Activation of the Coagulation Mechanism on Tumor Necrosis Factor-stimulated Cultured Endothelial Cells and Their Extracellular Matrix

THE ROLE OF FLOW AND FACTOR IX/IXa

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Infusion of tumor necrosis factor (TNF) into tumor-bearing mice led to intravascular clot formation with fibrin deposition in microvessels in the tumor bed in close association with the vessel wall, which could be prevented by active site-blocked factor IXa (IXai). This observation prompted us to examine the role of the intrinsic factor X activation complex on TNF-stimulated human endothelial cell monolayers and endothelial-derived matrix during exposure to purified coagulation factors or flowing blood. Treatment of endothelial cells in intact monolayers with TNF induced expression of the procoagulant cofactor tissue factor (TF) in a dose-dependent manner, and after removal of the cells, TF was present in the matrix. TNF-treated endothelial cell monolayers exposed to blood anticoagulated with low molecular weight heparin induced activation of coagulation. Addition of IXai blocked the procoagulant response on TNF-treated endothelial cells, and consistent with this, the presence of factor IX/VIIIa enhanced endothelial TF/factor VII(a) factor X activation over a wide range of cytokine concentrations (0–600 pm). When TF-dependent factor X activation on endothelial cells was compared with preparations of subendothelium, the extracellular matrix was 10–20 times more effective. IXai blocked TF/factor VII(a) mediated activated coagulation on matrix, but only at lower concentration of TF (<50 pm). Similarly, enhancement of factor Xa formation on matrix by factors IX/VIIIa was most evident at lower TNF concentrations. When anticoagulated whole blood flowing with a shear of 300 s⁻¹ was exposed to matrices from TNF-treated endothelial cells, but not matrices from control cells, fibrinopeptide A (FPA) generation, fibrin deposition, and platelet aggregate formation were observed. FPA generation could be prevented by a blocking antibody to TF and by active site-blocked factor Xa (Xai) over a wide range of TNF concentrations (0–600 pm), whereas IXai only blocked FPA generation at lower TNF concentrations (<50 pm). Activation of coagulation on matrix from TNF-stimulated endothelial cells was dependent on the presence of platelets, indicating the important role of platelets in propagating the reactions leading to fibrin formation. These observations demonstrate the potential of cytokine-stimulated endothelium and their matrix to activate coagulation and suggest the importance of the intrinsic system in factor Xa formation on cellular surfaces.

Tumor necrosis factor/cachetin (TNF) is a central mediator of the host response in diverse conditions ranging from Gram-negative sepsis to ischemia/reperfusion syndrome (1–3) in which abnormalities of coagulation, both localized and generalized, are a common component. TNF has been shown to induce the synthesis and expression of the procoagulant cofactor tissue factor (TF) by endothelium (4, 5) suggesting a mechanism whereby this cytokine can activate cellular clot-promoting mechanisms. Because fibrin formation is often localized to particular vascular beds, we have focussed on the role of endothelial cell TF in the activation of coagulation. TF, the major initiator of coagulation in vivo, binds factor VII/VIIa and promotes activation of factors IX and X (6–8). Although in many in vitro experimental systems employing purified proteins and phospholipids the activation rate of factor X exceeds that of factor IX, suggesting that factor IX could be bypassed, in vivo factor IX appears to have a central role in the hemostatic mechanism (8). This has led us to speculate that on cellular surfaces alternative mechanisms might be operative, providing insights into the possible role of factor IX/Xa in TF-mediated activation of coagulation.

