Several investigators have reported that tumor necrosis factor (TNF) can alter the production of plasminogen activator inhibitor type-1 (PAI-1) and plasminogen activators (PAs) by endothelial cells in vitro. We have examined the in vivo effects of recombinant human TNF administration on fibrinolysis as assessed by parameters in plasma during a 24-hour period of continuous TNF infusion to 17 cancer patients with active disease. The plasma levels of PAI activity increased sevenfold after 3 and 24 hours of TNF infusion. This was the result of an increase of PAI-1 antigen; PAI-2 antigen was not detectable. Plasma concentrations of tissue-type PA (t-PA) antigen increased twofold to fivefold after 3 and 24 hours of TNF infusion, whereas urokinase-type PA antigen levels in plasma remained unaltered. After 3 hours of TNF infusion the plasma levels of α2-antiplasmin were slightly decreased, 5% on average, suggesting that fibrinolysis continued. After 24 hours of TNF infusion a highly significant increase in fibrin- plus fibrinogen-degradation products, and separately of fibrin degradation products and fibrinogen degradation products, was found. This indicates that fibrinolysis persisted, at least partly, in the presence of high levels of PAI activity. Whereas PAI-1 production increased, t-PA production by human endothelial cells in vitro remains unaltered or even decreases on TNF addition. It has been shown previously that TNF infusion in our patients results in thrombin and fibrin generation. Therefore, it is possible that thrombin, not TNF, is the actual stimulus for t-PA production in our patients. We speculate that fibrin is formed during TNF infusions and that plasmin is generated by t-PA action immediately on the initial formation of (soluble) fibrin molecules. Such a process may explain the generation of degradation products of both fibrin and fibrinogen during infusion of TNF in patients.

The fibrinolytic process in plasma is regulated by plasminogen activators, particularly tissue-type plasminogen activator (t-PA) and plasminogen activator inhibitor(s), which are synthesized by the endothelium. The inflammatory mediators tumor necrosis factor (TNF), interleukin-1, and lymphotoxin have profound effects on endothelial cells and on their hemostatic properties. We and others have previously demonstrated that TNF increases the production of plasminogen activator inhibitor type-1 (PAI-1) by human endothelial cells in vitro, whereas t-PA production did not increase or even decreased. In cultured human endothelial cells, TNF also induces the synthesis of urokinase-type plasminogen activator (u-PA), which is secreted predominantly at the basolateral side of the cells. In addition to in vitro data, it was shown that injection of TNF in rats resulted in a marked increase in the plasma level of PAI inhibitor activity. However, no further evaluation of the fibrinolytic process in rats on TNF administration has been made, partly due to the species specificity of the necessary reagents.

Recently, several clinical trials have been conducted to evaluate the possible use of human recombinant TNF in the treatment of malignant disease. The effect of TNF on coagulation was extensively evaluated during one of these phase 1 clinical studies using TNF in patients with active malignancies. During infusion of TNF an increased rate of thrombin generation and fibrin formation was observed. In the same plasma samples we have now examined various parameters of the fibrinolytic system. In addition to the plasma concentration of the plasminogen activators and their inhibitors, we assayed consumption of α2-antiplasmin and the generation of fibrin- and fibrinogen-degradation products to evaluate the fibrinolytic process during infusion of TNF in humans.

Materials and Methods

Patient selection and therapy. Patients with histologic evidence of carcinoma, lymphoma, or sarcoma enrolled in phase I trials of recombinant human TNF at the Dana-Farber Cancer Institute (Boston, MA) were studied. The drug was administered by continuous intravenous (IV) infusion via a peripheral arm vein. Indomethacin (50 mg) was administered orally every 6 hours for four doses starting 12 hours before each infusion. Normal clinical measures of hepatic (total bilirubin < 1.5 mg/dL, SGOT < 60 IU/L) and renal function (serum creatinine < 1.5 mg/dL) were required before treatment. Specimen collection and informed consent procedures were approved by the Institute's Human Protection Committee.

Drug formulation. Recombinant human TNF was supplied by Asahi Chemical Industry America, Inc (New York, NY). The specific activity of this preparation is 2.3 × 10^6 U/mg protein. TNF preparations contained less than 100 pg of endotoxin/10^6 U as measured by the Limulus test. The drug was diluted with normal saline to a total volume of 100 to 300 mL for IV administration.

