Tumor Necrosis Factor α-induced Angiogenesis Depends on In Situ Platelet-activating Factor Biosynthesis

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Summary

Tumor necrosis factor (TNF) α, a potent inhibitor of endothelial cell growth in vitro, is angiogenic in vivo. Therefore, it was suggested that the angiogenic properties of this agent might be consequent to the production of secondary mediators. Since TNF-α stimulates the synthesis of platelet-activating factor (PAF) by monocytes and endothelial cells, we investigated the possible involvement of PAF in the angiogenic effect of TNF-α. Angiogenesis was studied in a murine model in which Matrigel was used as a vehicle for the delivery of mediators. In this model the angiogenesis induced by TNF-α was shown to be inhibited by WEB 2170, a specific PAF receptor antagonist. Moreover, in mice injected with TNF-α, PAF was detected within the Matrigel, 6 and 24 h after TNF-α injection. The synthesis of PAF within the Matrigel was concomitant with the early migration of endothelial cells and infiltration of monocytes. No infiltration of lymphocytes or polymorphonuclear leukocytes was observed. Synthetic PAF as well as PAF extracted and purified from mice challenged with TNF-α induced a rapid angiogenic response, inhibited by WEB 2170. These results suggest that the angiogenic effect of TNF-α is, at least in part, mediated by PAF synthesized from monocytes and/or endothelial cells infiltrating the Matrigel plug.

Angiogenesis is important in wound healing, tumor growth, and inflammation, and activated monocytes/macrophages are key angiogenesis effector cells in these settings (1, 2). The array of mediators involved in the initiation and progression of angioproliferative disease processes remains to be precisely defined. It has been recently reported that TNF-α/cachectin (TNF-α), a polypeptide hormone with a wide range of biological activities (3), is angiogenic in vivo (4, 5). Since TNF-α is a potent inhibitor of endothelial cell growth in vitro (4), it was suggested that the angiogenic properties of this agent might be consequent to the production of secondary mediators. Several in vitro studies (6–8) have established an interaction between TNF-α and platelet-activating factor (PAF), a phospholipid mediator of inflammation. PAF belongs to the structurally related family of acetylated alkyl phosphoglycerides and possesses diverse and potent biological properties (9–11). TNF-α induces PAF synthesis from several cell types including monocytes/macrophages, polymorphonuclear neutrophils, and endothelial cells (6, 7). PAF, in turn, was shown to stimulate the production of TNF-α by monocytes/macrophages (8). As the administration of PAF to experimental animals reproduces several aspects of TNF-α infusion (3), it seems possible that PAF mediates some of the biological properties of TNF-α. Aside from being the primary mediator of endotoxic shock and from being cytostatic or cytotoxic for certain tumor cells, TNF-α possesses a number of biological activities that are relevant to the development of inflammatory reactions (3), including the ability to stimulate in vivo the neoangiogenesis (4, 5). Whether these biological properties of TNF-α depend on interactive activities between TNF-α and PAF remains unknown. However preliminary results indicate that PAF mediates, at least in part, the increased permeability of endothelial monolayer (12) and the contraction of mesangial cells (13) induced by TNF-α as well as the delayed PMN adhesion to and migration across the endothelium activated by TNF-α (14). Other properties of TNF-α such as the cytotoxicity and the early adhesion of PMN to stimulated endothelium seem not to involve PAF as secondary mediator (15). The aim of this study was to evaluate whether the angiogenic property of TNF-α may depend on PAF synthesis. The results of this study indicate that the angiogenic effect of TNF-α in a murine model in which Matrigel is used as a vehicle for the delivery of mediators (16), depends, at least in part, on the synthesis of PAF. Indeed, PAF was synthesized within the Matrigel during the angiogenic response to TNF-α, furthermore, WEB 2170, a specific PAF receptor antagonist (17), inhibited this angiogenic response, and PAF itself was found to be angiogenic.