Previous studies of TNF-treated endothelial monolayers have demonstrated the presence of both TF and a binding site for factor IX/Xa, the latter promoting assembly of the intrinsic factor X activation complex on the cell surface (9–11). Therefore, we considered it likely that on endothelium, factor IX/Xa could contribute to activation of coagulation initiated by TF. In the current study, we demonstrate that active site-blocked factor IXa (IXai) does inhibit formation of intravascular thrombi in tumor-bearing animals after the infusion of TNF. In this context, TF/VII-mediated factor Xa...
formation on intact endothelial monolayers incubated with TF was enhanced by the addition of factor IX/VIII, whereas IXa blocked the activation of coagulation when anticoagulated blood was incubated with the stimulated endothelial cells. Unexpectedly, there was considerably greater TF activity in the medium of stimulated cells than that expressed on the cell surface. Activation of coagulation by matrix-associated TF was only blocked by IXa when the endothelial cells had been incubated with low concentrations of TFN. Matrix from stimulated endothelial cells effectively promoted fibrinopeptide A generation, fibrin deposition, and platelet aggregate formation in a flow system using low molecular weight heparin anticoagulated blood. These results suggest a contribution of the intrinsic system when coagulation is initiated on an intact vessel and point to the importance of TF in the subendothelial matrix as an initiator of coagulation.

EXPERIMENTAL PROCEDURES

Materials—Low molecular weight heparin (LMWH, Fragmin®) was from Kabivitrum, Stockholm, Sweden. All culture plastics were obtained from Nunclon, Nunc (Oslo, Norway). The bovine TPA© coverslips from Flow Laboratories Inc., Woodcock Hill, United Kingdom. The other tissue culture supplies (media, antibiotics, and trypsin) were from Gibco Biocult, Paisley, Scotland. All other chemicals obtained from commercial sources were of the highest purity grade available.

Preparation of Coagulation Proteins—Human factors IX and X, purified to homogeneity as described previously (12), were generously provided by Dr. W. Kiel (University of New Mexico, Albuquerque, NM). For preparation of active site-blocked coagulation factors, bovine factors IX and X were used (these were purified as described previously (13, 14)). Factor IX was activated with factor Xa (IXa) as described (10), and the product was chromatographed on a Mono Q column using fast protein liquid chromatography equipment (Pharmacia, Uppsala, Sweden) to remove the factor Xa. Based on sodium dodecyl sulfate-polyacrylamide gelelectrophoresis, the factor IXa preparations contained <5% residual factor IX. Factor X was activated by incubation with the purified coagulant protein from Russel's viper venom (1:100, w/w) (15) generously provided by Dr. Richard Hart (American Diagnostica, Greenwich, CT). The mixture was subjected to chromatography on QAE-Sephadex to obtain purified factor Xa (16). These preparations of factor Xa contained <1% residual factor X. Activated coagulation factors were inactivated during incubation with Glu-Gly-Arg-chloromethylketone (30-fold molar excess, Calbiochem) (17), and excess inhibitor was removed by dialysis. The active site-blocked factor IXa (IXai) and Xa (Xai) had no detectable procoagulant activity.

Monoclonal purified-factor VIII (Hemofil M), purchased from Baxter (Glendale, CA), was activated as described previously with α-thrombin (10). Purified human recombinant factor VIIIa was generously provided by Dr. U. Hedner (Novo Industri, Novo Alle, Denmark).

Radioimmunoassays for factor IX were performed by a modification of the method of Suzuki and Thompson (15) as described (19). These assays employed a monoclonal rabbit anti-bovine factor IX which did not cross-react with mouse factor IX antigen present in the animal's plasma but recognized bovine factor IX/IXa (detection limit was approximately 1 nM corresponding to 80% binding on the standard curve). The immunoactivity of bovine factor IX, IXa, and IXai appeared identical in this assay.

Infusion Studies with Tumor-bearing Mice—BALB/c mice were injected intradermally with methyl A sarcoma cells (106 cells/animal; generously provided by Drs. Hoffmann and Old, Memorial Sloan-Kettering Cancer Center, New York) (20). After tumors reached a size of approximately 1 cm, animals were injected intravenously with a tail vein with saline, TFN (3 µg/animal) (purified recombinant human TNF, 10 units/mg, was generously provided by Dr. P. Lo- medico, Hoffmann-La Roche, Nutley, New Jersey) or saline (3 µg/animal) with IXai. In case of the IXai infusions, the coagulation factor was diluted in saline and infused via a tail vein such that 5 µg/animal was given before the TNF and 5 µg/animal was given at the time of the TNF infusion. The level of IXai measured prior to killing the animals, assessed with the RIA ascribed above, was 20 pmol/ml of bovinecoagulated plasma (levels of factor IX in normal animals are in the range of 60–100 pmol/ml). Two hours after the injection, mice were anesthetized and subjected to whole body beating heart perfusion fixation, as described previously (21). Microvessels from tumor tissue were examined for evidence of thrombi.

