TUMOR NECROSIS FACTOR/CACHECTIN STIMULATES PERITONEAL MACROPHAGES, POLYMORPHONUCLEAR NEUTROPHILS, AND VASCULAR ENDOTHELIAL CELLS TO SYNTHESIZE AND RELEASE PLATELET-ACTIVATING FACTOR

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Platelet-activating factor (PAF)[1] is a mediator of inflammation with a wide range of biological activities (see reference 1 for review). PAF was initially recognized as a product of IgE-sensitized rabbit basophils (2) and was identified with 1-*O*-alkyl-2-*sn*-glyceryl-3-phosphorylcholine (3–5). It was subsequently shown that PAF is synthesized after appropriate stimulation by monocytes/macrophages (6–9), polymorphonuclear neutrophils (7, 10, 11), platelets (12) and endothelial cells (13–15). PAF induces aggregation and degranulation of platelets (2, 16), stimulates contraction of smooth muscle (17), promotes chemotaxis and granule secretion of neutrophils (18–19) and monocytes (20), increases vascular permeability, and alters the vascular tone (21).

It was recently suggested that PAF is a mediator of endotoxic shock (22–24) on the basis of the following observations: (*a*) PAF is produced during endotoxic shock and experimental sepsis by Gram-negative bacteria (23–25); (*b*) infusion of experimental animals with PAF results in hypotension, decrease in cardiac output, and hypovolemic shock (26–28); (*c*) three PAF receptor antagonists (CV3988, kadsurenone, and SRI 63072) inhibit or reverse endotoxin-induced hypotension and, in this way, prolong the survival of rats (22, 23, 29).

Tumor necrosis factor/cachectin (TNF) is a mediator of endotoxic shock (30). Because TNF administration to experimental animals reproduces several aspects of PAF infusion (30), it seems possible that PAF is synthesized in response to

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[1] *Abbreviations used in this paper:* acetyl-CoA, acetyl-coenzyme A; APNE, *N*-acetyl-D,L-phenylalanine-β-naphthyl ester; PAF, platelet-activating factor; PBDB, *p*-bromodiphenacylbromide; PC, L-2-phosphatidylcholine; PMN, polymorphonuclear neutrophils; TPCK, L-1-tosylamide-2-phenylethyl chloromethyl ketone.

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TNF. The aim of the present study was to establish whether TNF stimulates synthesis of PAF in cultures of different primary and tumor cell lines. The results obtained show that TNF-treated rat peritoneal macrophages and polymorphonuclear neutrophils (PMN), and human vascular endothelial cells synthesize and release PAF.

Materials and Methods

Cell Cultures. Peritoneal cells were obtained from Lewis rats of 250–300 g according to Bloom et al. (31). The peritoneum was washed with RPMI 1640 medium buffered with 5 mM Pipes containing 0.25% of lipid-free BSA. The cells were washed twice with this medium by 5 min centrifugation at 200 *g*, counted in a hemocytometer, and resuspended in MEM at 10^7 cells/ml. The cells were then plated in 3.5-cm-diam plastic dishes and incubated for 60 min at 37°C. Nonadherent cells were removed by three washes with MEM containing 0.25% BSA; 90–95% of the adherent cells were characterized as macrophages on the basis of positive staining for nonspecific esterase performed on formalin-fixed cells with α-naphtol-AS-D-chloroacetate, specific esterase staining (32), and by phagocytosis of complement-activated zymosan particles (0.2 mg/ml) or of latex beads after incubation for 30 min at 37°C. Rat PMN were cultured as described (7). Endothelial cells were isolated from the human umbilical cord vein, cultured in Iscove's medium supplemented with 15% fetal calf serum (FCS), and characterized as previously described (13). Primary cultures were plated in 3.5-cm-diam plastic dishes, washed, and refed with the same medium every 2 d and 12 h before PAF synthesis assays. These cells were used when confluent after 4–7 d of culture. Human melanoma SK-MEL-109 cells were cultured in F12 and MEM (1:1) with 8% FCS. HeLa cells and human osteosarcoma (HOS) cells were cultured in Dulbecco's medium with 10% FCS. Human foreskin fibroblasts were cultured in F12 medium with 10% FCS and grown to confluency before treatment with TNF.