Materials and Methods

Reagents. Matrigel basement membrane matrix was obtained from Becton Dickinson Labware (Bedford, MA). Human recombinant TNF-α, heparin (from porcine intestinal mucosa) and protamine sulfate were provided by Sigma Chemical Co. (St. Louis, MO). Synthetic C16 PAF (1-hexadecyl-2-acetyl-sn-glyceryl-3-phosphorylcholine) was obtained from Bachem Feinchemikalien (Bubendorf, Switzerland). WEB 2170 (Boehringer, Ingelheim KG, Germany) was used as PAF receptor antagonists (17). Anti–mouse T cell serum, anti-L3/T4 and Ly2 mAbs, and anti-Mac-1 FITC-conjugated mAbs were purchased from Cedarlane Laboratories Ltd., Hornby, ON, Canada). FITC-conjugated anti–rabbit IgG and anti–mouse IgG were obtained from Cappel Laboratories (Cochranville, PA). Rabbit anti–human von Willebrand Factor (vWF) was obtained from Sigma Chemical Co. Silica gel 60F254 TLC plates were obtained from Merck (Darmstadt, Germany). μPorasil HPLC columns were provided by Millipore Corp. Waters Chromatographic (Milford, MA).

Murine Angiogenesis Assay. Female C57 mice were used at 6-8 wk of age. Angiogenesis was assayed as growth of blood vessels from subcutaneous tissue into a solid gel of basement membrane containing the test sample (16, 18). Matrigel (8.13 mg/ml), in liquid form at 4°C, was mixed with the experimental substances and injected (0.5 ml) into the abdominal subcutaneous tissue of mice, along the peritoneal midline. Matrigel rapidly forms a solid gel at body temperature, trapping the factors to allow slow release and prolonged exposure to surrounding tissues. At various times, mice were subsequently killed and gels were recovered and processed for histology. Typically, the overlying skin was removed, and gels were cut out by retaining the peritoneal lining for support. Part of the tissue was fixed in 10% buffered formalin and embedded in paraffin. Sections cut at 3 μm and stained with hematoxylin and eosin were studied by light microscopy. Other sections, obtained from frozen tissue cut with a cryostat, were stained for nonspecific esterase activity (19, 20) or processed for immunofluorescence microscopy, performed as previously described (21). Vessel area and the total Matrigel area were planimetrically assessed from stained sections, as described by Kibbey et al. (18). Only those structures possessing a patent lumen and containing RBC were considered vessels. Results were expressed as percentage ± SEM of the vessel area to the total Matrigel area.

Experiment Protocol. The angiogenic effect of various doses of TNF-α (1–10 ng) in 0.5 ml of Matrigel was studied at different times and in the absence or presence of different doses of heparin (6.4–64 U/ml). In selected experiments, the effect of WEB 2170, (17) on TNF-α–induced angiogenesis was evaluated. WEB 2170 was included in the Matrigel plug (final concentration, 250 ng/ml) and injected intraperitoneally (10 mg/kg) 30 min before the subcutaneous injection and daily for 6 d. The direct angiogenic effect of synthetic PAF and PAF extracted and purified from Matrigel plugs of mice challenged with TNF-α was also studied.

PAF Assay. PAF extracted and purified by the Matrigel plugs obtained from mice was quantitated by bioassay on washed rabbits platelets (22) and by HPLC tandem mass spectrometry (MS) (23). PAF bioactivity, tested after extraction (24) and purification by TLC and HPLC (25), was characterized by comparison with synthetic PAF according to the following criteria: (a) induction of platelet aggregation by a pathway independent of both the ADP- and arachidonic acid/thromboxane A2–mediated pathway; (b) specificity of platelet aggregation as inferred from the inhibitory effect of PAF receptor antagonist WEB 2170 (5 μM); (c) TLC and HPLC chromatographic behavior and physicochemical characteristics such as

inactivation by strong bases and 5-min heating in boiling water; (d) MS spectra obtained at the same retention time of C16 synthetic PAF (29.9–30.2 min) with a fragmentation pattern characterized by a molecular ion (mass/atomic number ratio [m/z] 523) and a fragment corresponding to phosphocholine (m/z 183). The methods used were previously described in detail (23, 25).