Cell Cultures—Human umbilical vein endothelial cells were isolated from umbilical veins and cultured according to Jaffe et al. (22) and modified subsequently (23). The cells were typical characteristics such as the presence of von Willebrand factor. Routinely, endothelial cells of the second passage were subcultured on gelatin-coated Thermofax® coverslips. Before seeding the cells, the gelatin on the coverslips was fixed with 0.5% glutaraldehyde. The fixation step with glutaraldehyde did not influence the procoagulant activity on endothelial cells and the matrix upon stimulation. Moreover, virtually identical procoagulant activities on endothelial cells and their matrix upon stimulation were also observed when the endothelial cells were grown on fibronectin (generously provided by Dr. J. van Mourik, Central Laboratory of Bloodtransfusion of the Dutch Red Cross, Amsterdam, The Netherlands) instead of fixed gelatin. Cell monolayers, grown to confluence in 5–7 days, were used (approximately 60,000 cells/cm2). At confluence, the cell culture medium was refreshed 16 h before adding TNF.

Endothelial cells were stimulated for 4 h with TNF. TNF was dissolved in distilled water containing 1% bovine serum albumin (BSA) in a concentration of 600 nM and diluted in the cell culture medium to the indicated concentrations. Addition of TNF to the 0.1% BSA alone had no effect on endothelial procoagulant activity. To isolate the extracellular matrix, endothelial cells were exposed to the gelatin on the coverslips for 10 min at room temperature with gentle shaking. The cell layer was completely removed by this procedure leaving the extracellular matrix intact (24–27). Previous studies (28) have demonstrated that a similar procoagulant activity was found on the matrix when the extracellular matrix was isolated with 2 M urea or nitrocellulose-acetate paper stripping. The isolated extracellular matrix was washed three times with phosphate-buffered saline (10 mM phosphate, pH 7.4, and 150 mM NaCl) and used on the same day.

Factor IX Activation with Purified Coagulation Factors—Factor IX activation on TNF-stimulated endothelial cells monolayers (1.2 x 105 cells) and their isolated extracellular matrix was examined as follows. TNF-stimulated endothelial cell monolayers or matrices derived from TNF-stimulated endothelial cultures were washed three times with 2 ml of 10 mM HEPES, pH 7.4, 157 mM NaCl, 4 mM KCl, 5 mM CaCl2, 10 mM glucose, and 0.5 mg/ml BSA, and subsequently factor X (200 nM) in 1 ml of this HEPES buffer was incubated on the endothelial cell monolayers or matrices. Where indicated, factor IX (1 nM) and factor VIIa (10 unit/ml) were present. Factor VIIa was preactivated for 5 min with 0.01 unit/ml thrombin. This concentration of thrombin was sufficient to activate factor VII completely but did not influence the absorbance at 405 nm (10). TNF-stimulated endothelial cell monolayers or matrices. Where indicated, factor IX (1 nM) and factor VIIa (10 unit/ml) were present. Factor VIIa was preactivated for 5 min with 0.01 unit/ml thrombin. This concentration of thrombin was sufficient to activate factor VII completely but did not influence the absorbance at 405 nm (10). Addition of TNF to the indicated concentrations. Factor VIIa activity remained constant during the experiment which was started by addition of 1 nM factor VIIa. Three aliquots (50 µl) were collected over a 15-min time interval and added to 50 µl of 50 mM Tris-HCl, pH 7.9, 175 mM NaCl, 5 mM EDTA, and 0.5 mg/ml BSA. Factor X activity was measured with the chromogenic substrate MeO-Co-n-CH2-Gly-Arg-p-nitroanilide (Spectrozyme® FXa, American Diagnostica Inc., Greenwich, CT). 10 µl of 2 mM Spectrozyme was added to 100-µl samples in microtiter plates, and the absorbance at 405 nm was monitored with a Vmax reader (Molecular Devices, Menlo Park, CA). Factor X activation was correlated to a standard curve of purified factor X.