Collection and processing of blood samples. Venipunctures were performed automatically with 19- or 21-gauge butterfly infusion sets on the arm contralateral to that receiving the TNF infusion. A two-syringe technique was used and blood samples were drawn...
Fibrinolysis during TNF infusions in humans

Fig 1. Sequential changes of plasminogen activator inhibitors PAI-1 and PAI-2 (A), and t-PA and u-PA antigens (B) in response to 24-hour infusion of human recombinant TNF in three patients with carcinoma. Post, postinfusion samples were obtained 18 to 24 hours after the completion of the infusion.

A normal plasma pool was made by admixing equal volumes of plasma of 24 control subjects. This control population consisted of healthy laboratory personnel between the ages 20 and 50 years, with a negative history of bleeding and thrombosis, and who were not taking any medications at the time of sample collection.

Assays. All assays have been performed in duplicate or in triplicate.

t-PA antigen concentration in the plasmas was measured by enzyme immunoassay (EIA) (IMULYSE) purchased from Biopool (Umeå, Sweden) according to the manufacturer's description. In this assay, t-PA and t-PA inhibitor complexes are claimed to be detected with similar efficiency.

The level of u-PA antigen was determined with an enzyme-linked immunosorbent assay (ELISA) for urokinase as described by Binnema et al.11 The assay measures the inactive proenzyme single-chain u-PA (sc-u-PA), the active two-chain u-PA (tc-u-PA), and u-PA:PA inhibitor complexes with equal efficiency.

The plasmin-activatable u-PA activity (sc-u-PA) and active tc-u-PA were measured by a biologic immunoassay (BIA) as previously described.12 PAI activity was measured by the quantitative assay for plasminogen activator activity as described by Verheijen et al.13 It was determined by titration, with increasing amounts of t-PA, of a fixed volume of plasma or pooled plasma. The amount of PA inhibitor activity was calculated from the intersection of the asymptote of the titration curve with the X-axis. In all experiments this asymptote ran parallel to the control titration curve.

The plasma levels of PAI-1 antigen were determined by an EIA (IMULYSE, Biopool). According to the manufacturer, this assay determines mainly the unbound PAI-1 (both latent and active), whereas the t-PA:PAI-1 complex is recovered with about 10-fold lower efficiency. The plasma concentrations of PAI-2 antigen were determined by enzyme immunoassay (TintElize PAI-2; Biopool).

In addition to the assessment of the total amount of degradation
products from fibrin and fibrinogen (TDPs), specific determinations of fibrinogen-degradation products (FgDPs) and fibrin-degradation products (FbDPs) were made. All three assays are sandwich EIAs (Organon-Teknika, Turnhout, Belgium) based on a monoclonal-catch antibody (FDP-14) against a neoantigenic determinant in the E-domain of degradation products of fibrin and fibrinogen, but not with the intact parent molecules. For the specific assay of FgDPs, a horseradish peroxidase (HRP)-conjugated monoclonal antibody (MoAb) ("Y18"), reactive with covalently bound fibrinopeptide A, was used as tagging antibody. For the specific FbDP assay an HRP-conjugate of an MoAb against the fibrin D-domain ("DD13") was used as the tagging antibody. For the TDP assay a mixture of monoclonals Y18 and DD13 (both HRP conjugated) was used as a tagging antibody. These tests are not influenced by the rheumatoid factor; normal values for TDPs, FgDPs, and FbDPs in plasma are below 0.5 μg/mL.

α,-Antiplasmin activity was assayed spectrophotometrically. The presence of plasmin-α,-antiplasmin complexes was evaluated by crossed immunoelectrophoresis in 1% agarose gels, as previously described. The first dimension was run for 3 hours at 100 V; the second overnight at 100 V at about 10°C. The α,-antiplasmin rabbit antiserum was purchased from Diagnostica Stago (Asnières, France). The detection limit was estimated by addition of plasmin to plasma; it was 2.5% of conversion of α,-antiplasmin in plasmin-antiplasmin complex.

von Willebrand factor (vWF) was determined by ELISA as described previously.

