than either TNF or IL-1 (13). Endothelial cells were optimally stimulated by 1 μg/ml of elastase, but higher concentrations of this enzyme were somewhat inhibitory (Fig. 4 *B*). The amount of cell-associated PAF was greater than that of PAF released into the medium with all the elastase concentrations tested, but ~38% of the PAF produced was released from endothelial cells treated with 0.1 or 1 μg/ml of this protease (Fig. 4 *B*). These findings show that proteinases secreted by neutrophils and macrophages can directly stimulate these cells and vascular endothelial cells to produce PAF.

Another neutrophil proteinase, cathepsin G, was effective in inducing PMN to synthesize PAF (Fig. 5). Both cell-associated and released PAF peaked 10 min after addition of cathepsin G and decreased to basal levels after 60 min (Fig. 5 *A*). Relatively low cathepsin G concentrations were effective in inducing synthesis of PAF, but somewhat greater concentrations were required to obtain significant PAF release (Fig. 5 *B*). In subsequent experiments, 2 μg/ml of chymotrypsin induced the release of 5.3 ± 0.1 ng of PAF from PMN in 30 min. This finding suggests that proteinases with relatively broad substrate specificity can induce PAF production.

Elastase and cathepsin G have a variety of biological effects independent of their proteolytic activity. To test whether these enzymes can trigger PAF release after in-

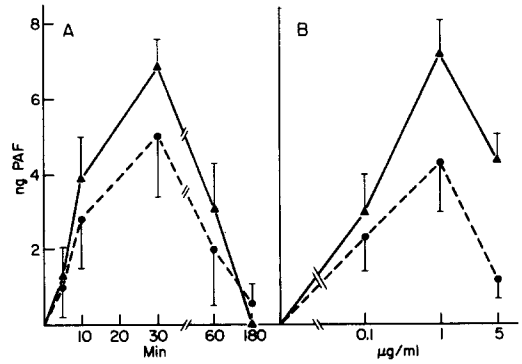

FIGURE 4. The effect of elastase on the production of cell-associated PAF (▲) and on the release of PAF (●) by vascular endothelial cells. Standard assays were incubated for the time indicated with 1 μg/ml of elastase (*A*) or for 30 min with the concentration of elastase indicated in the abscissa (*B*). The data shown represent the average of six experiments.

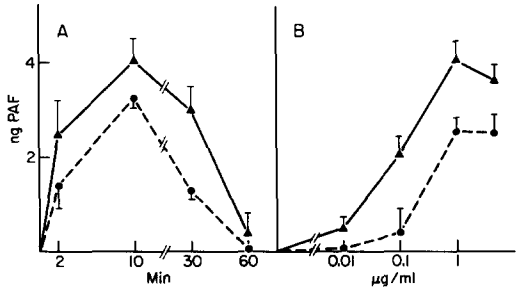

FIGURE 5. The effect of cathepsin G on the synthesis of cell-associated (▲) and released PAF (●) by PMN. 2.5 × 10^6 PMN were incubated for the time indicated with 1 μg/ml of cathepsin G (*A*) or for 10 min with the concentration of cathepsin G indicated in the abscissa. Experiments were carried out in triplicate, and the average ng PAF are shown.

activation of such activity, elastase and cathepsin G were treated at pH 6.5 either with 1 mM DFP or with 0.05 mM PMSF. When 4 μg/ml of these inactivated enzymes were added to PMN as described in Figs. 4 and 5, no release of PAF was detected after 10 or 30 min (data not shown). Therefore, we can exclude that antiproteinases inhibit PAF synthesis by interfering with binding of elastase or cathepsin G to critical target sites.