Blood Collection and Perfusion Studies—LMWH was diluted to medium to a concentration of 200 units/ml. Blood was collected by clean venipuncture in 1:10 (v/v) of this heparin saline. Perfusion studies with steady flow (29) were performed with a rectangular perfusion chamber which was described and characterized extensively elsewhere (30).

Blood was kept at room temperature before use in the perfusions. The standard perfusate was prepared with whole blood or with reconstituted blood. For this latter purpose, washed red blood cells were resuspended in plasma. Washed and packed red blood cells were added to a hematocrit of 0.4 (31). The final platelet count in reconstituted blood was 150,000 platelets/µl. The perfusion chamber was rinsed before the start of the perfusion with 25 ml of prewarmed (37 °C) 10 mM HEPES-buffered saline. Where indicated, factors IXa and X were added to the perfusates in concentrations of 150 nM. For the study of the...
fibrin deposition, peroxidase-labeled fibrinogen was added to the perfusates (15 ml) which were prewarmed for 5 min at 37 °C before the start of the perfusion and recirculated then for 5 min. Different wall shear rates were obtained by varying flow rate and chamber width. Since 15 ml of whole blood is exposed to 4.4-cm² cultured cells in this experimental setting and approximately 90,000 cells are present per cm², the cell to volume ratio is ±17,500 cells/ml of whole blood.

At the end of the perfusion, the chamber was thoroughly rinsed with 30 ml of HEPES-buffered saline. Fibrinopeptide A (FPA) samples were collected from the reservoir at the end of the perfusion. The coverslips were then removed from the chamber and rinsed with 2 ml of HEPES-buffered saline. The perfused part of the coverslip was examined for fibrin deposition as described previously. A previous characterization of this perfusion system has demonstrated that after a lag period of approximately 1 min, the FPA generation in the perfusate increases in a linear fashion up to 5-10 min and stabilizes at longer perfusion times of 10-20 min (28). Hence, the time point of 5 min best reflects steady state formation of FPA in whole blood and was selected for these experiments. It has also been shown (28) that preincubation of endothelial cells or matrix with IgG against human TF, or addition of IgG against human factor VIIa to the perfusate, inhibited the FPA generation in this experimental setting for over 80% (28). The cell to volume ratio in this experimental setting is ±240,000 cells/ml of whole blood.

Afterwards, the coverslips were fixed with 0.5% glutaraldehyde (33). The coverslips were subsequently exposed to osmium tetroxide (2%) as postfixation, dehydrated, and embedded in Epon as described (28, 33). For "en face" microphotographs of aggregate formation, the coverslips were fixed after perfusion and stained with May-Grunwald/Giemsa (30). Microphotographs were made with a Zeiss photomicroscope III at a 1575 magnification.

The Epon with the embedded matrix and adhering platelets were separated from the coverslip by thermoshock. 1-μm sections of the Epon-embedded matrices were prepared and stained with methylene blue and basic fuchsin (33) and evaluated for aggregate formation by light microscopy at 1000 magnification. The light microscope was interfaced with an image analyzer (AMS 40-1, Analytical Measuring Systems, Saffron Walden, United Kingdom). For each coverslip, at least 1400 points at a distance of 1 μm were selected at random and evaluated for aggregate formation.

