Clinical Trials with Human Tumor Necrosis Factor: In Vivo and In Vitro Effects on Human Mononuclear Phagocyte Function


INTRODUCTION

Following cloning and expression of the gene for TNF, this protein has been found to have many biological activities (for review, see Refs. 1 to 4). Several studies have suggested that TNF may be an important mediator of inflammation (5-9) and that it may significantly alter the procoagulant properties of vascular endothelial cells (10-13). Due to its tumor cell killing and other immunoregulatory properties, considerable interest has developed in the use of rHuTNF as a therapeutic agent. We have begun a Phase I trial to evaluate rHuTNF in the treatment of patients with refractory cancer. To understand the systemic effects and utility of rHuTNF as a therapeutic agent, we studied the effects of rHuTNF in vitro or in vivo on several parameters of human mononuclear phagocyte function. We correlated these findings with changes in related clinical parameters in these patients. We report enhanced PMA-stimulated hydrogen peroxide production and procoagulant activity expressed in human mononuclear phagocytes following in vitro or in vivo treatment with rHuTNF. In addition, patients treated with rHuTNF developed changes in standard clotting studies suggesting activation of the coagulation and fibrinolytic systems. These findings serve as indicators of the biological activity of rHuTNF and may help explain hematological alterations observed in this clinical trial. Moreover, these data have implications for further therapeutic strategies for rHuTNF.

MATERIALS AND METHODS

Materials. Fetal bovine serum of low endotoxin content was from Sterile Systems, Inc. (Logan, UT); human serum was obtained from normal, healthy donors. All serum was heat inactivated at 56°C for 30 min. RPMI was from Gibco (Grand Island, NY) and was supplemented with penicillin (100 units/ml) and streptomycin (100 µg/ml). Endotoxin content of all cell culture materials was measured by the Limulus amebocyte assay and found to be <200 pg/ml of lipopolysaccharide. rHuTNF was from Cetus Corporation (7 x 10^6 units/mg; Emeryville, CA) and Genentech (7 x 10^6 units/mg; San Francisco, CA) for in vitro studies and from Knoll Pharmaceuticals (6 x 10^6 units/mg; Whippany, NJ) for in vivo studies. Ficoll/sodium diatrizoate, lipopolysaccharide isolated from Escherichia coli, and PMA were from Sigma Chemical Company (St. Louis, MO). Rabbit brain thromboplastin standard was from General Diagnostics (Morris Plains, NJ).

Cell Preparation. For in vitro studies, human peripheral blood monocytes were prepared by collecting 50 to 150 ml of whole blood, anticoagulated with EDTA, from healthy volunteers. The blood was centrifuged at 200 x g for 20 min, and the platelet-rich plasma was removed.

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2 The abbreviations used are: TNF, tumor necrosis factor; rHuTNF, recombinant human tumor necrosis factor; PMA, phorbol myristate acetate; PT, prothrombin time; aPTT, activated partial thromboplastin time; RPMI, Roswell Park Memorial Institute Medium 1640; PBS, 2.7 mM KCl/0.88 mM KH2PO4/6.4 mM Na2HPO4/137 mM NaCl (pH 7.5).
monocytes were preincubated with rHuTNF for 30 min, washed with RPMI 1640 with 10% human serum, and then incubated for the specified time. Mononuclear cell preparations from in vivo studies were added to microtiter wells immediately before assay. The production of peroxide in response to 200 nM PMA was determined using horseradish peroxidase with phenol red and a microtiter plate reader as described previously (18). PMA (200 nM) was used since this dose produces maximal peroxide production by mononuclear phagocytes. All samples were done in triplicate and expressed as nmol of peroxide/mg of protein/h for in vitro studies and nmol of peroxide/10^6 monocytes/h for in vivo studies.