Effect of Chloromethyl Ketone Proteinase Inhibitors on PAF Synthesis and Release. Our working hypothesis is that a proteinase is activated on the plasma membrane of susceptible cells or released into the medium after TNF binds to its receptor. The experiments described above could not identify a specific proteinase involved in triggering PAF synthesis, since elastase and cathepsin G were almost equally effective. Another approach to identify a unique proteinase is based on the effect of specific inhibitors, such as MeO-Suc-Ala_2-Pro-ValCH_2Cl and *Z*-Gly-Leu-PheCH_2Cl that inhibit elastase and cathepsin G, respectively (26). TNF-induced PAF synthesis was measured in PMN treated with low concentrations (10 μM) of these proteinase inhibitors. The cathepsin G inhibitor was more effective than the elastase inhibitor in decreasing PAF synthesis and release (Table V). A similar result was obtained

TABLE V

Effect of Chloromethyl Ketone Proteinase Inhibitors on Synthesis of Cell-associated and Released PAF by Neutrophils

Treatment	Cell associated	Released
hTNF	8.0 ± 0.4	5.5 ± 0.3
hTNF + MeO-Suc-Ala_2-Pro-ValCH_2Cl	6.5 ± 1.9	6.1 ± 2.3
hTNF + *Z*-Gly-Leu-PheCH_2Cl	3.9 ± 1.3	1.0 ± 0.5
hTNF + *Z*-Gly-Leu-PheCH_2Cl (washed)	3.1 ± 0.1	1.2 ± 0.6
BYS-C3b	8.1 ± 3.5	8.5 ± 2.3
BYS-C3b + MeO-Suc-Ala_2-Pro-ValCH_2Cl	7.3 ± 3.1	7.4 ± 2.1
BYS-C3b + *Z*-Gly-Leu-PheCH_2Cl	2.6 ± 1.5	2.2 ± 1.1
BYS-C3b + *Z*-Gly-Leu-PheCH_2Cl (washed)	1.2 ± 0.7	2.7 ± 1.7

For each assay, 2.5 × 10^6 PMN were preincubated for 30 min at 22°C with 10 μM proteinase inhibitors, or with 0.1 mM inhibitors when these compounds were removed and the cells washed twice with fresh medium before induction of PAF synthesis. The cells were incubated 10 min at 37°C with 1 nM hTNF or for 20 min with 20 μl of BYS-C3b suspension. Each experiment was carried out in triplicate and the data shown are mean ng of PAF ± SD.

with PMN stimulated to synthesize PAF by phagocytosis of BYS-C3b (Table V). Addition of greater concentrations of the elastase inhibitor, up to 0.1 mM, did not result in further decrease of PAF synthesis (data not shown).

The inhibition of PAF production by the cathepsin G inhibitor was irreversible, as expected for a peptide containing a chloromethyl ketone group that binds covalently to serine proteinases. This was shown by adding 0.1 mM inhibitor to PMN for 30 min; after washing these cells, production of PAF stimulated by hTNF or phagocytosis of yeast spores was still markedly inhibited (Table V). The hTNF-mediated production of cell-associated PAF decreased also by 81% in endothelial cells treated with 0.1 mM cathepsin G inhibitor, whereas, it only decreased by 32% in cells treated with 0.1 mM elastase inhibitor (data not shown). These results suggest that a proteinase that is preferentially inactivated by the cathepsin G inhibitor is involved in promoting PAF synthesis induced either by TNF or by phagocytosis. However, this suggestion should be considered with some caution, since we do not know whether these inhibitors diffuse with different kinetics into PMN and vascular endothelial cells.

Similar results were obtained with less specific serine proteinase inhibitors. PMN were treated either with 1 mM DFP or with 50 μM PMSF for 10 min in TT-BSA, pH 6.5. The PMN were washed twice and then incubated with hTNF or with BYS-C3b, as described in Table I. PAF release was inhibited 96% and 90%, respectively, in PMN treated with DFP or PMSF and stimulated with hTNF. PAF release was inhibited 87% and 91%, respectively, in PMN treated with DFP or PMSF and stimulated by phagocytosis of CYS-C3b.