Statistical evaluation. The data are expressed as the mean ± SD; the significance was analyzed using the Student's t-test for paired data.

RESULTS

The changes in the plasma levels of the plasminogen activators t-PA and u-PA, and their inhibitors PAI-1 and PAI-2 during infusion of TNF are shown for three patients in Fig. 1. After a 3-hour infusion of TNF an increase in the circulating PAI activity was observed. During continuation of the infusion over a 24-hour period PAI activity further increased in all but one of the patients (seven of eight). As is shown in Fig. 1A, this PAI activity parallels the increase in PAI-1 antigen; no PAI-2 antigen was detectable. During the TNF infusion there was a rapid increase in the circulating t-PA antigen concentration, which was in all cases higher after 3 hours of infusion than after 24 hours (Fig 1B). No significant effect on the u-PA concentration in plasma was observed. Because rapidly induced increases in plasma t-PA concentration are commonly accompanied by increases in vWF, we also determined the vWF concentration. Plasma levels of vWF after 3 and 24 hours of TNF infusion in these three patients were 185% ± 43% and 281% ± 19%, respectively, of the preinfusion concentration (mean ± SD).

In another patient a sample was obtained at 75 minutes after the onset of the TNF infusion. At this time point t-PA antigen had increased fourfold (Fig 2), and vWF concentration was elevated 1.7-fold (not shown). Spontaneous fibrinolytic activity, but no PAI activity, was detected in the plasma (Fig 2). No increase in PAI-1 antigen was observed at this time point, indicating that the increase in t-PA preceded the rise in PAI-1. After a 24-hour infusion of TNF the level of PAI-1 exceeded that of t-PA, and an elevated PAI activity was found.

The data of 17 patients receiving TNF concentrations, which varied from 1 x 10^5 to 14 x 10^5 U TNF/m²/24 h, are summarized in Tables 1 and 2. In all these patients it has been established that during TNF infusion there was evidence of thrombin generation, as indicated by circulating F1+2, and fibrin formation, as indicated by fibrinopeptide A generation. After 3 and 24 hours, both t-PA antigen and PAI activity were significantly elevated. The plasma concentrations of overall u-PA antigen (Tables 1 and 2) and prourokinase (not shown) were not significantly altered.

The increase of both t-PA and PAI activity presented an opportunity of evaluating whether, under such conditions, fibrinolysis still occurs. To that end, we measured the circulating concentrations of the total of TDPs, FbDPs, and FgDPs by highly sensitive immunoassays. After the 24-hour TNF infusion a marked increase could be demonstrated not only in FbDPs but also in FgDPs. Hence, fibrinolysis must have continued during this infusion period. The levels of fibrinogen degradation products showed a tendency to increase also at 3 hours of infusion, but this increase was not statistically significant. The increase in fibrinogen degradation products was seen both in patients with ongoing fibrinolysis, as estimated from elevated levels of FbDPs and FgDPs at the start of the TNF infusion, and in patients with normal levels of FbDPs and FgDPs before the TNF infusion (Table 3).

As a second parameter of ongoing fibrinolysis we evaluated whether consumption of α,-antiplasmin occurred during TNF infusions and whether plasmin-α,-antiplasmin (PAP) complexes could be demonstrated. The samples of nine
patients receiving $3 \times 10^5$ to $12 \times 10^5 \text{U/m}^2/24 \text{h}$ TNF were investigated. In two patients, who had u-PA antigen levels of 8 and 16 ng/mL and t-PA antigen levels of 11 and 13 ng/mL, the basal level of $\alpha_2$-antiplasmin was 67% to 68% of the value of pooled human plasma. The range of $\alpha_2$-antiplasmin concentration was measured in seven of the nine patients and an increase by 24% to 37% of the preinfusion value. Plasma samples obtained after 3 hours of TNF infusion were examined for PAP-complexes by crossed immunoelectrophoresis. In five of seven patients with normal preinfusion values of $\alpha_2$-antiplasmin, a small amount of PAP complexes was detectable (not shown). In the two patients with decreased preinfusion $\alpha_2$-antiplasmin levels, no PAP-complexes could be demonstrated at this time point. This suggests that no intensive fibrinolytic activity occurred after 3 hours of TNF infusion and that the PAP-complexes were rapidly cleared.

