Tumor Necrosis Factor Induces Tissue Factor-Like Activity in Human Leukemia Cell Line U937 and Peripheral Blood Monocytes

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The induction of procoagulant activity (PCA) by human recombinant tumor necrosis factor (rTNF) was studied in human monoblastic leukemia cell line U937 and human peripheral blood monocytes. Using a one-step recalcifying clotting assay, PCA in cell lysates or whole cell preparations was measured by comparison to a rabbit brain thromboplastin standard. There was a dose- and time-dependent increase in PCA when U937 cells were cultured with rTNF. The effect of rTNF was not enhanced by recombinant human interferon-gamma (rIFNγ). Cychloheximide inhibited the expression of PCA by U937 cells, showing that protein synthesis was necessary to mediate the effects of rTNF. Whole cell preparations demonstrated that >80% of the PCA was expressed on the surface of the cells. The PCA functioned as a tissue factor-like substance, since it required coagulation factor VII and factor X. rTNF also increased PCA in human monocytes in a dose- and time-dependent manner. This effect was abrogated by boiling the rTNF for ten minutes, and was not inhibited by adding polymyxin-B to the cultures, making it unlikely that endotoxin accounted for the observed effects. These results suggest that TNF-induced expression of tissue factor by mononuclear phagocytes may modulate immunologic, inflammatory, and hemostatic processes.

MATERIALS AND METHODS

Sera, media, reagents. Fetal bovine serum (FBS) of low endotoxin content was from Sterile Systems, Inc (Logan, UT); it was heat-inactivated (HI-FBS) at 56°C for 30 minutes. RPMI 1640 cell culture medium was from Gibco (Grand Island, NY) and was supplemented with penicillin (100 U/mL) and streptomycin 100 μg/mL. Leucine-deficient HI-FBS and leucine-free Eagle’s minimum essential medium (MEM) were from Gibco. Human rTNF-α was from Cetus Corporation (Emeryville, CA). rIFN-γ was from Genentech (San Francisco). Polymyxin-B sulfate, cycloheximide, Ficol/Hypaque (specific gravity 1.077) and lipopolysaccharide (LPS) isolated from Escherichia coli were from Sigma Chemical Company (St Louis). Factor VII, factor IX, and factor X-deficient plasmas were from General Diagnostics (Morris Plains, NJ). Endotoxin content of all culture materials was measured by the limulus amoebocyte lysate assay and found to be <200 pg/mL of LPS.

Cell preparation. Cells of the human leukemia cell line U937 were maintained continuously by subculturing two to three times per week. For experiments, cells were washed twice with RPMI and viability checked by trypan blue exclusion (>95% viable before each experiment). Human peripheral blood monocytes were prepared by collecting 150 mL of whole blood, anticoagulated with EDTA, from healthy volunteers. The blood was centrifuged at 200 g for 20 minutes. To further the monocyte fraction, the cells were plated on plastic 35-mm tissue culture dishes at 5.0 x 10⁶ cells/mL in RPMI with 10% HI-FBS. RPMI alone was added to monocyte culture dishes. Various combinations of the following compounds were then added
to the culture dishes: rTNF, rIFN-γ, polymyxin-B, cycloheximide. The cells were cultured at 37°C for a specified time. In some experiments, the cells were washed, suspended in new culture medium, and incubated further with LPS in RPMI (with 10% HI-FBS in U937 cell cultures). U937 cells were collected by washing each well three times with RPMI. The cells were washed twice, counted, viability checked by trypan blue exclusion, and suspended in PBS. Monocytes were scraped from the culture dishes with a rubber policeman, washed twice, viability checked by trypan blue exclusion, and suspended in PBS. Monocyte viability after harvesting was >80%. For cell lysate studies, the cells were frozen at −70°C, then thawed and sonicated using a Heat Systems-Ultrasonics, Inc (Plainview, NY) sonifier with two 30-second bursts. For whole cell studies, the procoagulant assay was performed immediately after cell incubation. Protein concentrations in the cell preparations were determined using the method of Lowry et al.23 In these studies, 1.0 × 10^6 monocytes contained approximately 0.020 mg protein.