Procoagulant Activity Assay. Procoagulant activity of the cell preparations was determined from the one-step recallification clotting time, as described by others (19). Cell lysate (0.2 ml) was added to 0.1 ml of citrate-anticoagulated pooled normal human plasma and 0.1 ml of 25 mM CaCl_2 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, MD). A rabbit brain thromboplastin standard at 35.5 mg of protein/ml was used as a reference standard, to determine milliunits of activity in the cell preparations. Clotting times for this standard were: 10 milliunits = 136.0 s; 20 milliunits = 110.5 s; 40 milliunits = 88.0 s; 100 milliunits = 62.0 s; 200 milliunits = 48.5 s; 400 milliunits = 39.5 s; 1,000 milliunits = 32.5 s; 2,000 milliunits = 25.0 s; 10,000 milliunits = 20.0 s (see Fig. 1). Over this range, plotting log (clotting time) versus log (milliunits of thromboplastin activity) produced a straight line. A 50-s clotting time was produced by 190 milliunits of thromboplastin activity. Clotting times for the cell preparations were compared to clotting times for the standard, to determine milliunits of activity in the cell preparations.

Hematology Studies. Complete blood counts including platelet counts and differentials were obtained on patients at regular intervals from venous blood collected in EDTA, using a Coulter Counter (Hialeah, FL). PT and aPTT were determined using a Coag-A-Mate-X2 apparatus (General Diagnostics, Morris Plains, NJ). Fibrinogen levels were measured by clotting assay using thrombin and comparison to a normal pooled plasma standard containing 267 mg of fibrinogen/dl. Fibrin degradation products were measured using a Fibrinogen Degradation Products Detection Set (American Dade, Aguada, Puerto Rico) by a latex agglutination assay. D-dimer assay was performed as previously described (20) and reported as "negative" (<10 μg/ml) or "positive" (>10 μg/ml).

RESULTS

Hydrogen Peroxide Production. Peroxide production was measured in human peripheral blood monocytes and peritoneal macrophages following incubation in vitro with rHuTNF (0 to 1000 units/ml). Monocytes incubated alone produced very low levels of peroxide (<0.6 nmol/mg of protein/h); this baseline production did not change significantly following incubation with rHuTNF. Monocytes stimulated with PMA (200 nM) produced approximately 10-fold higher levels of peroxide (5.93 ± 2.13 nmol/mg of protein/h, mean ± SD for 11 experiments) (Fig. 2). PMA-stimulated peroxide production significantly increased following incubation with increasing doses of rHuTNF. Peroxide production continued to increase up to a rHuTNF concentration of 1000 units/ml (6000 pmol/liter). The maximal effect of rHuTNF may not have been reached at this dose. Similarly, macrophages cultured alone produced very low levels of peroxide, which did not change following incubation with rHuTNF (<0.6 nmol/mg of protein/h). Macrophages produced approximately 10-fold higher levels of peroxide following PMA stimulation (5.90 ± 3.30 nmol/mg of protein/h). PMA-stimulated peroxide production by macrophages significantly increased following incubation with rHuTNF. In contrast to that seen in monocytes, the maximal statistically significant effect of rHuTNF on macrophages was observed at a concentration of 10 units/ml; higher doses did not produce higher levels of peroxide production (Fig. 2).

Previous studies have suggested that rHuTNF has a very rapid effect on the production of superoxide anion by neutrophils (21). We evaluated the time course of the effect of rHuTNF on monocytes. PMA-stimulated peroxide production was enhanced 30 to 50% above background after only 5 min of incubation with rHuTNF (10 to 100 units/ml), although this increase was not statistically significant (Fig. 3). By 3 h of incubation, peroxide production was significantly increased and continued to increase up to 42 h of incubation. In order to determine if rHuTNF was required throughout the incubation, monocytes were preincubated with rHuTNF (0, 10, 100, or 1000 units/ml) for 30 min, washed thoroughly, and then incubated for the specified period. There was no significant increase in PMA-stimulated peroxide production at 180 or 900 min. At 1320 min, there was a significant increase for monocytes incubated with 1000 units/ml (126% of control). At 2520 min, there were increases for monocytes incubated with 10, 100, or 1000 units/ml (98%, 166%, and 292% of control, respectively). The maximal response was seen only for monocytes preincubated with rHuTNF at 1000 units/ml.