Synthesis and Release of PAF. For each assay, 2.5×10^5 or 10^6 cells were incubated at 37°C with either human or murine recombinant TNF (hTNF or mTNF), obtained respectively from Dr. Tatsuro Nishihara of the Suntory Institute for Biomedical Research, Osaka, Japan, and from Dr. Walter Fiers, University of Ghent, Ghent, Belgium. In some experiments, macrophages were incubated with the calcium ionophore A23187 and with complement-activated zymosan C (33), or preincubated for 10 min at 37°C with: acetyl-coenzyme A (acetyl-CoA); 2-lyso-PAF (Bachem Feinchemikalien, Bubendorf, Switzerland); EDTA; *p*-bromodiphenacylbromide (PBDB); L-1-tosylamide-2-phenylethyl chloromethyl ketone (TPCK); and *N*-acetyl-D, L-phenylalanine-β-naphthyl ester (APNE). To label PAF, macrophages were preincubated for 2 h with 15 μCi of [^{3}H]acetyl-CoA (1 Ci/mmol; Amersham Corp., Arlington Heights, IL) or with 2.5 μCi [^{3}H]2-lyso-PAF in 2 ml of MEM containing 0.25% BSA. This preincubation was previously found to yield maximal incorporation of the labeled precursors into PAF (34). After washing, the cells were stimulated with mTNF and the reactions were stopped by adding EDTA to 20 mM. The supernatant was removed and adherent cells were harvested with a rubber policeman in 1 ml of methanol. PAF was extracted from supernatant and cells according to Bligh and Dyer (35), with formic acid added to lower the pH of the aqueous phase to 3.0 (36). In parallel experiments, cell viability was monitored by Trypan blue exclusion and ranged between 89 and 92% after 4 h incubation with different doses of TNF, 95–99% after 1 h incubation with complement-activated zymosan, and 85–90% after 1 h incubation with A23187.

PAF Purification. The supernatants and the methanol fraction obtained from cells were extracted with a 1:1:0.9 (vol/vol/vol) mixture of chloroform, methanol, and water or medium, and the chloroform-rich phase was retained (37). To measure the overall recovery of PAF, 10 nCi of [^{3}H]PAF was added to the cells or supernatants and extracted as described above. The extracted lipids were analyzed by thin layer chromatography (TLC) on silica gel plates 60 F254 (E. Merck, Darmstadt, Federal Republic of Germany) developed with 65:35:6 (vol/vol/vol) chloroform, methanol, and water (37). 1-cm^2 sections

of the plates were scraped into glass tubes and the lipids were extracted three times for 30 min with 2 ml of 1:2:0.8 (vol/vol/vol) chloroform, methanol, and water. The silica was removed by centrifugation and the supernatant was adjusted to and extracted with 1:1:0.9 (vol/vol/vol) chloroform, methanol, and water. The chloroform phase was then removed and dried. The dried material was resuspended for quantitation and characterization of PAF in Tris-buffered Tyrode's solution (2.6 mM KCl, 1 mM $MgCl_2$, 1.37 M NaCl, 6 mM $CaCl_2$, 0.1% sucrose, and 1 mM Tris buffer, pH 7.4) containing 0.25% BSA (fraction V; Pentex Biochemicals, Kankakee, IL).

In studies with labeled precursors of PAF, the lipid extracts were chromatographed on 60 F254 TLC plates with 50:25:8.4 (vol/vol/vol/vol) chloroform, methanol, acetic acid, and water (34); 0.5 cm^2 sections were scraped, and radioactivity was counted after addition of scintillation liquid. Commercially available synthetic PAF and 2-lyso-PAF, L-2-phosphatidylcholine (PC) and sphingomyelin from bovine brain, and L-2-lyso-PC from bovine liver (Sigma Chemical Co., St. Louis, MO) were used as standards. The recovery of [^{3}H]PAF after extraction and purification was 96–98%.