### Table 1. Fibrinolytic Parameters in Plasma of Patients After Three Hours of TNF Infusion

<table>
<thead>
<tr>
<th>Parameter</th>
<th>No. of Patients</th>
<th>Plasma Concentration</th>
<th>Relative Change* (% of prevalue)</th>
<th>Statistical Significance†</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Pre-infusion</td>
<td>3-h Infusion</td>
<td></td>
</tr>
<tr>
<td>t-PA antigen (ng/mL)</td>
<td>9</td>
<td>15 ± 14</td>
<td>44 ± 34</td>
<td>340 ± 156</td>
</tr>
<tr>
<td>u-PA antigen (ng/mL)</td>
<td>9</td>
<td>6 ± 5</td>
<td>6 ± 6</td>
<td>115 ± 39</td>
</tr>
<tr>
<td>PAI activity (IU/mL)</td>
<td>8</td>
<td>5 ± 1</td>
<td>36 ± 21</td>
<td>739 ± 478</td>
</tr>
<tr>
<td>TDPs (µg/mL)</td>
<td>9</td>
<td>3 ± 4</td>
<td>5 ± 7</td>
<td>142 ± 64</td>
</tr>
<tr>
<td>FbDPs (µg/mL)</td>
<td>9</td>
<td>1.3 ± 1.9</td>
<td>2.0 ± 3.2</td>
<td>150 ± 62</td>
</tr>
<tr>
<td>FgDPs (µg/mL)</td>
<td>9</td>
<td>0.7 ± 0.5</td>
<td>0.8 ± 0.8</td>
<td>99 ± 32</td>
</tr>
</tbody>
</table>

Patients with various types of malignancies received a continuous infusion of human recombinant TNF ($3 \times 10^5$ to $10^6 \text{U/m}^2/24 \text{h}$). Values represent the mean ± SD.

Abbreviation: NS, not significant.

In the present report we demonstrate that the plasma levels of both t-PA and PAI-1 increase on infusion of TNF in patients, whereas the plasma levels of u-PA and PAI-2 are not affected. Concomitantly, the levels of FbDPs and FgDPs increase in the circulating blood.

DISCUSSION

The increase in PAI activity was totally accounted for by PAI-1. TNF induces PAI-1 messenger RNA and protein in human endothelial cells in vitro. In a previous study we indicated that the increased amount of PAI-1 induced by TNF is predominantly produced by the vascular tree, in particular the endothelium, and is not produced by blood platelets or hepatocytes. t-PA is synthesized in endothelial cells in vivo as well as in vitro, but in contrast to PAI-1, the in vitro synthesis of t-PA is not increased by TNF; it remains unaffected or even decreases. Bauer et al reported that infusion of TNF induces thrombin generation in the patients that we have studied. Thrombin is an active stimulator of both acute release of t-PA in vascular beds and of the synthesis of t-PA by human endothelial cells in vitro. We propose that thrombin generation may be the actual trigger for the increased level of t-PA during infusion of TNF. During the first hour(s) the t-PA level would be predominantly increased by the "acute" release mechanism, whereas prolonged TNF infusion increased the t-PA level by de novo synthesis. Additional experiments with inhibitors of factor
Xa and thrombin are necessary to verify this proposed mechanism.

The response of t-PA and PAI-1 to TNF infusion in our patients is similar to the response of these parameters to infusion of endotoxin in healthy volunteers. 38 This suggests that the observed effects do not merely reflect that pieces of the vascular bed in the patients have altered characteristics, caused by the underlying malignancy, but rather that our observations are representative of the normal vascular system. In the study of Suffredini et al 27 blood samples were taken every hour over a 5-hour period. It was demonstrated that the increase of the t-PA level preceded the rise in the t-PA antigen, resulting in an elevated plasma PA1 activity, was observed after continuation of the TNF infusion for 3 hours or more. To evaluate whether the fibrinolytic process is indeed active under these circumstances we have used three very sensitive new assays for the determination of FbDPs and FgDPs. Within 3 and 24 hours of TNF infusion there was a marked increase in circulating FbDPs, both in the patients that had no detectable fibrinogen degradation products and in those that showed ongoing fibrinolysis at the start of the infusion. This indicates that fibrinolysis is indeed active. It does not exclude the possibility that the increase in PAI-1 level retards the process.