Mononuclear cells (monocytes and lymphocytes) were isolated from 9 patients immediately before and 30 min after a 2-h i.v. infusion of rHuTNF (40 to 200 μg/m2). Using the percentage of monocytes in the preparations determined by light microscopy, peroxide production was determined per 10^6 monocytes per hour. Monocytes isolated before and after the
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Fig. 3. Time course of rHuTNF effect on PMA-induced peroxide production by human monocytes. Peroxide production for cultures with 10 and 100 units of TNF/ml expressed as the percentage of control value (culture with 0 units of TNF/ml). Columns, mean for 6 experiments; bars, SD. *, P < 0.01 when compared to control by Student's t test.

Fig. 4. PMA-stimulated peroxide production by human peripheral blood monocytes from patients treated with rHuTNF. Mononuclear cells were isolated immediately before (pre) and 30 min following (post) a 2-h i.v. infusion of rHuTNF. Individual patient values are indicated (Δ, 40 to 80 μg/m^2; ■, >80 μg/m^2); —, mean value for 9 patients. The mean post value is significantly greater than the mean pre value with P < 0.02 by the Student t test.

Fig. 5. Procoagulant activity (PCA) in human peripheral blood monocytes, isolated from 8 donors, incubated in vitro for 16 h with medium alone or rHuTNF (100 units/ml). Points, milliunits of procoagulant activity/million cells. —, mean of the individual values. The mean PCA value is greater than the mean medium value, with P < 0.01.

Fig. 6. Procoagulant activity (PCA) in human peritoneal macrophages, isolated from 8 women undergoing peritoneal laparoscopy. In 6 of these subjects, peritoneal macrophages were also obtained. When these cell preparations were incubated for 16 h in medium only, the monocytes contained 640 ± 400 milliunits of procoagulant activity/10^6 cells, and the peritoneal macrophages contained 1730 ± 1280 milliunits of procoagulant activity/10^6 cells (mean ± SD). After incubation for 16 h with rHuTNF (100 units/ml), the mean procoagulant activity increased in monocytes to 1160 ± 800 milliunits/10^6 cells, and in the peritoneal macrophages to 3620 ± 1960 milliunits/10^6 cells. These results are summarized in Figs. 5 and 6, respectively, showing the individual and mean values. There was a 1.3- to 3.5-fold (mean, 1.9-fold) increase in procoagulant activity in all monocyte samples following incubation with rHuTNF. Similarly, there was a 1.1- to 5.0-fold (mean, 2.1-fold) increase in procoagulant activity in all peritoneal macrophage samples following incubation with rHuTNF.

Procoagulant activity was then studied in mononuclear cells isolated from patients treated with rHuTNF. Due to the small number of cells available from these patients, mononuclear cells (monocytes and lymphocytes) were evaluated. Mononuclear cells isolated before the infusion contained 120 ± 160 milliunits/10^6 cells. Mononuclear cells isolated after the infusion contained 200 ± 120 milliunits/10^6 cells. Values for each patient and the mean are summarized in Fig. 7. We noted a decrease in procoagulant activity after the rHuTNF infusion in 2 of 3 patients treated with the lowest doses of rHuTNF (40 to 80 μg/m^2). Although there was a trend toward higher procoagulant activity values following rHuTNF infusion, the mean values were not statistically significantly different.