Results

Matrigel containing 64 U/ml of heparin and various doses of TNF-α, TNF-α plus WEB 2170, or vehicle alone, as control, was injected subcutaneously into mice. The histologic and morphometric analysis of Matrigel plugs was performed at death 6 d later. As shown in Fig. 1 A, TNF-α induced
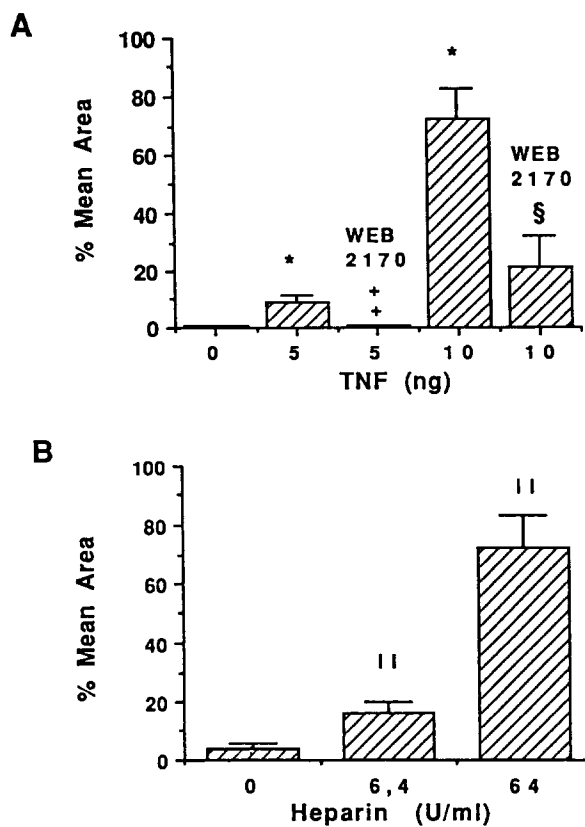

Figure 1. Effect of PAF receptor antagonist WEB 2170 (A) and heparin (B) on TNF-α–induced angiogenesis. (A) Matrigel containing 64 U/ml heparin was mixed with TNF-α, TNF-α + WEB 2170, or vehicle alone as a control and was injected subcutaneously into mice. (B) Matrigel containing 10 ng TNF-α without or with 6.4 or 64 U/ml heparin was used. After 6 d, mice were killed and the Matrigel plugs were excised and processed for light and immunofluorescence microscopy. Quantitation of neovascularization was performed on hematoxylin-eosin stained histologic sections as described in Materials and Methods, and results were expressed as percent ± SEM of the vessel area to the total Matrigel area. Each individual experimental group included five mice. ANOVA with Newman-Keul's multi-comparison test was performed: control vs. TNF-α 5 or 10 ng (*) $p <0.05$; WEB 2170 + TNF-α 5 ng vs. TNF-α 5 ng (‡) $p <0.05$; WEB 2170 + TNF-α 10 ng vs. TNF-α 10 ng (§) $p <0.05$; TNF-α without heparin vs. TNF-α + heparin 6.4 or 64 U/ml (∥∥) $p <0.05$. Similar results were obtained when quantitation of neovascularization was performed on slides stained by immunofluorescence with vWF antibodies.