Procoagulant activity assay. Procoagulant activity (PCA) of the cell preparations was determined from the one-step recalcification clotting time, as described by others.23 Cell lysate or whole cell suspension (0.2 mL) was added to 0.1 mL of citrate-anticoagulated pooled normal human plasma or factor-deficient plasma and 0.1 mL of 25 mmol/L CaCl₂ at 37°C. Each sample was run in duplicate. The time required for production of a fibrin clot was measured using a fibrometer (BBL Fibrosystems, Baltimore). A rabbit brain thromboplastin standard at 35.5 mg protein per milliliter (General Diagnostics, Morris Plains, NJ) was given a value of 100,000 mU/mL. Clotting times for this standard were: 10 mU, 136.0 seconds; 20 mU, 110.5 seconds; 40 mU, 88.0 seconds; 100 mU, 62.0 seconds; 200 mU, 48.5 seconds, 400 mU, 39.5 seconds, 1,000 mU, 32.5 seconds, 2,000 mU, 25.0 seconds; and 10,000 mU, 20.0 seconds. Over this range, plotting log (clotting time) vs. log (milliunits thromboplastin activity) produced a straight line (Fig 1). A 50-second clotting time was considered the cell preparations was determined from the one-step recalcification clotting time, as described by others.23 Cell lysate or whole cell suspension (0.2 mL) was added to 0.1 mL of citrate-anticoagulated pooled normal human plasma or factor-deficient plasma and 0.1 mL of 25 mmol/L CaCl₂ at 37°C. Each sample was run in duplicate. The time required for production of a fibrin clot was measured using a fibrometer (BBL Fibrosystems, Baltimore). A rabbit brain thromboplastin standard at 35.5 mg protein per milliliter (General Diagnostics, Morris Plains, NJ) was given a value of 100,000 mU/mL. Clotting times for this standard were: 10 mU, 136.0 seconds; 20 mU, 110.5 seconds; 40 mU, 88.0 seconds; 100 mU, 62.0 seconds; 200 mU, 48.5 seconds, 400 mU, 39.5 seconds, 1,000 mU, 32.5 seconds, 2,000 mU, 25.0 seconds; and 10,000 mU, 20.0 seconds. Over this range, plotting log (clotting time) vs. log (milliunits thromboplastin activity) produced a straight line (Fig 1). A 50-second clotting time was considered. Protein concentrations in the cell preparations were determined using the method of Lowry et al.23 In these studies, 1.0 × 10^6 monocytes contained approximately 0.020 mg protein.

Protein synthesis studies. U937 cells were washed four times with leucine-free RPMI, then cultured for 12 hours in a 96-well flat-bottom microtiter plate (Linbro/Titertek) in leucine-deficient MEM and 10% HI-FBS containing combinations of rTNF (100 U/mL) and cycloheximide (1.0 μg/mL). 3H-leucine (1.0 μCi/8.0 × 10^6 cells) was then added for an additional four-hour incubation. Ten percent cold trichloroacetic acid was added to precipitate the protein, and the precipitate was collected on glass fiber filter paper on a Belco Microharvester (Vineland, NJ). 3H-leucine in the precipitate was counted in Aquasol scintillation fluid using a Packard scintillation counter.

RESULTS

Leukemia cell line U937. Cells from the human monocytic leukemia cell line U937 contained low baseline levels of PCA (Fig 2). When these cells were cultured with rTNF for six hours, the cell lysates contained increased PCA per 10^6 cells. This increase in PCA was directly dependent on the concentration of rTNF in the culture. With an rTNF concentration of 100 U/mL (600 pmol/L), there was a fivefold increase in PCA over baseline values (four- to sevenfold in nine experiments). rIFN-γ has been shown to be synergistic with rTNF in enhancing certain effects of rTNF on some cell lines.8,9 When rIFN-γ (100 U/mL) was added to the U937 cell cultures, there was no significant enhancement of PCA, except at a rTNF concentration of 10 U/mL. With this concentration of rTNF, rIFN-γ enhanced the PCA 80% (P < .02) (Fig 2).