PAF Assay. After extraction and purification, PAF was detected by aggregation of washed rabbit platelets prepared as described (38), using an aggregometer. For PAF assay, 5×10^7 platelets were stirred at 37°C in 0.3 ml of Tris-buffered Tyrode's solution supplemented with 0.25% gelatin (Difco Laboratories, Detroit, MI), 0.01 mM indomethacin to inhibit cyclooxygenase, 312.5 mg/liter of creatine phosphate and 152.5 mg/liter of creatine phosphokinase to convert ADP into ATP. In this way, both arachidonic acid– and ADP–dependent platelet aggregation were blocked. PAF concentration was calculated from a calibration curve with 10 μl of various solutions containing synthetic PAF added to rabbit platelets at a final concentration of 1–15 ng/ml. A linear correlation between platelet aggregation and added PAF was observed in a concentration range of 1–10 ng/ml. PAF was quantitated after extraction and purification by TLC (37), and its concentration was calculated on the linear portion of the calibration curve from at least three dilutions of the same sample. This method allowed to quantitate (in nanograms per milliliter) biologically active material of standard PAF.

Characterization of PAF. Biologically active material extracted from cells and supernatant in different experiments was characterized by comparison with PAF obtained from sensitized rabbit basophils (37) and with synthetic PAF by the following criteria (39): (*a*) induction of platelet aggregation by a pathway independent from both ADP- and arachidonic acid/thromboxane A_2–mediated pathways; (*b*) specificity of platelet aggregation inferred from the inhibitory effect of 5 μM SRI63072 (29) and CV3988 (40), two different PAF receptor antagonists; (*c*) physicochemical characteristics such as inactivation by strong bases and phospholipase A_2, but resistance to phospholipase A_1 (41), acids, weak bases, and 5 min heating in boiling water. The methods used were previously described in detail (39).

Results

The experiments shown in Fig. 1 established that rat peritoneal macrophages adherent to plastic dishes release PAF in response to TNF. The macrophages were incubated for 1 h with 20 ng/ml of mTNF and PAF was assayed after purification from cells and medium as described in Materials and Methods. The macrophages produced PAF in amounts comparable to those obtained during phagocytosis of complement-activated zymosan C, and ~75% of maximal synthesis obtained with the calcium ionophore A23187 (Fig. 1). The level of cell-associated PAF was approximately equal to that in the medium, suggesting that TNF was a potent inducer of both PAF synthesis and release. The material extracted from cells and medium had biologic and physicochemical characteristics identical to those of synthetic PAF and of PAF released from IgE-sensitized rabbit basophils. It induced platelet aggregation in an ADP- and arachidonic

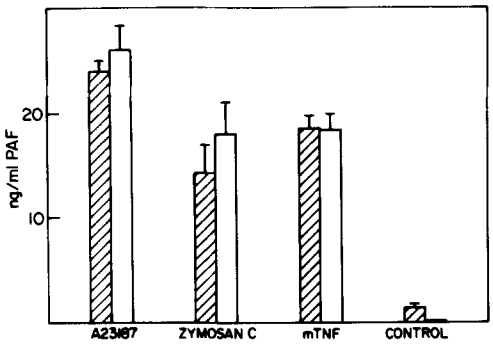

FIGURE 1. Synthesis and release of PAF by rat peritoneal macrophages treated for 1 h with 1 μg/ml of A23187, 0.2 mg/ml of zymosan C, 20 ng/ml of mTNF or untreated. 10^6 cells were used for each experiment. Cell-associated PAF, shaded columns; supernatant PAF, blank columns. In this and the following figures, PAF concentration is referred to 1 ml of cell culture medium and to the corresponding cell aliquot to allow comparison of the amount of PAF released with that remaining cell associated. Vertical bars indicate the standard deviation of the mean of different experiments ($n = 3$). The assay of PAF was carried out in duplicate for each experiment.

acid–independent way, which was specifically inhibited by the PAF receptor antagonists SRI63072 and CV3988 (29, 40). PAF activity was destroyed after base-catalyzed methanolysis (0–1% residual activity) or after treatment with phospholipase A_2 (0–3% residual activity), indicating the presence of an ester linkage at *sn*-2 (13, 37, 41–43). Treatment with phospholipase A_1 did not inhibit PAF activity, suggesting the presence of an ether bond at *sn*-1 (13, 41). The PAF activity was resistant to treatment with acids or weak bases (21, 37). After base-catalyzed methanolysis or digestion with phospholipase A_2, treatment with acetic anhydride restored 88.7 ± 7% ($n = 3$) of the biologic activity. The PAF obtained from macrophages had the same R_f (0.21) in TLC as synthetic PAF, using as solvent chloroform/methanol/water (65:35:6). No PAF activity was detected in any other TLC fraction. The R_f of PAF changed to 0.10 by using in TLC a solvent with different polarity (chloroform/methanol/water; 65:35:4), indicating that PAF extracted from macrophages is a polar lipid.