Activation of Coagulation on Endothelial Cells—Activation of the coagulation mechanism on endothelial cell monolayers was studied under static conditions. For this purpose, confluent endothelial monolayers (1.2 X 10⁶ cells) were stimulated for 4 h with TNF (0-600 pm). 0.5 ml of 20 units/ml LMWH anticogulated whole blood was incubated on the cell monolayer at 37 °C for 5 min. Where indicated, IXa and Xai were present in a concentration of 150 nM. Since 0.5 ml of whole blood is exposed to 1.2 X 10⁶ cells in this experimental setting, the cell to blood volume ratio in this experimental setting is ±240,000 cells/ml of blood. This is approximately 13-fold higher than the surface to volume ratio of the perfusion system. Samples (450 μl) were collected, added to 50 μl of anticoagulant mixture provided in the FPA kit, and assayed for FPA generation.

Fibrinopeptide A Assays—A radioimmunoassay kit (Byk-Sangtec, Dietzenbach, Federal Republic of Germany) was used for FPA measurements. Samples of 900 μl were collected before and after the experiments and added to 100 μl of anticoagulant mixture provided in the kit. Instructions of the manufacturer were followed. FPA values were expressed in nanograms/ml plasma. FPA generation was calculated from the increase in FPA level compared with the initial value just before experiments. All samples were assayed in duplicate. Base-line values were less than 2 ng/ml in all experiments. The results presented are the mean of at least three experiments.

RESULTS

TNF Infusion Studies—In vivo, infusion of TNF leads to disseminated intravascular coagulation at higher dose and localized intravascular thrombosis at lower doses (1). One striking instance of localized clot formation is the intravascular thrombosis observed in the tumor bed after the infusion of TNF (21). Previous studies (4, 5) have indicated that TNF induces the expression of procoagulant activity in cultured endothelial cells, suggesting a basis for the initiation of coagulation. In view of the paucity of TF in the intravascular space, a situation previously shown to favor activation of factor IX by the TF pathway (34, 35), and the presence of binding sites for factor IX/Xa on endothelial cells (9-11), we examined the role of factor IX/Xa in TNF-induced thrombosis.

After the infusion of TNF, intravascular thrombosis was observed localized to the tumor bed (Fig. 1A), as described previously (21). In contrast, when mice were infused with TNF and Xai, no fibrin deposition or thrombosis was evident by morphologic criteria (Fig. 1B). Mice infused with TNF and factor IX, the zymogen, developed thrombosis as in animals infused with TNF alone (data not shown). These data suggested that Xai prevented access of native factor IX/Xa to a limited number of cell surface binding sites in the intravascular space which contributed importantly to the development of thrombi. Based on previous reports demonstrating the presence of TF in the blood vessel wall of a renal cell carcinoma (36), as well as in the subendothelium of normal rabbit aorta and umbilical cords (37), we considered it likely that the infused TNF (either alone or in concert with tumor-derived mediators) was inducing TF in endothelium and that this TF would be found predominantly in the matrix. In support of this hypothesis, we have recently identified two tumor-derived polypeptides produced by murine meth A cells which induced TF in endothelium and interacted in a synergistic fashion with TNF (38, 39). To further examine mechanisms through which TNF-stimulated endothelial cells could activate coagulation, and the role of factor IX/Xa, studies were carried out with cultured endothelial cells and their extracellular matrices.

Factor X Activation on Endothelial Cells and Their Matrices in a Static System—Incubation of endothelial cells with TNF led to factor VIIa-dependent factor Xa formation due to the expression of TF (Fig. 2). Induction of TF activity occurred in a dose-dependent manner on both intact endothelial cell monolayers and subendothelium, with the rate of factor X activation 10-20 times greater on the matrix preparations than on intact endothelial cell monolayers. Activation of factor Xa occurred in a linear fashion over the experimental period examined. In control experiments where factor VIIa was absent, no factor Xa formation was observed (data not shown).

Addition of factor IX/VIIIa to TNF-treated endothelial cells incubated with factors VIIa and X enhanced factor Xa formation. The effect of factors IX/VIIIa on factor Xa for-
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FIG. 2. Effect of TNF on factor X activation on endothelial cells and matrix. Confluent endothelial monolayers were stimulated with various TNF concentrations (0–600 pM) for 4 h. Factor X (300 nM) was activated on cells (■) and matrix (○) by addition of factor VIIa (1 nM) as described under "Experimental Procedures." Samples were collected after 7- and 12-min incubation and assayed for factor X activity with the chromogenic substrate Spectrozyme. Values are calculated per cm² and are the average of three independent experiments.