The increase in PCA in U937 cells was also time dependent. In cells cultured with rTNF (100 U/mL), there was a rapid increase in PCA over the first six hours, to a maximum of 510 ± 45 mU PCA/10^6 cells. This activity returned to near baseline by 30 hours (Fig 3). When rIFN-γ (100 U/mL) was added to similar cultures, the maximum PCA/10^6 cells was decreased 28%, but the time course of the increase in PCA was not altered.

Protein synthesis studies demonstrated that cycloheximide at 1.0 μg/mL inhibited 3H-leucine incorporation by 96% in U937 cells (162.0 ± 7.3 pmol 3H-leucine incorporated per hour per 10^6 cells in control and cycloheximide-treated cells, respectively; mean from three experiments). This concentration of cycloheximide decreased cell viability only slightly, measured by trypan blue exclusion (>95% viable in control cultures, 77% ± 8% viable with cycloheximide). When rTNF (100 U/mL) and cycloheximide (1.0 μg/mL) were

Fig 1. Assay system for measuring procoagulant activity. Log(clotting time) for the thromboplastin standard was plotted on the horizontal axis, and log(milliunits thromboplastin activity) was plotted on the vertical axis. A similar standard curve was established for each experiment. A 50-second clotting time was produced by 190 mU thromboplastin activity.

Fig 2. Dose-response relationship of PCA in U937 cells incubated with rTNF. rTNF was added at increasing concentrations to a six-hour culture. rIFN-γ (100 U/mL) or cycloheximide (1 μg/mL) was added to some cultures. The PCA assay was performed in duplicate on cell lysates. Values represent mean ± SD for nine experiments.
added to U937 cells, 3H-leucine incorporation was inhibited by 97% (6.1 pmol/h/10⁶ cells), cell viability was decreased slightly (72% ± 10%), and there was no increase in PCA noted in these cells (Fig 2).

PCA was measured in whole U937 cells immediately following six-hour culture, to determine how much PCA was expressed on the surface of these cells. Cells cultured only with media had very low PCA (119 ± 28 mU/10⁶ cells, mean ± SD for three experiments). Following culture with rTNF (100 U/mL), whole cells contained 563 ± 11 mU PCA/10⁶ cells, a fivefold increase over control. Following culture with rIFN-γ (100 U/mL), whole cells expressed very little PCA (118 ± 23 mU/10⁶ cells), similar to control cells. Following culture with rTNF and rIFN-γ, whole cells expressed 612 ± 102 mU PCA/10⁶ cells, similar to cells cultured with rTNF only. The supernatants from these cultures were also evaluated and contained very low levels of PCA (<62 mU/10⁶ cells).

The nature of the PCA induced by rTNF was evaluated using human plasmas deficient in coagulation factor VII, factor IX, or factor X. In the one-stage clotting assay, lysates of U937 cells incubated with rTNF (100 U/mL) for six hours clotted pooled normal human plasma (384 mU PCA/10⁶ cells), but did not clot factor VII-deficient plasma (<16 mU PCA/10⁶ cells) or factor X-deficient plasma (<16 mU PCA/10⁶ cells). Factor IX-deficient plasma clotted similarly to normal human plasma (313 mU PCA/10⁶ cells). These values are the mean of duplicate measurements of PCA from one representative experiment. Similar values were obtained in two additional experiments.

In other experiments, U937 cells were cultured with or without rTNF (100 U/mL) for six hours, then washed and cultured for an additional six hours with LPS (1 µg/mL). LPS enhanced the PCA/10⁶ cells 1.7-fold compared with cells cultured in media only (control). With rTNF, LPS did not further enhance the PCA/10⁶ cells in the cell lysates (data not shown). Thus, LPS induced PCA in U937 cells, but rTNF-treated cells could not be further stimulated by LPS.

