

Complement-mediated Regulation of Tissue Factor Activity in Endothelium

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Summary

Inflammation and immunity may be associated with endothelial cell (EC) injury and thrombus formation. We explored the mechanisms through which a humoral immune response directed against the endothelium might promote coagulation. Using the interaction of anti-EC antibodies and complement (C) with cultured EC as a model, we studied the expression and function of tissue factor, a cofactor for factor VIIa-mediated conversion of factor X to Xa. Exposure of EC to anti-EC antibodies and C in sublytic amounts stimulated the synthesis of tissue factor over a period of 16–42 h. Cell surface expression of tissue factor activity required activation of C and assembly of the membrane attack complex, because expression was inhibited by soluble CR1 and was not detected in the absence of C8. Elaboration of tissue factor messenger RNA was observed over a period of 8–30 h and required protein synthesis. Expression of tissue factor was not a direct consequence of the action of C on the EC but was a secondary response that required as an intermediate step the release of interleukin 1 α , an early product of the EC response to C activation. These findings suggest that, after the assembly of membrane attack complex on EC, the production of tissue factor and initiation of coagulation in a blood vessel depend on the production of interleukin 1 α and on its availability to stimulate affected EC.

Vascular endothelium plays a central role in regulating hemostasis and thrombosis. Hemostatic balance is achieved through the interplay of endothelial cell (EC)¹-associated anticoagulant functions, such as heparan sulfate-antithrombin III (1), thrombomodulin (2), and tissue factor pathway inhibitor (3), and procoagulant functions, such as tissue factor (4) and plasminogen activator inhibitor (5). In response to inflammatory mediators such as histamine, IL-1, and TNF (6–9), and noxious agents such as endotoxin (10–12), endothelium adopts a procoagulant posture in which the assembly of prothrombinase is enhanced, inhibition of thrombin is attenuated, and endothelium becomes adherent toward platelets (13). These changes are also associated with the secretion of cytokines and the increased expression of cell adhesion molecules and may be called collectively EC “activation.”

Presumably, the regulation of coagulation and hemostasis is perturbed in immune reactions. In the Arthus reaction, for example, deposition of immune complexes in small blood vessels is followed by formation of fibrin thrombi (14). While the procoagulant changes in the Arthus reaction may be caused in part by the action of neutrophils on endothe-

lium (15), it is not unlikely that the direct action of C on endothelium also plays a role. Consistent with this concept, the pathology of vasculitides such as systemic lupus erythematosus, serum sickness, and rheumatoid arthritis (16–19), in which neutrophils are less critically involved, is also characterized by fibrin deposits in affected vessels. Perhaps the most extreme example of an immune-mediated reaction associated with widespread coagulation is acute vascular rejection of xenografts, in which the reaction of antidonor antibodies and C with endothelium over a period of hours or days results in widespread intravascular coagulation and thrombosis (20).

One mechanism by which immune-mediated inflammation might cause prothrombotic changes is alteration of the barrier properties of endothelium. Exposure of endothelium to IL-1, TNF, and C5b67 causes the formation of intercellular “gaps” (21–23) that expose tissue factor in the underlying matrix to plasma clotting factors. Tissue factor then interacts with coagulation factor VIIa to promote assembly of the prothrombinase complex (Va and Xa), which in turn catalyzes conversion of prothrombin to thrombin. In many cases, these gaps are found to be transient; thus, while they may explain acute changes in hemostatic function (21–23), they may not account for the more persistent procoagulant condition of blood vessels in immune complex-

¹Abbreviations used in this paper: EC, endothelial cell; mRNA, messenger RNA; RT, reverse transcriptase.

mediated diseases. However, such ongoing changes might result from the synthesis and elaboration of tissue factor by EC. EC exposed to IL-1 and TNF up-regulate expression of tissue factor and manifest increased procoagulant activity through this mechanism (6, 8). The goal of studies reported here was to determine whether endothelium would respond to C by synthesizing tissue factor, as it does after exposure to other inflammatory mediators. To this end, we assessed the response of porcine EC to anti-EC antibodies and C, modeling the conditions thought to lead to acute vascular rejection of an organ graft. We report here that C does induce the expression of tissue factor as determined by up-regulation of messenger RNA (mRNA) and elaboration of membrane-associated tissue factor activity. However, unlike the response to IL-1 and TNF, EC's up-regulation of tissue factor is not a primary response to C, but, rather, it requires the synthesis and release of IL-1 α as an intermediate step.