Morphological studies were also performed, allowing direct visualization of fibrin fibrils and platelet aggregates (Fig. 6). Micrographs from matrix derived from TNF-stimulated endothelial cells showed platelet aggregates with connecting fibrin fibrils covering much of the coverslip in the presence of TNF alone (Fig. 6A). In the presence of IXai, there was a reduction

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Generation of FPA, fibrin deposition, and platelet aggregate formation on the matrix depended on the concentration of TNF to which the endothelium originally had been exposed (Fig. 5, A–C). The latter indices of activation of coagulation steadily increased in a dose-dependent manner, being half-maximal at 10–20 pM and maximal by 50–100 pM.

To investigate the pathway contributing to activation of coagulation, we examined the role of factor IXa and factor Xa. In a previous study (28) we have demonstrated that initiation of coagulation in this setting was initiated by the procoagulant cofactor TF initially synthesized by the endothelial cells in response to TNF. The contribution of factors IX/IXa and X/Xa were assessed using active site-blocked forms that have been shown to recognize cellular and phospholipid surfaces similar to the native enzymes (10, 16). Although they are devoid of enzymatic activity, propagation of the procoagulant response involved formation and function of factor IXa at the lower concentrations of TNF (5–50 pM), as indicated by inhibition of platelet aggregate, FPA, and fibrin formation in the presence of factor IXa (150 nM, Fig. 5, A–C). The inhibitory effect of factor IXa was dependent on the amount of active site-blocked enzyme added and was half-maximal at 75 nM IXa. In contrast, Xa inhibited coagulation more effectively at all concentrations of TNF (Fig. 5, A–C) pointing to the importance of effective factor Xa assembly into the prothrombinase complex for activation of coagulation. The inhibitory effect of Xa was half-maximal at 15 nM using this experimental system. Separate experiments, in which the effect of IXa and Xa was tested after 3 min of perfusion, instead of 5 min, gave similar results (data not shown).

Morphological studies were also performed, allowing direct visualization of fibrin fibrils and platelet aggregates (Fig. 6). Micrographs from matrix derived from TNF-stimulated endothelial cells showed platelet aggregates with connecting fibrin fibrils covering much of the coverslip in the presence of TNF alone (Fig. 6A). In the presence of IXa, there was a reduction
in the size and number of platelet aggregates and evidence of fibrin deposition was almost completely absent, except for the presence of small fibrin fibrils near platelet aggregates (Fig. 6B). Addition of Xai completely blocked the formation of platelet aggregates and fibrin deposition on matrix completely (Fig. 6C). In the latter case only platelets adhered to the matrix were visualized.

Role of Platelets in Activation of Coagulation on Matrix Derived from TNF-treated Endothelial Cells—Although activation of coagulation is initiated by TF associated with the matrix, propagation of the procoagulant response could involve the multiple cell types present in blood. This led us to compare FPA generation in plasma with that observed in whole blood after perfusion over matrices derived from TNF-treated endothelium. On the matrix, FPA generation was reduced in plasma at all TNF concentrations, with the most marked inhibition at lower TNF concentrations (Fig. 7A). To more specifically establish the contribution of blood cells to FPA generation, perfusion studies with reconstituted blood were performed over matrices derived from endothelial cells stimulated with 50 pM TNF (Fig. 7B). Reconstitution of plasma with erythrocytes to a hematocrit of 0.4 did not enhance FPA generation, but when platelets were added the procoagulant response was restored to that observed in blood (Fig. 7B).