Peripheral blood monocytes. Human peripheral blood monocytes were cultured with increasing doses of rTNF for 16 hours. Cells cultured with media only (control) contained low baseline PCA. With increasing rTNF concentrations, the PCA in monocyte lysates increased significantly (Fig 4). The induction of PCA reached a plateau with increasing doses of rTNF, with half-maximal stimulation occurring at a concentration of 1,000 U/mL (6,000 pmol/L). The increase in PCA was also time dependent (Fig 5). When rIFN-γ (100 U/mL) was added, there was no significant increase in PCA compared with baseline values. rIFN-γ did not alter the effect of rTNF in inducing PCA in monocytes (data not shown).

Bacterial endotoxin has been shown to induce PCA in mononuclear phagocytes. Even though we were careful to avoid endotoxin in our experiments, we were concerned that endotoxin contamination could induce the PCA observed in monocytes. Two experiments were performed to evaluate this concern. First, the media and rTNF were boiled for ten minutes before addition to the cell cultures. Boiling of the media alone (control) had no effect on the baseline PCA in

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**Fig 3.** Time course of increased PCA in U937 cells incubated with rTNF and/or rIFN-γ. Final concentrations of additives were: rTNF (100 U/mL) and rIFN-γ (100 U/mL). The PCA assay was performed in duplicate on cell lysates. Values represent mean ± SD for three experiments.

**Fig 4.** Dose-response relationship of PCA in human peripheral blood monocytes incubated with rTNF. Increasing concentrations of rTNF were added to a 16-hour culture. Cell lysates were evaluated by the PCA assay. PCA induction by rTNF was saturable in monocytes, with a half-maximal stimulation at a concentration of 1,000 U TNF/mL. Values represent mean ± SD for seven experiments.

**Fig 5.** Time course of increased PCA in monocytes incubated with rTNF (800 U/mL) or media alone (control), mean for three experiments.
the monocyte cell lysates (324 ± 20 mU/10⁶ cells v 332 ± 40 mU/10⁶ cells, respectively). Boiling of the rTNF (100 U/mL) completely abrogated its effect in inducing PCA in a 16-hour culture (native rTNF, 1,268 ± 192 mU/10⁶ cells; boiled rTNF, 432 ± 184 mU/10⁶ cells). Second, polymyxin-B was added to monocyte cultures for 16 hours with rTNF (100 U/mL). Polymyxin-B had no effect on the baseline PCA in cells cultured with media only (365 ± 20 mU/10⁶ cells). Polymyxin-B did not inhibit the effect of rTNF, but it did slightly enhance it (2,100 ± 272 mU PCA/10⁶ cells without polymyxin-B, and 2,360 ± 180 mU PCA/10⁶ cells with polymyxin-B).

When monocytes were cultured for 16 hours with rTNF (100 U/mL), then washed and incubated an additional six hours with LPS (1 µg/mL), there was a dramatic increase in PCA in all cell lysates (six- to 20-fold). With increasing doses of rTNF, slightly higher levels of PCA following LPS incubation were observed. However, these differences were not statistically significantly different (Fig 6).