Materials and Methods

Materials. DMEM, L-glutamine, penicillin, streptomycin, phenol, guanidinium thiocyanate, and Moloney murine leukemia virus reverse transcriptase (RT) were from Life Technologies (Gaithersburg, MD). Sodium barbital, diethyl pirocarbonate, gelatin (type B, from bovine skin), BSA (fraction V), glutaraldehyde, *Limulus* amoebocyte lysate, endotoxin (LPS, *Escherichia coli* serotype 0111: B4; stimulation index 4.5/1 μ g), and alkaline phosphatase-conjugated antibodies came from Sigma Chemical Co. (St. Louis, MO). FCS was from HyClone Laboratories, Inc. (Logan, UT). Human serum immunodepleted of C8, purified human C8, purified human rC5a, and mouse mAbs specific for a neoantigen on iC3b were from Quidel (San Diego, CA). Pyrogen-free inulin was from ICN Biomedicals, Ltd. (Costa Mesa, CA). Spectozyme FXa (MeO-CO-D-CHG-Gly-Arg-pNA.AcOH) was purchased from American Diagnostica Inc. (Greenwich, CT). Purified human factor VIIa, factor X, and factor Xa were obtained from Enzyme Research Laboratories, Inc. (South Bend, IN). Deoxynucleotide triphosphates, oligo d(T)₁₆, and AmpliTaq DNA polymerase were from Perkin-Elmer Cetus Corp. (Norwalk, CT). Random hexanucleotide primer was from Boehringer Mannheim Corp. (Indianapolis, IN), and rNasin was from Promega Corp. (Madison, WI). Human rIL-1 α was from Genzyme (Cambridge, MA). Oligonucleotide primers were synthesized by Operon Technologies, Inc. (Alameda, CA). [α -³²P]dCTP was from DuPont-New England Nuclear (Wilmington, DE). Soluble CR1 was a gift of T-Cell Sciences (Cambridge, MA). IL-1 receptor antagonist and anti-human IL-1 α neutralizing antibodies were from R&D Systems, Inc. (Minneapolis, MN). Recomb protein A/G was obtained from Pierce (Rockford, IL).

EC Culture. Porcine aortic EC were isolated, characterized, and cultured as described previously (23, 24). EC monolayers (passages 3–6) were grown to confluence on gelatin-coated 6-well, 24-well, and 96-well plates and were maintained in DME with 10% FCS for 48–72 h before each experiment.

Anti-EC Antibodies and C. Samples of human, porcine, and rabbit blood were used as sources of antibodies and C. Blood was collected in sterile, pyrogen-free containers and allowed to clot at 4°C, and the serum fraction was separated and stored in plastic vessels. The *Limulus* amoebocyte lysate test (25) was used to detect endotoxin contamination of the sera and purified serum compo-

nents. Human serum used as a source of anti-EC antibodies was heated to 56°C for 30 min to inactivate C. The titer of antibodies in serum was determined by ELISA as described previously using the cultured porcine EC as a target (26). Anti-porcine EC antibodies were isolated from human serum by adsorption to cultured EC followed by thermal extraction as described previously (27). A rabbit antiserum was raised against porcine EC, and the IgG antibodies in that serum were affinity isolated using a Recomb protein A/G column as described by the manufacturer. For some experiments, a human serum that contained very low or undetectable levels of natural anti-porcine EC antibodies (28), or that was immunodepleted of IgM and C1q restored (29), was used as a source of human C. C activation was determined by measuring deposition of iC3b neoantigen on EC by ELISA, using mouse monoclonal anti-human iC3b antibodies as described (23).

Procoagulant and Tissue Factor Activity of EC. To determine procoagulant activity, monolayers of confluent EC ($\sim 10^5$) were either scraped to obtain a suspension or kept intact and examined for procoagulant activity (11). Single-stage clotting assays were performed by adding to the cells 0.1 ml clotting buffer consisting of 7.7 mM sodium barbital, 12.8 mM sodium acetate, and 140 mM NaCl, pH 7.4. 0.1 ml citrated human or porcine plasma and 20 mM calcium chloride were added, and the time required for the first visual evidence of fibrin clot at 37°C was determined. Tissue factor activity expressed by porcine EC was assessed based on the generation of factor Xa from human factor X in the presence of 10 nM human factor VIIa, as described (30). Tissue factor activity was determined in the extracellular matrix by treating EC monolayers for 10 min with 0.1 M NH₄OH to remove EC and expose the extracellular matrix (31). The generation of factor Xa, as a measure of tissue factor expression, was compared with a standard curve generated using purified factor Xa.