Evidence that PAF was synthesized de novo by mTNF-treated rat peritoneal macrophages was obtained by following the incorporation of radioactive precursors. TLC analysis of the lipid fraction extracted 1 h after addition of mTNF from macrophages preincubated with [^{3}H]acetyl-CoA showed one main peak of radioactivity that comigrated with synthetic PAF; this peak was absent from the lipid fraction of untreated macrophages (Fig. 2*A*). Another experiment showed that exogenous 2-lyso-PAF could be a substrate for PAF synthesis. TLC analysis of the lipid fraction of macrophages preincubated with [^{3}H]2-lyso-PAF plus 0.1 mM unlabelled acetyl-CoA and then treated with mTNF showed three peaks of radioactivity (Fig. 2*B*). The first peak comigrated with 2-lyso-PAF, the second peak with PAF, and the third peak with PC. In the lipid fraction of untreated cells the PAF peak was absent, indicating that [^{3}H]2-lyso-PAF was only converted into other phospholipids comigrating with PC. It should be pointed out that, in these experiments, efficient conversion of 2-lyso-PAF into PAF required the addition of acetyl-CoA, since only a small peak of radioactivity comigrated with PAF when the lipid fraction of cells preincubated with [^{3}H]2-lyso-PAF alone and treated with mTNF was analyzed by TLC (data not shown).

Time Course and Dose-Response of PAF Synthesis. The following experiments measured the time course of PAF synthesis and release into the supernatant by macrophages treated with 20 ng/ml of mTNF (Fig. 3). After 30 min PAF was mainly cell associated, whereas after 1 h it was present in equal amounts in cells

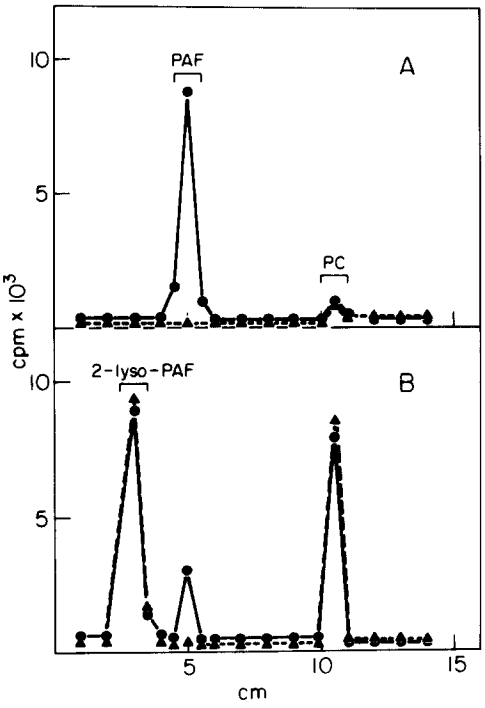

FIGURE 2. Representative TLC analysis of PAF synthesized by rat peritoneal macrophages stimulated for 1 h with 20 ng/ml of mTNF after 2 h preincubation with 15 μCi/ml of [^{3}H]acetyl-CoA (*A*) or with 0.1 mM unlabelled acetyl-CoA and 2 μCi/ml [^{3}H]2-lyso-PAF (*B*). The lipids were extracted from cells and supernatant (35) and analyzed by TLC using as solvent chloroform/methanol/acetic acid/water (50:25:8:4). The plates were divided into 30 sections of 0.5 cm each and counted as described in Materials and Methods. The pattern obtained with TNF-stimulated cells (●) and control unstimulated cells (▲) is shown. In parallel experiments, labeled PAF, PC, and 2-lyso-PAF were chromatographed as standards; the position of these compounds is indicated. Three experiments were performed with similar results on cell-associated and supernatant lipids.