Discussion

The plasma proteinase inhibitors PI and AC impair synthesis and release of PAF induced by TNF and IL-1. PI and AC inhibit PAF synthesis at concentrations ~100- and ~10-fold lower than those present in serum (~2 mg/ml and ~0.45 mg/ml, respectively; see reference 25 for references). Therefore, it is unlikely that PMN, macrophages, or endothelial cells can synthesize and release significant amounts of PAF when exposed to the vast excess of antiproteinases present in serum. Synthesis of PAF may require the inactivation of these proteinase inhibitors, or it may be limited to zones of close contact between cells where antiproteinases are excluded. PI can be inactivated by oxidation of two methionyl residues in the reactive site (25). Such inactivation is observed in rheumatoid synovial fluid, where PI containing methionine sulfoxide residues is recovered (25). Therefore, this oxidized antiproteinase would not inhibit PAF synthesis by PMN or macrophages participating in the rheumatoid inflammatory response. However, no comparable mechanism to inactivate AC is known and there are several other antiproteinases in serum that might inhibit PAF synthesis, but have not yet been examined.

The hypothesis that PAF synthesis may occur in zones of close cell contact is supported by recent observations on the fibrinogenolysis catalyzed by neutrophil elastase (26). This enzyme is active in the presence of physiological concentrations of its most effective inhibitor, PI (25), as shown by the appearance of a specific fibrinopeptide in plasma (26). The fibrinogenolytic activity of free elastase is inhibited by low concentrations of serum, whereas, the activity of PMN migrating across fibrinogen-coated filters is incompletely blocked, even in the presence of undiluted serum (26). Weitz et al. (26) suggest that the access of antiproteinases is prevented in zones of

close contact between PMN and fibrinogen as an explanation for the presence of circulating fibrinopeptide.

In view of these findings, it seems possible that PAF is only synthesized and released in zones of the cell plasma membrane that adhere to other cells or to extracellular matrix. Appropriate stimuli, such as TNF or IL-1, may induce competent cells to produce PAF by interacting with specific receptors over the whole cell surface, but synthesis and release of PAF may be restricted to zones that exclude antiproteinases. According to this hypothesis, PAF production may be localized and its effects may be limited to target cells in close contact with producer cells. Furthermore, the presence in serum of the acetylhydrolase that rapidly degrades PAF supports the notion that PAF acts in a localized way.

The antiproteinases have no effect on PAF synthesis stimulated by the calcium ionophore A23187, which may involve activation of protein kinase C (27). This suggests that antiproteinases do not directly inhibit the biosynthesis of PAF, but interfere with some step in a pathway leading to PAF synthesis. We propose that after TNF or IL-1 bind to their receptors, a proteinase becomes activated at the cell surface or is released in the medium. This hypothesis is based on the observation that PMN preincubated with plasma antiproteinases and then washed respond normally to hTNF. However, PMN preincubated with the chloromethyl ketone proteinase inhibitors and then washed do not respond to hTNF (Table V). These inhibitors react with zymogens and may diffuse into cells, whereas, plasma antiproteinases react slowly with zymogens (25) and cannot diffuse into cells.

The hypothesis that proteinases are involved in the induction of PAF synthesis by TNF or IL-1 led us to assay their effect on PMN, RPM, and endothelial cells. Neutrophil elastase and cathepsin G stimulated PAF synthesis to levels comparable with those observed after addition of TNF or phagocytosis. It seems possible that these enzymes cleave some protein on the cell surface and that PAF is synthesized subsequently to this proteolysis. Furthermore, proteinase-treated PMN, RPM, and endothelial cells synthesize PAF faster than cells treated with TNF, since PAF production peaks in PMN ~10 min after TNF addition (our unpublished observations), in RPM at 30 min (12), and in endothelial cells at 4–6 h (13). This finding suggests that proteinases can shortcut the pathway that leads from the binding of TNF or IL-1 to their receptors to synthesis of PAF.

The present experiments have not identified a specific proteinase that may be activated in TNF-treated cells. PI is inhibitory for PAF synthesis at lower concentrations than AC, the antiproteinase that shows the fastest time of association with cathepsin G (25). However, the chloromethyl ketone inhibitor specific for this enzyme decreases PAF synthesis more effectively than the corresponding elastase inhibitor (Table V). Therefore, these experiments are inconclusive, and the hypothetical proteinase activated in cells treated with TNF or IL-1 has not yet been identified.