a dose-dependent angiogenic response that was absent in controls and significantly reduced in mice receiving Matrigel containing TNF-α and WEB 2170 (250 ng/ml) followed by daily intraperitoneal injection of WEB 2170 (10 mg/kg die). The optimal angiogenic effect of TNF-α required the concentration of 64 U/ml of heparin (Fig. 1 B), as described for the basic fibroblast growth factor (16). However, the neoangiogenesis was also observed at a concentration of 6.4 U/ml of heparin, compatible with that observed in inflamed tissues (26). In preliminary time course experiments, it was established that maximal vascularization of Matrigel occurred at day 6. After 24 h, penetration into the Matrigel of cords of vWF-positive endothelial cells (Fig. 2, A and B) and of scattered Mac-1 (Fig. 2 A, inset) esterase positive cells was observed in mice treated with TNF-α but not in controls (Fig. 2 C). Canalization of vessels progressively increased in the following days and reached its maximal at day 6 (Figs. 2 D

and 3 A), with formation of linear or microaneurismatic structures containing RBCs and leukocytes (Fig. 3 A). Sections of the gel were stained with anti-vWF antibodies to confirm the presence of endothelial cells in association with the vessels (Fig. 2 D). Infiltration into the Matrigel of cells positive for pan-T lymphocyte markers or for L3/T4 or Ly2 antigens as well as of PMN was never observed. In contrast, scattered Mac-1-esterase positive cells were also infiltrating the Matrigel at day 6, indicating the presence of few monocytes/macrophages in the extravascular matrix. As shown in Fig. 3 B, WEB 2170 markedly reduced the vascularization of Matrigel induced by TNF-α at day 6. In selected experiments, PAF was extracted from Matrigel 6 h, 24 h, and 6 d after the beginning of the experiment. PAF was detected at 6 and 24 h in mice injected with Matrigel containing TNF-α but not in controls (Fig. 4). When the animals were injected with Matrigel containing 5 ng synthetic PAF, an angiogenic response was observed (Fig. 5, Fig. 3, C and D). Moreover, the amount of biological PAF produced after the stimulation