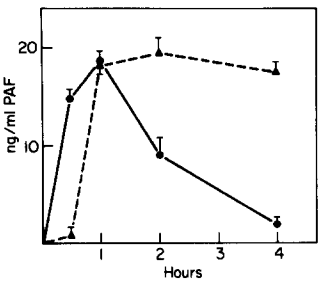

FIGURE 3. Time course of PAF synthesis and release by rat peritoneal macrophages stimulated with 20 ng/ml of mTNF. Cell-associated PAF (●) and PAF released in the supernatant (▲) are shown; 10^6 cells were used for each time point.

and supernatant. Cell-associated PAF decreased afterwards, whereas that in the supernatant increased sharply after 1 h but declined slightly after 4 h (Fig. 3). This indicated that maximal synthesis of PAF occurred within 1 h of TNF addition, and that PAF was gradually released into the supernatant from 1 to 4 h. Some PAF was also inactivated during this time, since its total amount (cell associated plus supernatant) decreased. The synthesis of PAF and its release by macrophages in response to different doses of mTNF or hTNF was also measured (Fig. 4). PAF was extracted from cells and supernatant 1 h after TNF addition. The amount of cell-associated PAF was approximately equal to that found in the supernatant at all TNF concentrations tested. Significant PAF synthesis was detected with 1 ng/ml of mTNF and nearly maximal synthesis with 10 ng/ml. Fivefold greater concentrations of hTNF were required to obtain a similar response.

The Effect of Precursors and Enzyme Inhibitors on PAF Synthesis. The effect of precursors of PAF and of inhibitors of cyclooxygenase or phospholipase A_2 was

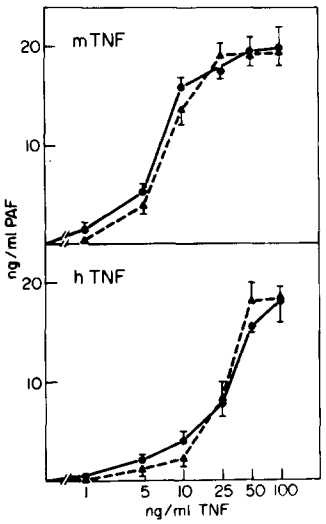

FIGURE 4. Dose response of PAF synthesis and release by rat peritoneal macrophages stimulated for 1 h with mTNF (*A*) or hTNF (*B*). The cell-associated PAF (●) and that released into the supernatant (▲) by 10^6 cells treated with different concentrations of TNF are shown.

TABLE I

Effect of PAF Precursors and Enzyme Inhibitors on the Synthesis of PAF by Rat Peritoneal Macrophages

Compound added	Concentration	PAF	
		Cell associated	Released
	μM	*μg/ml*	
Acetyl-CoA	0	15.5 ± 3.3	15.8 ± 3.7
	1	15.2 ± 3.3	16.2 ± 3.3
	10	20.5 ± 3.5	22.2 ± 1.8
	50	23.1 ± 2.5	23.7 ± 2.6
	100	24.4 ± 2.4	26.4 ± 3.3
2-Lyso-PAF	1	15.8 ± 2.9	16.8 ± 3.3
2-Lyso-PAF + acetyl-CoA	1 + 100	27.8 ± 1.7	28.5 ± 3.4
APNE	100	5.3 ± 2.5	2.4 ± 0.6
TPCK	10	0.9 ± 0.9	<0.1
EDTA	100	0.7 ± 0.6	<0.1
Indomethacin	10	16.3 ± 1.5	15.3 ± 1.4
PBDB	1	4.7 ± 1.5	3.5 ± 1.4

For each experiment, 10^6 rat peritoneal macrophages were preincubated for 10 min with the compounds indicated and then incubated for 1 h with 20 ng/ml of mTNF. Cell-associated PAF and that released into the culture medium were measured as described in Materials and Methods. Cell viability was between 85 and 92% in experiments with different inhibitors. The mean ± SD of different experiments (n = 3) is indicated.

investigated in the experiments shown in Table I. Acetyl-CoA stimulated in a dose-dependent manner the synthesis and release of PAF by peritoneal macrophages treated for 1 h with 20 ng/ml of mTNF. It should be pointed out that no PAF activity was recovered from the supernatant of untreated macrophages in control experiments (n = 8) carried out in the absence of acetyl-CoA. In contrast, minimal amounts of PAF (1.2 ± 0.8 ng/ml) were extracted from macrophages treated with 0.1 mM acetyl-CoA. When these cells were preincu-