Role of Wall Shear Rate on Activation of Coagulation on Matrix Derived from TNF-stimulated Endothelial Cells—In view of the range of shear rates present in vivo, where the procoagulant response must occur, it was important to compare activation of coagulation observed at 300 s⁻¹ with that observed at a higher wall shear rate (1300 s⁻¹) and in the absence of shear (0 s⁻¹). In both the static and the high shear system, exposure of blood to matrix derived from stimulated endothelial cells led to FPA generation (Fig. 8, A–B). Activation of coagulation was dependent on the TNF concentration to which the cells originally had been exposed and was blocked at low concentrations of TNF by IXai and at all TNF concentrations by Xai.

When studies of platelet aggregate formation were performed at 1300 s⁻¹, the results were also comparable with those observed at 300 s⁻¹. Activation of coagulation was dependent on the TNF concentration up to 50 pM and was blocked by IXai at low TNF concentrations and by Xai at all TNF concentrations (data not shown). Platelet aggregate formation in the static system was not measurable since in the absence of flow, formation of platelet aggregates is negligible.

Activation of Coagulation on TNF-stimulated Endothelial Cell Monolayers—Although endothelial cells expressed only low amounts of TF (Fig. 2), making it difficult to study in the flow system, they were capable of initiating activation of coagulation. When endothelial cell monolayers (1.2 × 10⁵ cells) were treated with TNF (0–600 pM) and then exposed to anticoagulated blood, generation of FPA occurred in a dose-dependent manner. FPA generation could be prevented by factor Xai (150 nM) over a wide range of TNF concentrations. In contrast to previous studies with matrix, IXai (150 nM) also blocked FPA generation at all concentrations of TNF examined (0–600 pM) (Fig. 9).

DISCUSSION

In the response of tumor vasculature to TNF, intravascular fibrin deposition, progressing to occlusive thrombosis, is initiated when the integrity of the endothelial cell monolayer is still intact (21). We therefore considered that TNF-mediated induction of endothelial cell TF (4, 5) could initiate coagulation in undamaged vessels of the tumor bed. In view of the presence on the vessel surface of specific binding sites for factor IX/Xa, which can promote assembly of the intrinsic factor X activation complex (9–11), the possibility was considered that factors IX/VIII contribute to the factor Xa formation that leads to fibrin deposition in this setting. In support

FIG. 5. Effect of IXai and Xai on TNF-induced FPA generation, fibrin, and platelet aggregate formation. Confluent endothelial cells were exposed to various TNF concentrations for 4 h, and the matrix was perfused with 20 units/ml LMWH anticoagulated whole blood for 5 min at 300 s⁻¹. Control perfusates (+) were compared with perfusates containing IXai (□) and Xai (▲). A, platelet aggregate formation was measured in cross-sections from Epon-embedded matrices. Platelet aggregates between 2 and 5 µm are shown and are expressed in percent surface coverage. Values are mean ± S.E. (n = 4); B, FPA generation in the perfusate was measured with an RIA and expressed in nanograms/ml; C, fibrin deposition on the matrix was measured with Fg-PO as described under “Experimental Procedures” and expressed in micrograms/cm². Values are mean ± S.E. (n = 4).
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FIG. 6. Effect of IXai and Xai on en face morphology after perfusions. Endothelial cells were treated for 4 h with 12.5 pm TNF, and the matrix was perfused with 20 units/ml LMWH anticoagulated whole blood for 5 min at 300 s⁻¹. Control perfusates were compared with perfusates containing IXai or Xai. The perfused coverslips were fixed and stained with May-Grunwald/Giemsa. Preparates were photographed with a Zeiss photomicroscope III (× 575). Representative fields are shown under each condition.