Analysis of Tissue Factor, IL-1 α , and β -actin mRNA by PCR. RNA from porcine aortic EC was isolated by the guanidinium thiocyanate method (32). The cDNA synthesis and PCR reactions were performed in a PCR machine (model 9600; Perkin-Elmer Cetus Corp.) in reaction conditions recommended by the manufacturer. The amplified products were autoradiographed and quantitated using a scanner (model 9600; Relisys, Milpitas, CA) and software (Image 1.43; National Institutes of Health Shareware, Bethesda, MD). The PCR conditions for β -actin and tissue factor were 94°C for 20 s, 55°C for 20 s, and 72°C for 75 s, for 25 and 30 cycles, respectively. The sequences of primers for β -actin were 5'ATGTTTGAGACCTTCAACAC3' and 5'CACGTCACACTTCATGATGGA3' (33), and for tissue factor were 5'TTC-AAGAC[A/C]ATT[T/C]TGGAGTGG3' and 5'AGGGGAGT-TGGTAAAC3' (34). The design of primers for tissue factor was based on the sequence of porcine tissue factor, which we obtained by cloning tissue factor cDNA from porcine EC treated with endotoxin (not shown here). The PCR conditions for porcine IL-1 α were 94°C for 20 s and 63°C for 20 s for 30 cycles. The sequences of primers for IL-1 α were 5'GCAGCCACCGGG-AAGATTCTG3' and 5'GTACATACGGCCTGTCAACAC3' (accession No. M86730). All PCR products, except β -actin, were confirmed by sequencing (not shown). To compare relative levels of tissue factor, and IL-1 α mRNA to β -actin mRNA, a semi-quantitative RT-PCR strategy was used. First, cDNA for each sample was examined for β -actin mRNA, and concentrations of cDNA were adjusted to generate approximately equal β -actin PCR products. This information was used as a guide to determine the amount of cDNA used for examination of other specific genes. Tissue factor and IL-1 α PCR products, from EC stimulated with anti-EC antibodies plus C, human rIL-1 α , or endo-

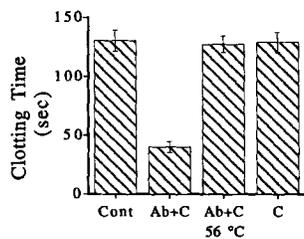


Figure 1. Induction of coagulant activity in EC by anti-EC antibodies and C. Coagulant activity was measured by determining clotting time of porcine plasma applied to porcine EC. EC treated for 16 h with 10% human serum containing natural anti-EC antibodies and C (*Ab+C*) showed increased coagulant activity compared with control cells (*Cont*).

EC exposed to heat-inactivated serum (*Ab+C*, 56°C) or to human C (serum lacking anti-porcine EC antibodies, *C*) had coagulant activity similar to that of controls.

toxin, were generated from ~500–1,000 ng of cDNA, in accord with the β -actin PCR results.

Results

Induction of Procoagulant Activity in EC by Antibodies and C. To ascertain whether humoral reactions might induce procoagulant activity in endothelium, porcine EC monolayers were exposed to human serum containing natural anti-porcine EC antibodies and C. Fig. 1 depicts a representative experiment in which an increase in coagulant activity, indicated by a decrease in plasma clotting time, was observed 16 h after exposure of porcine EC to 10% human serum, a concentration that yields no detectable cytotoxicity (23). EC treated with the same serum that had been heated to 56°C to inactivate C did not decrease plasma clotting time, suggesting that anti-porcine EC antibodies alone do not induce procoagulant activity (Fig. 1). Furthermore, a serum that lacked anti-porcine EC antibodies but had intact C was ineffective; therefore, the manifestation of procoagulant activity in this system is neither a function of serum response seen in fibroblasts (35, 36) nor a response to contaminants such as endotoxin (8, 10).

Contribution of Tissue Factor to Procoagulant Activity of EC. The contribution of tissue factor to the increased coagulant activity of EC was evaluated by measuring the conversion of factor X to Xa in the presence of excess factor VIIa after stimulation of EC by anti-EC antibodies and C. In repeated experiments, exposure of 10^4 porcine EC to human natural antibodies and C caused a very significant increase in conversion of X to Xa (Fig. 2). We concluded that the conversion of factor X to Xa in this system was not catalyzed by a cellular protease, because it was not observed in the absence of factor VIIa.

Dose Response and Kinetics of Tissue Factor Activity in EC in Response to Anti-EC Antibodies and C. Tissue factor activity of EC exposed to serum containing anti-EC antibodies

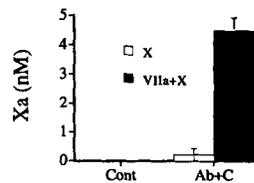


Figure 2. Role of tissue factor in expression of procoagulant activity. EC monolayers were exposed to 10% human serum containing natural anti-EC antibodies and C (*Ab+C*) for 16 h or were untreated (*Cont*) and were examined for expression of tissue factor activity based on conversion of factor X to

Xa in the presence of factor VIIa, as described in Materials and Methods. Stimulated EC mediated generation of Xa only in the presence of VIIa and X (■) but not in the presence of X alone (□).

and C depended on the concentration of serum used. Fig. 3 A shows a representative response. Tissue factor activity observed in porcine EC after stimulation with a sublytic concentration of human serum containing anti-EC antibodies plus C increased progressively during the course of 42 h (Fig. 3 B). The rate of tissue factor activity was a reflection of the amount of anti-EC antibodies and C used to stimulate EC.