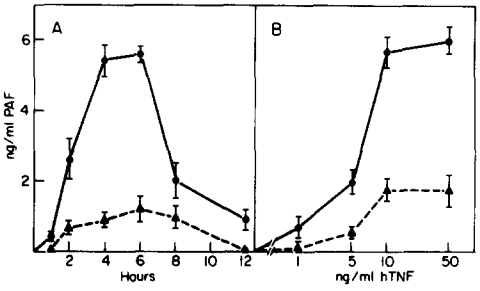

FIGURE 5. Time course (*A*) and dose response (*B*) of PAF synthesis and release by human endothelial cells stimulated with hTNF. The cell-associated PAF (●) and that released in the supernatant (▲) after treatment with 10 ng/ml of hTNF or after 4 h incubation with different concentrations of hTNF is shown; 2.5 × 10^5 cells were used for each time point and hTNF concentration. The vertical bars indicate the standard deviation of the mean of different experiments (n = 5).

bated with 2-lyso-PAF before mTNF addition, no significant increase in PAF production was observed (Table I). However, addition of both 2-lyso-PAF and 0.1 mM acetyl-CoA increased significantly the synthesis and release of PAF. In the absence of mTNF, these compounds increased the level of cell-associated PAF to 2.2 ± 0.6 ng/ml, but did not cause PAF release into the medium (data not shown).

Two inhibitors of serine proteases that block the cytocidal activity of TNF in human and murine cells (44) reduced both synthesis and release of PAF (Table I). The effect of the reversible inhibitor APNE, which is a substrate of proteases (44), was less pronounced than that of TPCK, an alkylating agent that is an irreversible inhibitor of proteases (45). TPCK is, on a molar basis, the most effective antagonist of the cytocidal activity of TNF (44). This inhibitor abolished the release of PAF into the medium. A similar effect was obtained with the cation chelator EDTA (Table I) and with ethylene-*bis*-(oxyethylenenitrilo)-tetracetic acid, a chelator with high specificity for Ca^{2+} (data not shown), suggesting that synthesis and release of PAF may require Ca^{2+} entry into TNF-treated cells. The inhibitor of cyclooxygenase indomethacin had no effect on PAF synthesis (Table I). In contrast, the inhibitor of phospholipase A_2 PBDB drastically reduced synthesis and release of PAF. These findings suggest that PAF is produced upon TNF-mediated activation of phospholipase A_2.

Production of PAF by Human Vascular Endothelial Cells and Rat PMN. Cultures of human endothelial cells obtained from the umbilical vein were treated for different times and with different concentrations of hTNF. Significant PAF synthesis was observed after 2 h of treatment, and reached its maximum after 4 h, declining sharply afterwards (Fig. 5*A*). Most of the PAF synthesized was cell associated, since only ~20% was found in the supernatant fraction. The dose-response of PAF synthesis showed nearly maximal synthesis, with 10 ng/ml of hTNF and significant synthesis with 1–5 ng/ml (Fig. 5*B*). Only ~20% of the PAF synthesized was found in the supernatant fraction at all concentrations of hTNF tested. We also assayed synthesis and release of PAF in rat PMN treated for 1 h with 20 ng/ml of mTNF: 5.7 ± 1.7 ng of PAF were found associated with 10^6 PMN, and 3 ± 1.1 ng were released per milliliter of medium. In contrast, no detectable amounts of PAF were recovered after 1–4 h of treatment with 10 ng/ml of hTNF from human foreskin fibroblasts and tumor cells such as SK-MEL-109 melanoma, HOS osteosarcoma, and HeLa cells, or from their culture media (data not shown). It appears from these results that synthesis and release of PAF are a specialized cell response to TNF. The reasons for the

different efficiency of PAF release between macrophages and endothelial cells are presently unknown.