FIG. 7. Role of blood cells in FPA generation. A, endothelial cells were exposed to increasing TNF concentrations (0–600 pm), and perfusions over the isolated matrix were performed with 20 units/ml LMWH whole blood (+) or with 20 units/ml LMWH platelet-poor plasma (○) for 5 min. Platelet-poor plasma (PPP) was obtained by centrifugation (3000 rpm, 30 min, 4 °C) and contained <10,000 platelets/μl and no detectable erythrocytes. Blood samples were collected and assayed for FPA generation with an RIA. Values are expressed in nanograms/ml and are the mean of three independent experiments. B, endothelial cells were exposed to 50 pm TNF for 4 h, and perfusions were performed for 5 min over the isolated matrix. Reconstitution of blood was performed as described under “Experimental Procedures.” The final hematocrit was 0.4 and the final platelet count 120,000. FPA generation after perfusions with whole blood (wb), platelet-poor plasma (PL), platelet-poor plasma containing washed erythrocytes (PL+P), and platelet-poor plasma containing washed erythrocytes plus platelets (PL+E+P) were compared. Values are expressed in nanograms/ml and are mean ± S.E. (n = 4).

of this hypothesis, infusion of IXai prevented thrombus formation in the tumor vasculature of mice infused with TNF. Furthermore, the effect of IXai was observed at a concentration less than that of the zymogen, factor IX, present in mouse plasma (20 nM versus 60–100 nM). This suggested that IXai was blocking participation of the relatively small amounts of endogenously formed factor IX in the intrinsic factor X activation complex, presumably, by competing for a limited number of high affinity sites on cell surfaces such as the endothelial cell (9–11).

These results led us to focus initially on the role of the intrinsic system in TF-mediated activation of coagulation on the surface of stimulated endothelial cells. Since changes in permeability of the endothelial cell monolayer accompany thrombin generation (94), it was important to compare the TF activity present on the surface of an intact monolayer with that of a matrix preparation from the same TNF-treated endothelial cells. The extracellular matrix of cultured endothelial cells closely resembles the vascular basement membrane in composition and structural array of organization (24–27) and is used as a model system to study the interaction of blood cells and coagulation factors with the subendothelium (40, 42–44). In our experiments, only small amounts of TF activity were observed on intact endothelial cell monolayers, compared with that found in the matrix following removal of the endothelial cells. These observations led us to compare characteristics of the activation of coagulation on TNF-treated endothelium with that observed on the matrix.

The greater activation of coagulation observed on matrix allowed for studies using a perfusion system with whole blood anticoagulated with LMWH. This system, which has been described in detail elsewhere (28), employs the LMWH to slow down fibrin formation by promoting inactivation of

FIG. 8. Effect of IXai and Xai at different wall shear rates. Confluent endothelial monolayers were exposed to increasing TNF Concentrations (0–600 pm TNF) for 4 h, and the isolated matrix was either exposed to 0.5 ml 20 units/ml LMWH blood for 5 min (wall shear rate = 0 s⁻¹) (A) or perfused with LMWH blood for 5 min at a wall shear rate of 1300s⁻¹ (B). Control perfusates (+) were compared with perfusates containing IXai (○) and Xai (▲). Blood samples were collected and assayed for FPA generation with an RIA. Values are expressed in nanograms/ml and are mean ± S.E. (n = 3).

FIG. 9. Effect of IXai and Xai on endothelial cell surface-mediated FPA generation. Endothelial cells (1.2 × 10⁵ cells) were treated with increasing concentrations TNF (0–600 pm TNF). 0.5 ml of 20 units/ml LMWH blood was incubated on the endothelial surface for 5 min. Control samples (+) were compared with samples containing IXai (○) or Xai (▲). Samples were collected and the FPA generation was determined with an RIA and expressed in nanograms/ml. The mean of duplicate experiments is shown.
endothelial cell monolayers might also result from the low affinity of TF to the endothelial cell surface thereby favoring factor Xa activation (34). These observations indicate that cellular binding sites for coagulation factors, especially those on the endothelium and the platelet, can significantly influence activation of coagulation on stimulated endothelial cells and their matrices. Furthermore, the matrix derived from stimulated endothelial cells appears to promote coagulation much more effectively than intact monolayers. This finding emphasizes a link between the pathogenesis of thrombosis and perturbations in vessel wall barrier function which lead to increased endothelial cell monolayer permeability, thereby allowing access of plasma coagulation proteins to the TF containing matrix.

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