Role of Antibodies and Activation of C in Induction of Tissue Factor Activity in EC. As shown in Fig. 4, the procoagulant activity of porcine EC stimulated with human serum was dependent on the amount of C activation, as assessed by deposition of iC3b neoantigen on the cells, a linear function of IgM recognizing α -galactose on porcine EC (23, 27, 37). Thus, a serum with a significant level of anti-EC antibodies decreased clotting time as a function of IgM and C activation (Fig. 4 A), whereas a serum free of these antibodies neither activated C nor decreased coagulation time at any concentration (Fig. 4 B). The requirement for C activation was also demonstrated by the findings that (a) neither purified anti-EC antibodies nor C alone induced a change in clotting time by EC; and (b) procoagulant activity was not observed in EC treated with serum in which C activity was abolished by heating to 56°C or with serum combined with sCR1, which inhibits C3 convertases (Fig. 4 C) (38). Thus, the interaction of IgM with EC does not by itself alter the coagulant activity of EC as studied here, but IgM binding is needed to activate C, which, in turn, increases the procoagulant activity of EC.

Several lines of evidence suggested that induction of tissue factor activity in this system was not mediated by C5a (Fig. 5). First, exposure of EC to recombinant C5a in the absence or presence of anti-EC antibodies did not stimulate tissue factor activity as measured by conversion of factor X to Xa. Second, exposing EC to native C5a, generated by activation of C by pyrogen-free inulin, failed in repeated experiments to stimulate porcine EC expression of tissue fac-

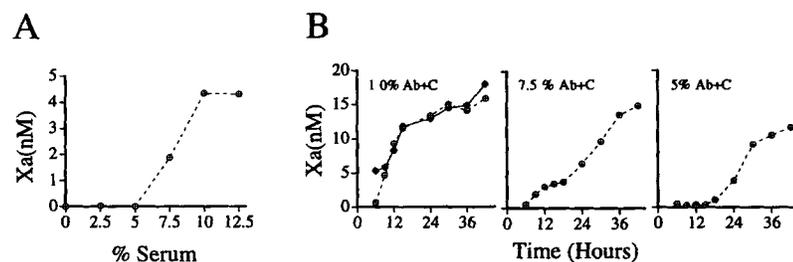


Figure 3. Dose response and kinetics of procoagulant activity induced by natural antibodies and C. Monolayers of EC were treated with human natural anti-EC antibodies plus C, and tissue factor activity was determined based on conversion of factor X to Xa in the presence of factor VIIa. (A) Dose response of coagulant activity induced by natural anti-EC antibodies and C. EC were treated for 16 h with various concentrations of antibodies and C. (B) Kinetics of coagulant activity induced by natural antibodies and C. EC treated with antibodies and C (—○—) or with 1,600 U/ml human rIL-1 α (—◆—) for indicated periods.

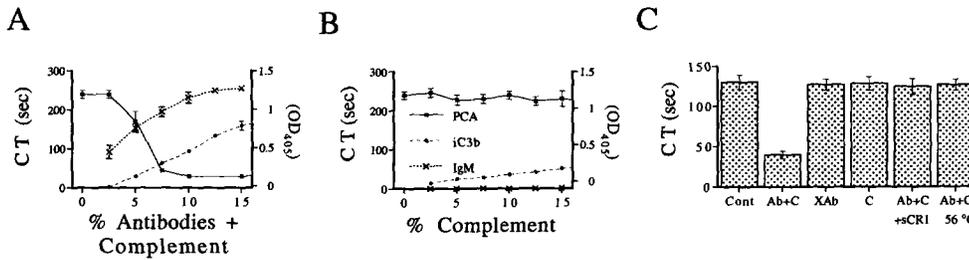


Figure 4. Role of C activation in induction of coagulant activity in EC. Cultured porcine EC treated with human natural anti-EC antibodies and C were examined for coagulant activity determined by the clotting time (CT, PCA) of porcine plasma. (A) Induction of coagulant activity as a function of IgM binding and C activation. EC were treated with inactivated EC. EC treated under conditions in which C was not activated had the same coagulant activity as controls (Cont).

creasing amounts of anti-EC antibodies and C and examined for coagulant activity. Binding of IgM to EC (IgM) and activation of C (iC3b) on EC was measured by ELISA. (B) Lack of coagulant activity in the absence of IgM binding and C activation. EC were treated with serum lacking anti-EC antibodies and examined as in A. (C) Requirement for complement activation in induction of coagulant activity. EC were exposed for 16 h to affinity-purified human natural anti-EC antibodies (XAb), serum lacking human anti-EC antibodies (C), antibodies and C to which sCR1 was added (Ab+C+sCR1), or serum that was heated to 56°C (Ab+C, 56°C). EC treated under conditions in which C was not activated had the same coagulant activity as controls (Cont).

tor activity. However, expression of tissue factor by EC in this system required assembly of the membrane attack complex, because pooled human serum depleted of C8 plus purified anti-EC antibodies did not mediate tissue factor activity, whereas this C8-depleted serum reconstituted with purified C8 did so.