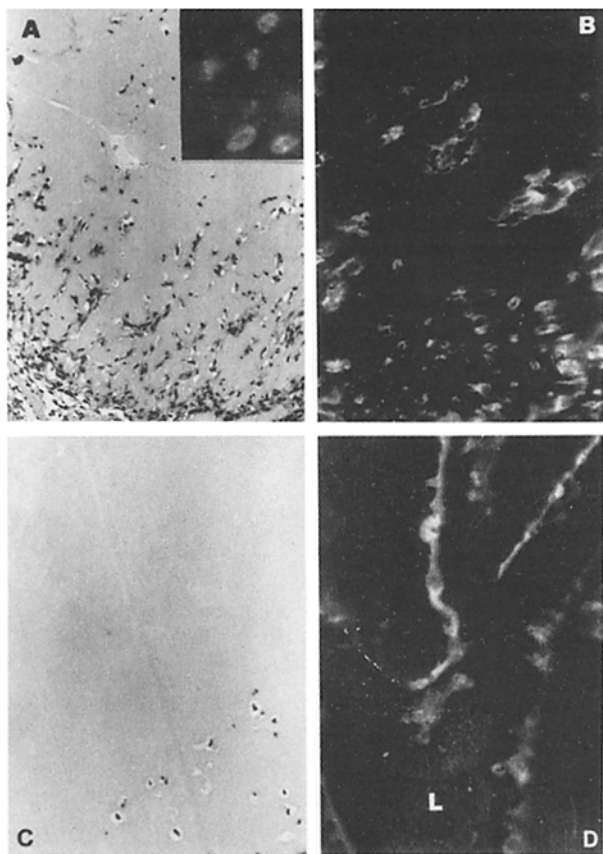
Figure 2. Histological analysis of Matrigel plugs. Hematoxylin-eosin (A) and vWF antibody–stained section (B) of Matrigel containing 64 U/ml heparin and 10 ng TNF-α excised 24 h after injection. Penetration of cords of vWF-positive endothelial cells into Matrigel is evident. Scattered Mac-1–positive monocytes (inset, A) are seen in the Matrigel containing TNF-α at 24 h. (C) Hematoxylin-eosin–stained section of control Matrigel without TNF-α, excised 24 h after injection. (D) Endothelial cells, stained by indirect immunofluorescence for vWF, underlining the lumen of a large branched vessel in sections of Matrigel containing 64 U/ml heparin and 10 ng TNF-α excised 6 d after the injection. (A) ×100, inset ×400; (B) ×250; (C) ×100; (D) ×250.
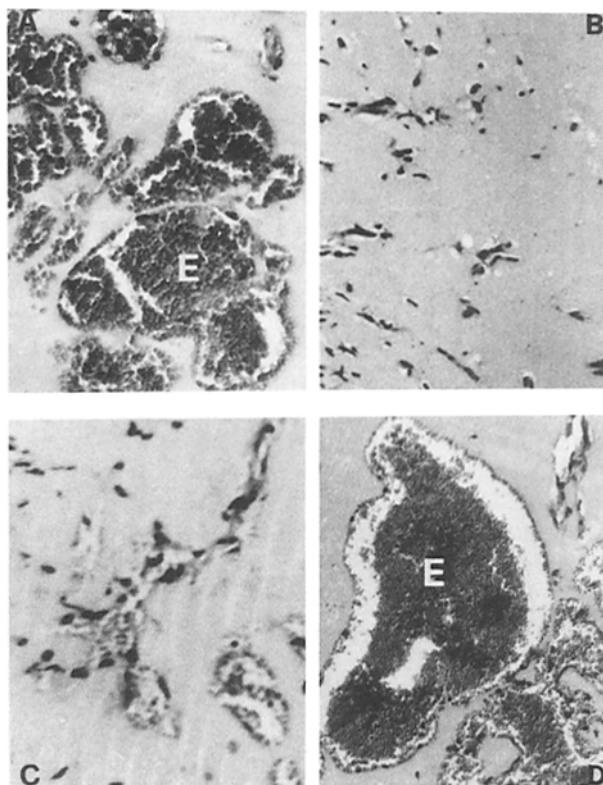
Figure 3. (A) Hematoxylin-eosin–stained sections of Matrigel containing 64 U/ml heparin and 10 ng TNF-α excised 6 d after the injection. Canalized vessels and microaneurismatic structures containing RBCs and leukocytes are seen. (B) Inhibitory effect of WEB 2170 on neovascularization of Matrigel containing 64 U/ml heparin and 10 ng TNF-α excised 6 d after injection. (C and D) Sections of Matrigel containing 64 U/ml heparin and 5 ng of synthetic PAF, excised 24 hours (C) and 6 d (D) after the injection. By light microscopy canalized vessels are seen already at 24 h and tend to form large aneurismatic vessels containing erythrocytes (E) after 6 d. (A–D, ×100).
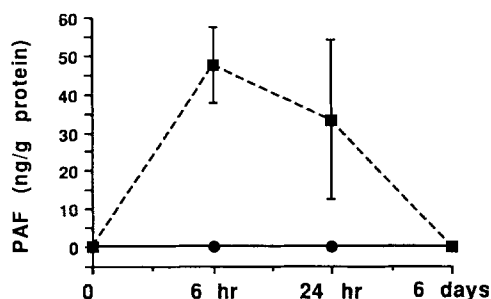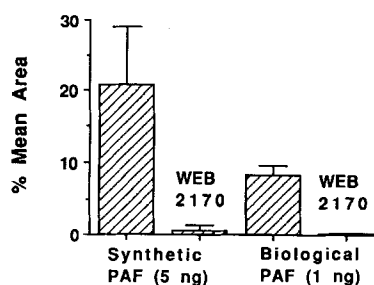
Figure 4. Quantitation of PAF extracted and purified from Matrigel containing 64 U/ml heparin and 10 ng TNF-α (■) or the vehicle alone (●) excised at different times after the injection. PAF was extracted, purified, and quantitated as detailed in Materials and Methods. Results are expressed as mean ± SEM of five experiments for each time studied.