**DISCUSSION**

Tissue factor is a membrane-bound phospholipid-protein complex found in several cell types, including fibroblasts, endothelial cells, some leukemia cell lines, and mononuclear phagocytes. Many substances, including endotoxin, immune complexes, phorbol esters, thrombin, and some components of the complement system can induce tissue factor activity in these cells.15-18,24 Tissue factor is a potent activator of coagulation factor VII, and the tissue factor-VIIa complex activates factor X.27,28 In this study we present evidence that PCA is induced in the human monoblastic leukemia cell line U937 and peripheral blood monocytes by TNF. The PCA induced in U937 cells in this study was tissue factor-like, since it required factor VII and factor X to initiate coagulation. Recent studies by Nawroth and Stern, and Bevilacqua et al have shown that TNF elicits tissue factor expression in cultured human endothelial cells in a dose- and time-dependent manner.11,12 The time course and requirement for protein synthesis in their studies are similar to the results reported here for U937 cells. Recent studies by Gregory et al have suggested that induction of PCA in monocytes is related to a previously unidentified, unique cytokine.29 In their experiments, TNF (as high as 5,000 U/mL) did not induce PCA in monocytes. It is possible that the effect of TNF on monocytes is dependent on a number of factors, including the specific conditions of the monocyte isolation and culture, producing the different results with similar doses of TNF.

Our experiments confirm that endotoxin stimulates tissue factor activity in U937 cells and peripheral blood monocytes.13,30,31 Induction of PCA, as well as the lymphocyte mitogenic and macrophage “activating” properties of endotoxin, have been attributed to the lipid A portion of the lipopolysaccharide.32-34 Lipid A is resistant to heating to 100°C for 20 minutes, while many native polypeptides are denatured by this treatment. Also, the lipid A portion of some bacterial endotoxins is bound by polymyxin-B, a cationic polypeptide antibiotic, abrogating its effect on lymphocyte mitogenesis, induction of macrophage tumoricidal activity, and induction of PCA.32,43 Our studies with heat-denatured TNF and polymyxin-B strongly suggest that the induction of tissue factor-like activity by TNF is not due to contaminating endotoxin.

Previous studies have shown that the majority of tissue factor activity in mononuclear phagocytes can be isolated from the plasma membrane.44,47 In this study, >80% of the tissue factor-like activity induced in U937 cells by TNF was expressed on the surface of intact cells, compared with cell lysates. Very little or no activity was released into culture supernatants by these cells.

Expression of tissue factor-like activity by mononuclear phagocytes in response to many stimuli, such as immune complexes, viruses, and lectins, may require or be enhanced by interaction with lymphocytes.39,40 Other stimuli may induce tissue factor expression directly.41 In our monocyte cultures, some contaminating lymphocytes were present, and these may have contributed to the observed effects of TNF. However, in the U937 leukemia cell cultures, no lymphocytes were present, and therefore they could not play a role in the TNF-induced expression of tissue factor-like activity. Other factors not tested here may further modify the effects of TNF on these cells.

The molecular events involved in induction of tissue factor-like activity in mononuclear phagocytes by TNF are unknown. In other cell systems, protein synthesis has been shown to be a necessary event for tissue factor induction.11,12,42 Likewise, our results indicate that protein synthesis is necessary for the TNF induction of PCA in U937 cells. U937 cells, other leukemia cell lines, and normal human hematopoietic cells (except erythrocytes and platelets) have been shown to possess cell surface receptors for TNF.10,43 IFN-γ has been shown in some cell systems to induce TNF surface receptors.44-46 and IFN-γ has been shown to be synergistic with TNF in enhancing certain effects of TNF on these cell lines.9,10 In this study, IFN-γ was not found to enhance the effects of TNF on induction of tissue factor-like activity in U937 leukemia cells or monocytes, except at a TNF concentration of 10 U/mL with U937 cells.

The data presented here suggest a new way in which...
immunologic and inflammatory events may modulate the hemostatic system: by TNF-induced expression of tissue factor-like activity on the surface of mononuclear phagocytes. This may represent an important role for mononuclear phagocytes in a variety of disease processes. In particular, mononuclear phagocytes could mediate the hemorrhagic necrosis of some tumors attributed to TNF. TNF may also mediate tissue factor production by mononuclear phagocytes in disease processes such as disseminated intravascular coagulation related to gram-negative bacterial sepsis or some malignancies. Understanding the molecular events in the modulation of mononuclear phagocyte hemostatic properties may offer new therapeutic strategies in these diseases.

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