Hematological Parameters. Hematological studies of the patients treated with a single infusion of rHuTNF revealed a trend toward a lower WBC count and a lower platelet count 4 h after the infusion (see Fig. 8), although these values were not statistically significantly different. The absolute monocyte count was significantly lower (P < 0.001 by Student's t test) 4 h after the infusion. This improved by 24 h. Routine clotting studies

infusion produced very low baseline levels of peroxide (0.074 ± 0.006 and 0.054 ± 0.004 nmol/10^6 monocytes/h, respectively). Monocytes isolated before the infusion and stimulated with PMA (200 nM) produced significantly more peroxide (0.895 ± 0.230 nmol/10^6 monocytes/h) than unstimulated monocytes. Monocytes isolated after the infusion produced even more peroxide with PMA stimulation (2.02 ± 0.99 nmol/10^6 monocytes/h; see Fig. 4). We noted that PMA-stimulated peroxide production remained essentially the same in 2 patients, and in 1 patient the amount of peroxide produced decreased; all 3 of these patients were treated with the lowest doses of rHuTNF (40 to 80 μg/m^2). The monocytes of all 5 patients receiving >80 μg/m^2 had increased ability to produce peroxide (Fig. 4). For all 9 patients, the mean PMA-induced peroxide production was 2.3-fold higher in monocytes isolated after the rHuTNF infusion compared to monocytes isolated before the infusion. PMA-induced peroxide production by monocytes isolated from patients after the rHuTNF infusion was elevated to a degree comparable to that of normal subject monocytes treated in vitro with rHuTNF.

Procoagulant Activity. Peripheral blood monocytes were isolated from 8 women undergoing peritoneal laparoscopy. In 6 of these subjects, peritoneal macrophages were also obtained. When these cell preparations were incubated for 16 h in medium only, the monocytes contained 640 ± 400 milliunits of procoagulant activity/10^6 cells, and the peritoneal macrophages contained 1730 ± 1280 milliunits of procoagulant activity/10^6 cells (mean ± SD). After incubation for 16 h with rHuTNF (100 units/ml), the mean procoagulant activity increased in monocytes to 1160 ± 800 milliunits/10^6 cells, and in the peritoneal macrophages to 3620 ± 1960 milliunits/10^6 cells. These results are summarized in Figs. 5 and 6, respectively, showing the individual and mean values. There was a 1.3- to 3.5-fold (mean, 1.9-fold) increase in procoagulant activity in all monocyte samples following incubation with rHuTNF. Similarly, there was a 1.1- to 5.0-fold (mean, 2.1-fold) increase in procoagulant activity in all peritoneal macrophage samples following incubation with rHuTNF.

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Fig. 7. Procoagulant activity (PCA) in human peripheral blood mononuclear cells (monocytes and lymphocytes) from patients treated with rHuTNF. Mononuclear cells were isolated immediately before (pre) and 30 min following (post) a 2-h i.v. infusion of rHuTNF. Points, milliunits of procoagulant activity/million cells; mean value for each group of data. Individual patient values are indicated (Δ, 40 to 80 μg/m2; Θ, >80 μg/m2). The mean pre and post values are not significantly statistically different by Student's t test.

revealed no significant change in the mean PT or mean fibrinogen levels 24 h after the infusion. However, the mean aPTT did increase significantly following the infusion (25.5 to 30.1 s, P < 0.01 by Student's t test; see Fig. 8). Fibrin degradation products were <10 μg/ml in all measurements before and after the infusion. The D-dimer assay was positive (>10 μg/ml) in 1 of 7 patients before the infusion and 4 of 7 patients 24 h after the infusion. All 4 of the patients with positive D-dimer assays were treated with >80 μg/m2 of rHuTNF.