Not only do the levels of FbDPs increase, but there also is a parallel increase in the levels of FgDPs amounting to about 30% of the level of FbDPs. This increase in FgDPs was not expected here because no change in u-PA is seen. Several possibilities may explain the generation of FgDPs. Firstly, at the initial fibrin generation both FbDPs and FgDPs may be generated. Probably most likely occurs on infusion of TNF can directly or indirectly (via the generation of thrombin and Fg.) fibrinogen degradation products (FgDP), fibrinogen degradation products (FbDP), and total fibrinogen degradation products (TDP) were determined by EIA as described in Materials and Methods. Patients were divided in three groups according to their pre-infusion plasma level of FbDP: I, less than 0.5 μg/ml; II, 0.5 to 2.2 μg/ml; III, ≥6.2 μg/ml.

Table 3. Degradation Products of Fibrin and Fibrinogen in Plasma of Patients Receiving TNF Infusions

<table>
<thead>
<tr>
<th>Type of Degradation Product</th>
<th>Patients</th>
<th>Pre-infusion</th>
<th>3-h or 4-h Infusion</th>
<th>24-h Infusion</th>
</tr>
</thead>
<tbody>
<tr>
<td>FbDP</td>
<td>I (n = 5)</td>
<td>&lt;0.5</td>
<td>0.5 ± 0.5</td>
<td>2.5 ± 0.9</td>
</tr>
<tr>
<td></td>
<td>II (n = 6)</td>
<td>1.4 ± 0.6</td>
<td>1.7 ± 0.6</td>
<td>3.9 ± 2.3</td>
</tr>
<tr>
<td></td>
<td>III (n = 1)</td>
<td>6.2</td>
<td>10.3</td>
<td>16.5</td>
</tr>
<tr>
<td>FgDP</td>
<td>I (n = 5)</td>
<td>&lt;0.5</td>
<td>&lt;0.5</td>
<td>0.9 ± 0.5</td>
</tr>
<tr>
<td></td>
<td>II (n = 6)</td>
<td>0.6 ± 0.5</td>
<td>0.8 ± 0.2</td>
<td>1.9 ± 1.4</td>
</tr>
<tr>
<td></td>
<td>III (n = 1)</td>
<td>1.6</td>
<td>2.7</td>
<td>3.2</td>
</tr>
<tr>
<td>TDP</td>
<td>I (n = 5)</td>
<td>1.2 ± 0.4</td>
<td>1.4 ± 0.5</td>
<td>4.9 ± 0.5</td>
</tr>
<tr>
<td></td>
<td>II (n = 6)</td>
<td>2.7 ± 0.9</td>
<td>3.4 ± 0.6</td>
<td>8.7 ± 6.5</td>
</tr>
<tr>
<td></td>
<td>III (n = 1)</td>
<td>12.7</td>
<td>22.5</td>
<td>28.0</td>
</tr>
</tbody>
</table>

Patients with various types of malignancies received 3 x 10^8 to 14 x 10^8 U TNF/m^2/24 h. Fibrin degradation products (FbDP), fibrinogen degradation products (FgDP), and total fibrinogen degradation products (TDP) were determined by EIA as described in Materials and Methods. Patients were divided in three groups according to their pre-infusion plasma level of FbDP: I, less than 0.5 μg/ml; II, 0.5 to 2.2 μg/ml; III, ≥6.2 μg/ml.
The technical assistance of R. Laterveer, J. Opdenberg, N. Los, and P. Turin is gratefully acknowledged.

REFERENCES


In Fig 3 we have summarized the suggested events that take place during TNF infusion.


24. van Hinsbergh VWM: Regulation of the synthesis and secretion of plasminogen activators by endothelial cells. Haemostasis 18:307, 1988


Progress of fibrinolysis during tumor necrosis factor infusions in humans. Concomitant increase in tissue-type plasminogen activator, plasminogen activator inhibitor type-1, and fibrin(ogen) degradation products

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