The induction of tissue factor in endothelium was not restricted to stimulation of EC by xenoreactive natural antibodies and C. Incubation of porcine EC with rabbit IgG specific for porcine EC in combination with human C also induced significant tissue factor activity (Fig. 6). Moreover, tissue factor expression in porcine EC was induced in response to rabbit anti-porcine EC antibodies plus porcine serum (homologous C), although the amount of tissue factor produced under these conditions was less than the amount produced by human C (Fig. 6). On the other hand, preimmune IgG either alone or in combination with porcine or human C did not mediate tissue factor activity (Fig. 6).

Cellular Localization of Tissue Factor Activity. Cellular localization of tissue factor was evaluated by comparing the ability of intact EC to generate conversion of factor X to Xa with that of "extracellular matrix." As Fig. 7 A shows, most of the tissue factor activity induced in cultured por-

tor activity was observed on the surface of intact EC and not in the extracellular matrix. Since the method used to expose extracellular matrix leaves behind some of the basal cell membrane, it is likely that tissue factor activity was present on both the apical and basal membranes. Tissue factor activity also was not localized to the intracellular compartment, because stimulated EC subjected to repeated cycles of freezing and thawing had the same level of tissue factor activity as control cells (Fig. 7 B).

Expression of Tissue Factor mRNA by EC Induced by Antibodies and C. The effect of anti-EC antibodies and C on the level of tissue factor mRNA in EC was evaluated by PCR. Fig. 8 A depicts a representative experiment showing expression of tissue factor mRNA in EC stimulated by natural antibodies and C. Tissue factor mRNA was first detected 8 h after stimulation, reaching maximum levels at 12 h. These findings differ from the results obtained after EC were stimulated by endotoxin, where tissue factor mRNA was detected as early as 2 h. Serum factors other than antibodies and C did not mediate this response, since neither serum lacking anti-EC antibodies nor serum in which C was inactivated up-regulated tissue factor mRNA (Fig. 8 B).

Induction of Tissue Factor by Antibodies and C Is Not an Early Response. As demonstrated in Fig. 8, endotoxin induced tissue factor mRNA more rapidly than did C, suggesting that a mechanism of induction by optimum amounts of C differs from endotoxin-mediated immediate early responses (39, 40). That tissue factor mRNA is not elaborated as an immediate early gene in EC stimulated with C was substantiated by the finding that tissue factor mRNA in our system was not increased in the presence of cycloheximide. However, cy-

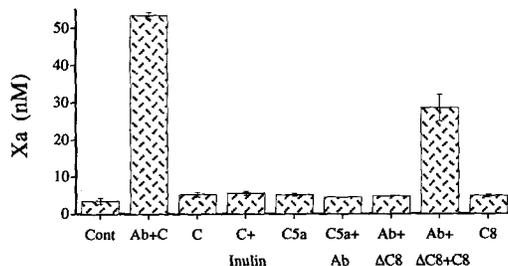


Figure 5. Role of membrane attack complex in expression of tissue factor by EC. Porcine EC were stimulated with various components of C for 18 h and examined for tissue factor activity based on conversion of factor X to Xa in the presence of factor VIIa. EC exposed to intact C, C activated by 10 mg/ml inulin (C+Inulin), or 500 ng/ml recombinant human C5a in the absence or presence of antibodies (C5a + Ab) had tissue factor activity similar to that of untreated EC (Cont). EC treated with 0.4 μg/ml purified rabbit anti-porcine EC antibodies (Ab+) plus human serum immunodepleted of C8 (ΔC8) did not exhibit tissue factor activity; however, EC treated with these reagents plus purified C8 did so.

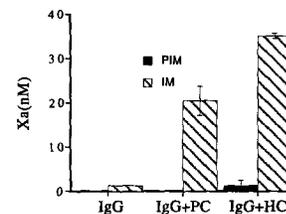


Figure 6. Induction of tissue factor activity in EC by rabbit anti-EC IgG. Tissue factor activity in porcine EC was examined after treatment of the cells with rabbit anti-porcine EC antibodies in combination with porcine C or human complement. EC were incubated with 0.4 μg/ml purified preimmune (PIM) or immune (IM) rabbit IgG in the absence of C or in combination with either 10% porcine C (PC) or 10% human C (HC) for 18 h and examined for tissue factor activity.