DISCUSSION

Our studies demonstrate that rHuTNF enhances PMA-stimulated peroxide production in human monocytes and peritoneal macrophages in vitro. This effect is seen at rHuTNF concentrations as low as 10 units/ml (60 pmol/liter). Other compounds have been shown recently to enhance PMA-stimulated peroxide production by human monocytes, including 1,25-dihydroxyvitamin D3 (22, 23) and γ-interferon (24, 25). There is evidence to suggest that the enhancement of peroxide secretion by these compounds may be a receptor-mediated event (22). Cells from human monoblastic leukemia cell line U937 and human promyelocytic leukemia cell line HL-60 contain cell surface receptors for TNF, with comparable binding affinities and numbers of binding sites (8, 26, 27). Recent studies have also demonstrated cell surface receptors for TNF on normal human hematopoietic cells (27). Although the mechanism by which rHuTNF enhances peroxide production by monocytes and macrophages is unknown, the concentration of rHuTNF inducing this effect in vitro is compatible with the receptor affinity measured in human hematopoietic cells (27). This concept is compatible with other data demonstrating that rHuTNF in doses as low as 100 pmol/liter will induce superoxide anion generation in human neutrophils (9) and enhance the PMA-stimulated respiratory burst in neutrophils (21). Gamble et al. have shown that a brief exposure (5 min) of human neutrophils or human umbilical vein endothelial cells to recombinant human TNF is sufficient to induce maximal adherence of neutrophils to endothelial cells (28). This effect developed rapidly in neutrophils and did not require protein or RNA synthesis. In endothelial cells, this effect developed over 4 h and required protein and RNA synthesis. We have found that a 30-min exposure of monocytes to rHuTNF will enhance PMA-stimulated peroxide production 42 h later. However, this effect develops more slowly compared to monocytes exposed continuously to rHuTNF. Significantly higher concentrations of rHuTNF are required in a brief exposure to achieve maximal peroxide enhancement. This suggests that maximum biological effects may be achieved by a brief, high dose of TNF or a continuous, lower dose. In other recent studies, recombinant human TNF-α alone did not trigger peroxide release from human monocytes during a 4-h incubation (29). These data, using a different assay system, are consistent with our findings.

Fig. 8. Hematological data determined for patients treated with rHuTNF. Values were determined immediately before (0), 4 h after (4), 24 h after (24), or 3 days after (72) a 2-h i.v. infusion of rHuTNF. Individual patient values are indicated by Δ; due to overlap, several patients may be indicated by one Δ. —», mean value for each group of data. n indicates the number of patients evaluated in each panel. a, WBC count, n = 9; b, absolute monocyte count, n = 9; c, platelet count, n = 9; d, prothrombin time, n = 6; e, activated partial thromboplastin time, n = 6; f, plasma fibrinogen, n = 7. The absolute monocyte count 4-h value differs significantly from the 0-time value in b (P < 0.001). The aPTT 24-h value differs significantly from the 0-time value in e (P < 0.01).
for in vitro treatment of monocytes with rHuTNF alone.

Several cytokines have been reported to modify human mononuclear phagocyte function following in vivo administration. α-Interferon has been shown to augment monocyte Fc-receptor expression (30), but not to modify superoxide secretory capacity (31). In vivo administration of γ-interferon will enhance PMA-stimulated peroxide release in human monocytes (24). Interleukin 2 will enhance peroxide releasing capacity of human monocyte-derived macrophages in vitro, although this activity can be blocked by a monoclonal antibody against γ-interferon (32). Recombinant murine TNF added to in vitro cultures will induce changes in murine peritoneal macrophage function, such as enhanced peroxide secretory capacity and Fc-receptor expression (33). Recombinant human TNF will enhance PMA-stimulated peroxide production when added to cultures of murine peritoneal macrophages (34). We now show that i.v. rHuTNF enhances PMA-stimulated peroxide production by human monocytes isolated 30 min after a single 2-h infusion. This finding in vivo is consistent with the time course for the in vitro effect of rHuTNF reported here. Recombinant human TNF may have a number of biological effects when administered to patients, including induction of other cytokines. It is possible that the changes in monocyte function we have observed may be due to the direct effects of rHuTNF or indirect effects through changes in other biological parameters.