Synthesis of PAF induced by proteinases may play a significant role in the inflammatory response of endothelial cells to activated PMN and macrophages. These cells release elastase, which induces synthesis and release of PAF. This factor in turn may promote chemotaxis of PMN and monocytes (16–18) and increases vascular permeability (19) by autocrine or paracrine mechanisms. Furthermore, both PI and AC inhibit PAF synthesis stimulated by phagocytosis in RPM (Table II). Similarly, a cathepsin G inhibitor decreases phagocytosis-stimulated PAF synthesis in PMN (Table

V). These findings suggest that PAF production induced by TNF and IL-1 shares some common step(s) with the stimulation of PAF synthesis by phagocytosis. Thus, it seems possible that antiproteases may interfere with a general mechanism for PAF synthesis in response to a variety of physiological stimuli. Such a mechanism presumably requires proteinase(s) activity.

The synthesis of PAF requires phospholipase A_2-like activity (28). It has been proposed that the peripheral membrane proteins lipocortins block phospholipase A_2 activity by sequestering phosolipid substrates rather than by a direct interaction with this enzyme (29). Cirino et al. (30) recently reported that perfusion of isolated guinea pig lungs with recombinant human lipocortin I inhibits the release of thromboxane that is formed by the lipoxygenase pathway from arachidonic acid. Therefore, lipocortins applied extracellularly can inhibit phospholipase A_2-like activity. An hypothesis consistent with our findings is that proteolytic cleavage of lipocortins or related proteins on the cell surface may promote activation of phospholipase A_2. Alternative explanations for the role of proteinases cannot be excluded at this time. For example, these enzymes may inactivate an inhibitor of lyso-PAF acetyltransferase. Experiments directed at testing these hypotheses are currently in progress.

It is relevant to point out that the present findings provide evidence for a link in a chain of regulatory mechanisms that may control the production of mediators of inflammation. TNF induces fibroblasts and other cells to secrete IFN-β_2 (31). This cytokine is identical to hepatocyte-stimulating factor and induces synthesis of all major acute phase proteins, including plasma antiproteinases, in human hepatoma cells and rat primary hepatocytes (32). Therefore, TNF stimulates production of mediators of inflammation such as PAF, but at the same time, via IFN-β_2/hepatocyte-stimulating factor, induces synthesis of antiproteinases that inhibit production of this mediator.

Summary

TNF and IL-1 stimulate the synthesis and release of platelet-activating factor (PAF) by neutrophils and vascular endothelial cells. Serum inhibits PAF production even after inactivation of an acetylhydrolase that degrades PAF. Human plasma was fractionated by gel filtration chromatography, and two inhibitory fractions were detected, one containing PAF-acetylhydrolase activity and the other α_1-proteinase inhibitor. Low concentrations of this antiproteinase and of human plasma α_1-antichymotrypsin inhibited TNF-induced PAF synthesis in neutrophils, macrophages, and vascular endothelial cells. Both antiproteinases also inhibited PAF production stimulated by phagocytosis in macrophages and induced with IL-1 in neutrophils or with TNF in vascular endothelial cells. These results suggest that a proteinase activated on the plasma membrane or secreted by these cells is involved in promoting PAF synthesis. Indeed, addition of elastase to macrophages, neutrophils, and endothelial cells stimulated synthesis and release of PAF much faster than TNF. A similar stimulation was observed in incubations with cathepsin G. To identify a proteinase activated in TNF-treated cells, neutrophils and endothelial cells were incubated with specific chloromethyl ketone inhibitors of elastase and cathepsin G. Synthesis of PAF was significantly inhibited by low concentrations of the cathepsin G inhibitor. The finding that antiproteinases are inhibitory at concentrations 100-fold lower than those present in plasma raises questions as to the ability of TNF and IL-1 to stimulate neutrophils

in circulation or endothelial cells to synthesize PAF. We propose that PAF production is limited to zones of close contact between cells, which exclude antiproteinases.

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