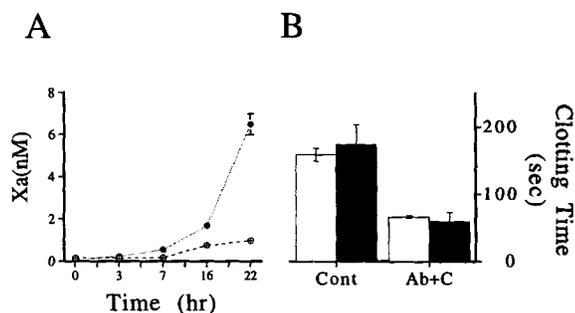


Figure 7. Cellular localization of tissue factor. Porcine EC treated with human natural anti-EC antibodies and C and examined for tissue factor activity. (A) Comparison of tissue factor activity on cell surface (●) versus extracellular matrix (⊕). EC were treated with 20% human serum for indicated periods of time and examined for tissue factor activity based on conversion of factor X to Xa in the presence of factor VIIa. Tissue factor activity was detected on the cell surface. (B) Comparison of coagulant activity of intact EC (□) and that of EC cytoplasmic components (■). EC treated for 20 h with 20% serum were subjected to repeated freezing and thawing, and coagulant activity was determined by the clotting time of porcine plasma applied to EC. Most coagulant activity is on the cell membrane.

cloheximide increased tissue factor mRNA induced with IL-1 α (Fig. 9).

Release of EC-stimulating Factors. Since the induction of tissue factor mRNA by antibodies and C required protein synthesis, as the experiments shown in Fig. 9 demonstrate, it was possible that the induction of tissue factor might in fact be a secondary response to some primary product of EC. We tested this idea by asking whether the medium from EC treated with anti-EC antibodies and C contained factors other than C that would induce EC to express tissue factor activity. When such medium was removed 1 or 5 h after stimulation of EC and the monolayers were "chased" by fresh medium, procoagulant activity notably decreased (Fig. 10 A). This decrease was not caused by the removal of C; the conditioned medium had little C activity (Fig. 10, inset). Also, although it contained little C activity, the conditioned medium from EC stimulated with antibodies and C for 5 h, induced procoagulant activity in new EC monolayers (Fig. 10 B). The latter was not the effect of residual C activity in the conditioned medium because addition of sCR1 to the conditioned medium did not inhibit the ability of the medium to induce tissue factor activity. Thus, conditioned medium, generated by addition of antibodies and

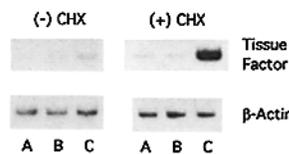


Figure 9. Role of protein synthesis in expression of tissue factor mRNA. EC were untreated (A), exposed to 10% human natural antibodies and C (B), or exposed to 800 U/ml IL-1 α (C) in the presence (+) or absence (-) of cycloheximide (CHX) for 4 h. RNA was then extracted, and the cDNA for tissue factor and β -actin was amplified by PCR as described in Materials and Methods. In contrast to EC treated with IL-1 α , EC treated with anti-EC antibodies and C do not show an increase in tissue factor mRNA in the presence of cycloheximide, indicating that tissue factor is not an early response.

C to EC, contains an intermediary agent responsible for the induction of tissue factor activity.

A series of experiments revealed that the identity of this intermediate agent was IL-1 α . First, treatment of porcine EC with antibodies and C induced the expression of IL-1 α mRNA within 2 h (Fig. 11 A), and expression of IL-1 α mRNA in this way was an immediate early response because it increased markedly when cycloheximide was present (Fig. 11 B). Second, IL-1 receptor antagonist eliminated the procoagulant response of EC treated with antibodies and C (Fig. 12 A). Third, as shown in Fig. 12 B, addition of anti-human IL-1 α antibodies to EC treated with antibodies and C in a dose-dependent manner decreased expression of tissue factor activity.

Discussion

The maintenance of hemostatic balance by endothelium is compromised in various types of humoral immune responses. For example, in the Arthus reaction, local deposition of immune complexes is associated with prominent evidence of thrombosis in the microvessels (41). Similarly, necrotizing vasculitis, systemic lupus erythematosus, serum sickness, and other clinical vasculitides are characteristically associated with intravascular coagulation (16, 17). Whereas neutrophils or antiphospholipid antibodies may initiate thrombosis, in some cases the synthesis of tissue factor and elaboration on the surface of EC probably contribute to a procoagulant diathesis. The frequent association of coagulation with assembly of C complexes in the microvessels also suggests a potential role of C in up-regulation of tissue factor activity in EC. Consistent with that concept, we report that assembly of the membrane attack complex on EC induces the synthesis and release of IL-1 α , which, as a potent