Figure 5. Quantitation of neovascularization performed on hematoxylin-eosin-stained sections of Matrigel containing 64 U/ml heparin and 5 ng synthetic PAF (five experiments) or 1 ng biological PAF extracted and purified from five mice injected with Matrigel containing TNF-α, and effect of WEB 2170 (see Materials and Methods). Mice were killed 6 d after the injection of Matrigel.

with TNF-α and purified from the Matrigel plugs was sufficient to elicit a direct angiogenic response (Fig. 5). This angiogenic response was completely prevented by the receptor antagonist WEB 2170 (Fig. 5). The time course study indicated that the angiogenic effect of PAF occurred earlier than with TNF-α, as canalized vessels containing erythrocytes and leukocytes were already observed at 24 h (Fig. 3 C). The angiogenic effect of PAF was not associated with an inflammatory infiltration of Matrigel.

Discussion

The results of this present study indicate that PAF is a mediator of the angiogenic activity of TNF-α. PAF may function either as an intercellular mediator or as an intracellular messenger (11). Some of its actions are achieved at concentrations as low as 10^{-12} M and include events relevant for the development of several pathological and physiological processes, such as inflammation, shock, embryogenesis, and cell differentiation (9–11, 27–29). It was recently shown that PAF acts through a specific receptor that belongs to the family of "serpentine" receptors containing seven α-helical domains that weave in and out of the plasma membrane (30, 31). The receptor interacts with a G protein which activates a phosphatidylinositol-specific phospholipase C (32). Potent antagonists of PAF receptor have been described and are currently used in studies of the pathophysiology of PAF.

In vitro studies have established an interaction between TNF-α and PAF (6–8). TNF-α stimulates the synthesis of PAF by monocytes/macrophages, polymorphonuclear neutrophils, and endothelial cells (6, 7). PAF, in turn, stimulates TNF-α production by monocytes/macrophages (8). It is therefore possible that these two mediators may reciprocally interact during the development of several pathological and physiological processes. The present observation that PAF is synthesized within Matrigel during vascularization induced by TNF-α and that a specific PAF receptor antagonist, WEB 2170, inhibits the angiogenic process, suggests that this phos-

pholipid may act as a secondary mediator for TNF-α. Moreover, PAF itself was shown to possess angiogenic activity. This study does not clarify the cellular source of PAF synthesized after stimulation with TNF-α. However, the monocytes as well as the endothelial cells, which were the two cell populations detectable at 6 and 24 h when PAF was synthesized within the Matrigel, are both possible candidates for the synthesis of PAF induced by TNF-α (6, 7). At the doses of TNF-α used in this study, inflammatory cells other than monocytes were not detected within the Matrigel plugs. Therefore, it is unlikely that PMN or lymphocytes are involved in the biosynthesis of PAF. Previous studies (5) have demonstrated that TNF-α can act as attractant for monocytes and that TNF-α–mediated angiogenesis is monocyte-dependent. The present observation that the angiogenic response increases seven times when TNF-α is doubled from 5 to 10 ng, further supports the contention that TNF-α acts via secondary mediators that are produced when TNF-α exceeds a threshold concentration. Previous in vitro experiments have established that 10 ng/ml of TNF-α is an effective dose for initiating PAF synthesis from both macrophages and endothelial cells (6). PAF synthesized by infiltrating monocytes/macrophages and/or endothelial cells may either act as a chemoattractant or as a mediator that amplifies and propagates the reaction. Moreover, since cells that synthesize PAF have receptors for this phospholipid, it is possible that the PAF receptor antagonist interferes with an autocrine modulation of the cell response to angiogenic cytokines. In addition, PAF stimulates arachidonate hydrolysis from phospholipids, thus initiating the synthesis of eicosanoids which, in turn, may induce additional PAF and TNF-α production (8, 33–35). In conclusion, the results of this study indicate that PAF mediates, at least in part, the in vivo angiogenic effect of TNF-α. The definition of the relative contribution of the direct angiogenic effect of PAF and/or of its role in amplifying the response to other angiogenic cytokines, requires further investigations.

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