Tissue factor is a membrane-bound phospholipid complex found in mononuclear phagocytes and other cells. rHuTNF and interleukin 1 have recently been shown to induce tissue factor in human endothelial cells in vitro (10, 11). rHuTNF and interleukin 1 also alter the synthesis of plasminogen activator and plasminogen activator inhibitor, and they decrease Protein C activation in endothelial cells (12, 13). We have previously shown that rHuTNF will induce procoagulant activity in human monocytes and the human monoblastic leukemia cell line U937 in vitro in a dose- and time-dependent manner (35). This procoagulant activity is tissue factor like; the activity will not clot Factor VII- or Factor X-deficient plasma, but clots Factor VIII-deficient plasma like normal plasma. In this study we confirmed this previous finding and extend these results to include human peritoneal macrophages in vitro. We note that freshly isolated peritoneal macrophages express approximately 3-fold more procoagulant activity/IO6 cells than freshly isolated monocytes, a difference which may reflect a different degree of differentiation of these cells. Based on our previous in vitro time course studies in monocytes (35), we expected a significant increase in expression of procoagulant activity after 2 h of exposure to rHuTNF, with maximum expression at approximately 12 h. However, in our in vivo hematological studies, the absolute monocyte count was extremely low in our subjects 4 h and 12 h after the rHuTNF infusion, and sufficient monocytes could not be isolated at those times for study. This is similar to observations in other Phase I. i.v. trials of rHuTNF (36, 37). Therefore, we studied mononuclear cells obtained 30 min after rHuTNF infusion. Although there was no significant increase in the mean procoagulant activity before and after the infusion, there was a trend toward higher activity. Decreases in procoagulant activity in 2 of 3 patients treated with the lowest doses of rHuTNF made these results more difficult to interpret and weakened the significance of the trend.

Our hematological studies indicate significantly lower absolute monocyte counts and prolonged aPTT in patients treated in vivo with rHuTNF, as well as four patients with the presence of fibrin D-dimer. One recent clinical trial with recombinant human TNF in patients with advanced cancer found a similar decrease in the absolute monocyte count following i.v. or s.c. TNF administration (38). No consistent changes in hemostatic parameters were noted, although several patients developed increased fibrin split products, and one patient developed a deep venous thrombosis (38). Using a 5-day continuous i.v. infusion of recombinant human TNF, another recent study demonstrated leukopenia and thrombocytopenia (39). In this study, hemostatic parameters remained normal for all patients (39). Previous studies in animal systems suggested that procoagulant activity expressed by mononuclear phagocytes may initiate disseminated intravascular coagulation (40-43). Schwartz et al. have shown that human monocyte procoagulant activity induced by bacterial lipopolysaccharide initiates platelet aggregation and thrombin generation (44). Although the effects of HuTNF on the hemostasis system are not completely understood, this cytokine may alter the hemostatic properties of many cells, including mononuclear phagocytes, endothelial cells, and tumor cells. This might result in low-grade activation of the clotting system with platelet consumption and fibrinolysis. Such a process could remain clinically silent and be detected only by assays such as the D-dimer assay, which has been shown to be a more sensitive test for activation of fibrinolysis than measurement of fibrin degradation products (20).

These studies have several implications. (a) Biological effects of rHuTNF on host cells can be detected within 30 min after a single 2-h i.v. infusion of this cytokine given at a dose range of 40 to 200 µg/m2. This is manifest as an enhancement of mononuclear phagocyte PMA-stimulated peroxide production. (b) In vitro studies indicate that longer infusions may increase biological response. If the degree of therapeutic benefit is directly related to the magnitude of biological response, then infusions longer than 2 h will be necessary for maximal effect. However, such alterations may have adverse clinical consequences on the hemostasis system, which remain to be defined. Several patients treated with rHuTNF have experienced thrombotic events, including deep venous thrombosis of the lower extremity.4 (c) The coagulation abnormalities seen with rHuTNF may be due not only to activated endothelial cells but perhaps to a complex interaction among endothelial cells, monocytes, tumor cells, and other cells.

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