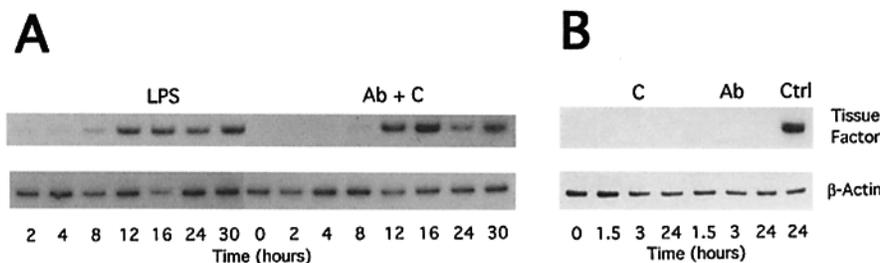


Figure 8. Induction of tissue factor mRNA in EC treated with human natural antibodies and C. RNA from EC was extracted, and the mRNA for tissue factor and β -actin were amplified by PCR as described in Materials and Methods. (A) Comparison of tissue factor mRNA expression in EC induced by human natural antibodies and C versus endotoxin (LPS). EC were treated with 25% human antibodies plus C or 25 μ g/ml LPS for indicated periods and examined for expression of tissue

factor and β -actin mRNA. (B) Comparison of tissue factor mRNA expression in EC induced by human natural antibodies and C versus C or antibodies alone. EC were either treated for 24 h with 20% antibodies and C (Ctrl), or were exposed for indicated periods to 20% C (C) or 20% antibodies (Ab). Tissue factor mRNA is detected only in EC treated with antibodies and C.

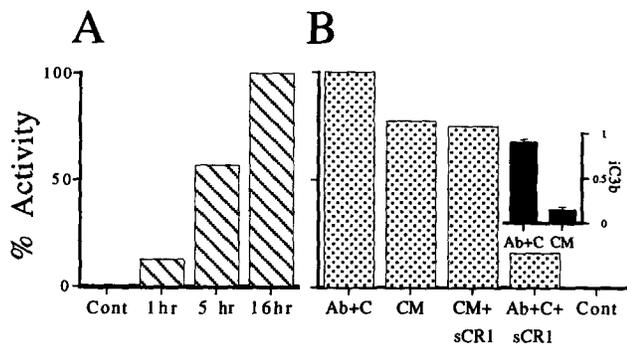


Figure 10. Release from EC of a factor that stimulates production of tissue factor. EC were treated with anti-EC antibodies and C and then examined for coagulant activity based on clotting time of porcine plasma applied to porcine EC. (A) Dependence of tissue factor expression on conditioned medium. EC were treated with 20% antibodies and C for 16 h, or for 1 h and 5 h, after which conditioned medium was chased with DMEM + 1% FCS and incubation was continued for 15 and 11 h, respectively. Removal of conditioned medium containing antibodies and C from EC inhibited tissue factor expression. (B) Stimulation of tissue factor production by conditioned medium. EC were treated for 16 h with antibodies and C (Ab+C) or with conditioned medium obtained from EC treated with antibodies and C for 5 h (CM) in the presence or absence of sCR1. C activity was assessed by deposition of iC3b and is shown in Ab+C and CM. Conditioned medium from EC treated with antibodies and C induced procoagulant activity in new EC independent of C activity.

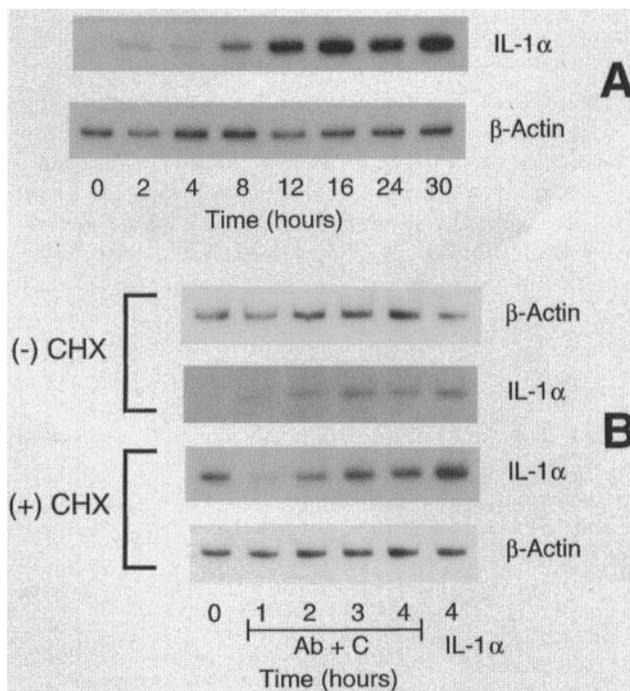


Figure 11. Up-regulation of IL-1 α mRNA mediated by human natural antibodies and C. RNA from EC was extracted and the cDNA for IL-1 α and β -actin were amplified by PCR as described in Materials and Methods. (A) Kinetics of IL-1 α mRNA expression by EC. Porcine EC were treated with 25% human natural anti-EC antibodies plus C for indicated periods and examined for expression of IL-1 α and β -actin mRNA. (B) Early expression of IL-1 α mRNA in EC. EC were stimulated with 10% anti-EC antibodies and C or 800 U/ml IL-1 α in the absence or presence of cycloheximide (CHX) for the indicated periods. EC treated with human natural anti-EC antibodies plus C show an increase in IL-1 α mRNA in the presence of cycloheximide.