Discussion

TNF was initially detected in the serum of endotoxin-treated animals as the mediator of the necrosis of some transplantable tumors in mice (46). It was subsequently reported that TNF is cytostatic or cytotoxic for certain tumor cells (47). However, several lines of evidence indicate that this is not the only biologic activity of TNF. This factor can stimulate fibroblast proliferation (48, 49), is pyrogenic (50), affects lipid metabolism (51), and modulates several functions of endothelial cells (52, 53). Furthermore, recent reports indicate that TNF is a mediator of inflammatory reactions. TNF is chemotactic for monocytes and PMN (54), stimulates phagocytosis (55), adherence to endothelium (52), and superoxide production by these cells (56, 57), and induces procoagulant activity in cultured human vascular endothelial cells (58). Furthermore, data from experimental animals suggest that TNF production plays a primary role in endotoxin shock (59).

A variety of cells that play a role in inflammation have been shown to produce PAF. This factor may be synthesized and released, after appropriate stimulation, from cells of the monocyte/macrophage series (6–9), PMN (7, 10, 11), platelets (12), and endothelial cells (13–15). Therefore, PAF may be involved in a variety of immunopathological reactions triggered by different cellular effectors. In addition to its role in acute inflammatory reactions, PAF may be a mediator of shock when it is released intravascularly in massive amounts within a short period of time (23–27, 37). The present study shows that, after stimulation with TNF, rat peritoneal macrophages, human endothelial cells, and rat PMN synthesize and release PAF.

Appropriate stimuli are necessary to synthesize and release PAF (1, 60). The calcium ionophore A23187 (6, 33) and the phagocytosis of zymosan (61, 62) or of immunocomplexes (9) triggers PAF production from monocytes/macrophages. The present study shows that the amount of PAF synthesized by TNF-treated rat peritoneal macrophages is comparable to that produced in response to phagocytic stimuli. The response to TNF is rapid, since already at 30 min considerable amounts of PAF are detected in these cells (Fig. 3). The subsequent decrease of cell-associated PAF in macrophages continuously incubated with mTNF indicates that its synthesis is a transient response. This can be either a consequence of downregulation of TNF receptors or of other regulatory mechanisms that limit the synthesis of PAF.

Previous studies on a variety of inflammatory cells (8, 13, 63–65), including zymosan-stimulated macrophages (33), show that PAF is synthesized by two enzymatic steps: (*a*) hydrolysis of 2-lyso-PAF by phospholipase A_2; and (*b*) acetylation of 2-lyso-PAF at position 2 by an acetyl transferase. The first step requires Ca^{2+} and is inhibited by PBDB (8). The second step is detected by the incorporation of acetate into PAF by cells incubated with labelled acetyl-CoA (8, 13, 33, 66–68). The biosynthesis of PAF by TNF-stimulated macrophages involves these two metabolic steps (Fig. 2). The requirement for Ca^{2+}, suggested by the effect of EDTA, and the inhibitory effect of PBDB (Table I) indicates

that phospholipase A_2 is involved in 2-lyso-PAF hydrolysis from membrane lipids. Addition of this compound to TNF-stimulated macrophages does not result in increased PAF synthesis, whereas acetyl-CoA stimulates PAF synthesis. These results are in agreement with previous findings (33), which suggest that the limiting step for PAF biosynthesis is the concentration of activated acetate available as acetyl-CoA.

The reduced synthesis of PAF observed in the presence of the protease inhibitors APNE and TPCK suggests that some proteolytic activity is required for TNF stimulation of PAF synthesis. These compounds protect human and murine cells incubated with the inhibitor of protein synthesis cycloheximide from the cytocidal activity of TNF (44). It seems therefore possible that this activity and the synthesis of PAF are elicited through some common step in response to the signalling of TNF-receptor complexes. Phospholipase A_2 activity is thought to be regulated in vivo by potent inhibitory membrane-associated proteins designated calpactins or lipocortins (69). Synthesis of PAF may possibly require some proteolytic cleavage of these proteins to activate phospholipase A_2.

Identical amounts of PAF are synthesized by rat peritoneal macrophages in response to optimal concentrations of either mTNF or hTNF (Fig. 4). However, mTNF stimulates these macrophages at lower concentrations than hTNF. This finding may be explained by the species-specificity of TNF. By measuring binding of radiolabelled mTNF and hTNF to homologous and heterologous receptors of murine and human cells, Smith et al. (70) have shown that mTNF binds with almost equal affinity to both cell types, whereas hTNF binds with higher affinity to human cells than to murine cells. This difference in binding to receptors is reflected by a correspondingly lower cytostatic activity of hTNF on murine L cells than on human HeLa cells (70). In the present experiments carried out on rat cells, mTNF appears to be more effective than hTNF. Therefore, it seems likely that TNF receptors on rat cells bind mTNF with greater affinity than hTNF. This concept is supported by the finding that maximal PAF synthesis by human endothelial cells is obtained with hTNF concentrations similar to those that induce maximal response in rat peritoneal macrophages with mTNF, namely 10 ng/ml (Figs. 4*A* and 5*B*). This TNF concentration (0.6 nM) results in occupancy of a large fraction of cellular receptors, based on a K_d at 4°C of 2×10^{-10} for the hTNF and mTNF receptors (70).

The synthesis of PAF in response to TNF appears to be a specialized response of cells involved in inflammation. Large amounts of PAF are synthesized by macrophages and endothelial cells, but macrophages appear to release PAF rapidly, whereas 80% of the PAF synthesized remains associated with endothelial cells (Fig. 5). The time course of PAF synthesis is also slower for endothelial cells than for macrophages. However, the endothelial cells synthesize PAF for a few hours, in contrast to macrophages, which show a transient synthesis. The observation that in a continuous incubation with TNF endothelial cells respond differently from macrophages supports the hypothesis that PAF synthesis is regulated by specific mechanisms rather than by downregulation of TNF receptors due to receptor-mediated endocytosis. These findings have to be confirmed with other macrophage and endothelial cell systems, but they suggest that regulatory networks control the duration of PAF synthesis and release in response

to TNF. The possibility that PAF itself could promote TNF synthesis was tested in rat peritoneal macrophages. Treatment of these cells with 5–50 ng/ml of PAF for 1–4 h did not result in appreciable release of mTNF into the culture medium (our unpublished observations). This finding suggests that TNF and PAF production are not interrelated.

The presence of TNF in the serum of patients with meningococcal meningitis and/or septicemia has recently been reported (71). TNF was detected in 10 of 11 patients who died, but in only 8 of 68 survivors (71). Clinical signs of severe meningococcal disease are leukopenia, thrombocytopenia, and hypotension. These symptoms may be caused by release of PAF mediated by the TNF in serum. It seems likely that PAF release may play a significant role in both local and systemic inflammatory responses to TNF production. Further studies are necessary to establish the relevance of TNF-mediated PAF synthesis in different immunopathologic conditions.

Summary

Murine tumor necrosis factor (mTNF) stimulates production of platelet-activating factor (PAF) by cultured rat peritoneal macrophages in amounts comparable to those formed during treatment with the calcium ionophore A23187 or phagocytosis of zymosan. The cell-associated PAF that was released into the medium was identical to synthetic PAF, as determined with physicochemical, chromatographic, and enzymatic assays. Furthermore, de novo synthesis of PAF by macrophages was demonstrated by the incorporation of radioactive precursors such as [^{3}H]acetyl–coenzyme A or [^{3}H]2-lyso-PAF. Macrophages incubated with mTNF for 4 h synthesized PAF only during the first h of treatment. At this time, the amount of cell-associated PAF was approximately equal to that released into the medium. The cell-associated PAF decreased afterwards, whereas that in the medium did not correspondingly increase, suggesting that some PAF was being degraded. The response of rat macrophages to different doses of mTNF and human TNF (hTNF) was examined. Maximal synthesis of PAF was obtained with 10 ng/ml of mTNF and 50 ng/ml of hTNF. This finding may be explained by a lower affinity of hTNF for TNF receptors of rat cells. The hTNF stimulated production of PAF by human vascular endothelial cells cultured from the umbilical cord vein. The time course of PAF synthesis was slower than that observed with macrophages, with maximal production between 4 and 6 h of treatment. Optimal synthesis of PAF was obtained with 10 ng/ml of hTNF. Only 20–30% of the PAF synthesized by endothelial cells was released into the medium, even after several hours of incubation. Synthesis of PAF in response to TNF was also detected in rat polymorphonuclear neutrophils, but not in human tumor cells and dermal fibroblasts. Therefore, production of PAF is a specialized response that is transient in macrophages continuously treated with TNF, and that appears to be controlled by unidentified regulatory mechanisms.

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