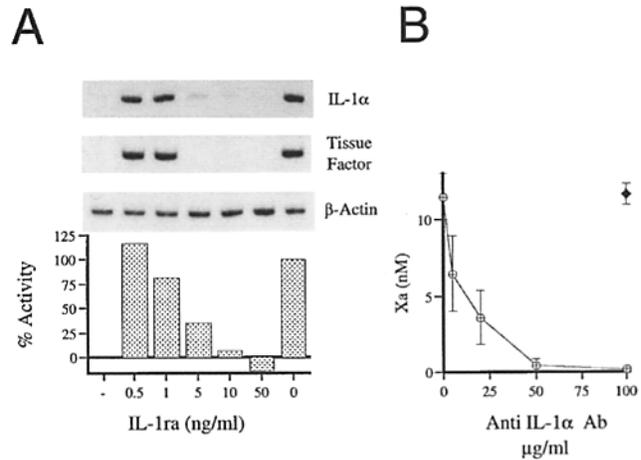


Figure 12. Role of IL-1 in C-mediated tissue factor expression. (A) Inhibition of tissue factor expression by IL-1 receptor antagonist. Porcine EC were treated for 16 h with 10% human natural anti-EC antibodies and C in the presence of increasing concentrations of IL-1 receptor antagonist and then examined for coagulant activity by determining clotting time of porcine plasma applied to porcine EC or expression of tissue factor and IL-1 α mRNA by RT-PCR, as described in Materials and Methods. (B) Inhibition of tissue factor expression by anti-IL-1 α antibodies. Porcine EC were treated with 10% human natural anti-EC antibodies and C in the presence of increasing concentrations of anti-human IL-1 α antibodies (\oplus) or 100 μ g/ml of control (noninhibitory) antibodies (\blacklozenge) for 16 h. Porcine EC were then examined for tissue factor activity based on conversion of factor X to Xa in the presence of factor VIIa.

intermediate, causes a remarkable increase in tissue factor activity. Our findings suggest that the initiation of intravascular coagulation and generation of fibrin leading to formation of occlusive thrombi and necrosis in humoral immune reactions may be caused in part by expression of tissue factor in endothelium in response to the host's humoral immunity.

The system used in our experiments involved stimulation of EC by heterologous serum as a model for the reaction that might occur in a xenogeneic organ graft undergoing acute vascular rejection, a type of rejection that develops over a period of days after engraftment and that is characterized by widespread intravascular coagulation (20). The prominent formation of fibrin thrombi in this setting suggests that porcine tissue factor might be able to bind to human VIIa, initiating coagulation. Indeed, although porcine tissue factor shows only 75% homology to its human counterpart (not shown) and is not conserved in the factor VIIa binding site, our studies show that it interacts very efficiently with human factor VIIa.

Expression of IL-1 and subsequent modulation of tissue factor activity in this system probably reflect signaling by the membrane attack complex (for review, see reference 42). Assembly of the membrane attack complex in sublytic amounts on B lymphoblastoid cells increases ceramide (43), and on Ehrlich's ascites cells induces protein kinase C activation, and Ca²⁺ influx and increases the intracellular concentration of cAMP (44). While protein kinase C activation in monocytes and increased intracellular levels of Ca²⁺ in EC play a direct role in the expression of tissue factor

(45–47), ceramide and cAMP have been implicated in up-regulation of IL-1 in various types of cells (48, 49). Although the formation of membrane attack complex on EC is clearly requisite for synthesis of tissue factor in the model we studied, membrane attack complex assembly may not be sufficient. Thus, other components of plasma may contribute to this process.

Based on the findings reported here, a model for association between humoral immunity and coagulation might be proposed. In a humoral immune reaction, the membrane attack complex induces EC to elaborate IL-1 α , perhaps through channels provided by membrane attack complexes. Elaborated this way, IL-1 α can act on EC as well as monocytes to up-regulate procoagulant activity. Blood flow may be a critical determinant in this process. Rapid blood flow may carry IL-1 α away from EC, limiting procoagulant changes, while vasoconstriction, perhaps mediated by

thromboxane A₂ (another product of EC bearing membrane attack complexes [50]) may retard the removal of IL-1 α , promoting coagulation. The procoagulant effects of tissue factor synthesized by EC in response to antibodies and C are probably amplified by other changes. For example, C5b67 and/or membrane attack complex perturb the boundary provided by endothelium (23), thus exposing the tissue factor associated with smooth muscle cells to plasma coagulation factors. Complement-mediated injury also releases heparan sulfate proteoglycan from EC (51), which may decrease the availability of antithrombin III and thus impair control of thrombin generated in these reactions. IL-1 α may also interrupt barrier function of endothelium, and, migrating through endothelium, it may stimulate smooth muscle cells and fibroblasts to elaborate procoagulant and inflammatory functions (for review, see reference 52).

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