Tissue Factor Is a Major Stimulus for Vegetation Formation in Enterococcal Endocarditis in Rabbits

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Abstract. We examined the possible mechanisms of local initiation of coagulation in vegetation formation in enterococcal endocarditis by using a rabbit model. Contact activation and tissue factor expression by freshly excised aortic valves were assessed using assays developed for use with cultured cells. Bacteria alone lacked procoagulant activity and contact activation of plasma by excised valves did not occur. 4-d infected but not control valves expressed significant tissue factor activity (231 ± 17 mU vs. 51 ± 7 SE), which did not correlate with numbers of bacteria in vegetations. Tissue factor activity was also present in valves from rabbits infected for 1 and 2 d, as well as those from granulocytopenic and monocytopenic animals. Our findings suggest that tissue factor, expressed by host cells in response to infection, is a major stimulus for fibrin deposition in vegetation development.

Introduction

Recent investigations have led to an understanding of the initiating event in the pathogenesis of infective endocarditis, the adherence of circulating organisms to sterile fibrin-platelet thrombi in the surface of damaged valves (1). However, the factors responsible for subsequent vegetation development have not been elucidated. The coagulation system clearly plays a critical role at this stage, as warfarin anticoagulation prevents vegetation formation (2). This study examines the mechanisms by which cardiac valve infection may initiate local activation of coagulation. First, the intrinsic coagulation pathway might be activated by exposed connective tissue components of the damaged valve (3) or by bacterial cell wall elements (4). Secondly, tissue factor expression by host cellular elements could initiate the extrinsic pathway (5). Third, it is known that some bacteria can directly activate coagulation proteins (6), although most endocarditis-associated organisms cannot. We determined the relative role played by each of these mechanisms in an experimental model of enterococcal endocarditis.

Methods

Materials. Streptococcus fecalis strains cor and hol (isolated from patients with endocarditis) were maintained at −70°C before use. Catheters were made from PE 90 polyethylene tubing (Clay Adams, Parsippany, NJ). Mechlorethamine HCl was from Merck Sharp & Dohme (West Point, PA). Human Factor VII was provided by Dr. George J. Broze, Jr. (Washington University, St. Louis, MO) and activated as described (7); its specific activity was 53 U/µg. Factor X and prothrombin were purified from human plasma as described (8); Factor X-specific activity was 160 U/mg. Bovine Factor VII/X-deficient plasma, rabbit brain cephalin, bovine serum albumin (BSA-Fraction V), and rabbit brain thromboplastin were from Sigma Chemical Co. (St. Louis, MO). Polystyrene petri dishes (35 × 10 mm) were supplied by Falcon Labware (Oxnard, CA). The radiolabeled protease substrate benzoyl-L-prolyl-L-phenylalanyl-L-arginyl-^{14}C-anilide and Omnifluor were products of New England Nuclear (Boston, MA). Unlabeled benzoyl-L-prolyl-L-phenylalanyl-L-arginyl-anilide was from Vega-Fox Biochemical Div., Newbery Energy Corp. (Tucson, AZ).

Establishment of endocarditis. White New Zealand rabbits (male and female, 1.8–2.5 kg) were anesthetized and the left heart catheterized as described (9). 1 d later, 4×10^7 log phase *S. fecalis* in 1 ml saline (or saline alone for controls) were injected via the catheter, which was then removed. Over 95% of animals thus infected developed endocarditis, manifested by bacteremia and temperature > 39.6°C within 4 d. 37 rabbits were infected and 11 were not infected. Four more rabbits thus treated were rendered leukopenic by a single intravenous injection of mechlorethamine HCl (2.5 mg/kg) 3 d before infection; two were infected and two controls were not. Leukocyte counts were determined on the day of infection and at the time of death, 24 h later, being <600/mm³ on each occasion. 18 additional rabbits were treated in the following manner: 5 were neither catheterized nor infected; 3 were not catheterized but were injected intravenously with the bacterial inoculum (these did

not develop endocarditis); and 10 uninfected rabbits had the catheter left in place for a total of 3 or 5 d before death.

Tissue factor assay. Aortic valves were excised from rabbits at 20 min and 1, 2, or 4 d after catheter removal. Animals were killed by rapid intravenous injection of 250 mg pentobarbital several minutes after administration of 800 U i.v. heparin. Hearts were immediately removed, opened, and the aortic valve flushed gently with 20 mM Tris-HCl, 150 mM NaCl, 2 mg/ml BSA pH 7.4 (assay buffer). All three cusps with adherent vegetations were excised using fine forceps and iris scissors, avoiding inclusion of valve ring substance and placed in a petri dish containing 1 ml assay buffer. Tissue factor activity was then measured as has been described for cultured cells (10), incubating valves with purified Factors VII$_a$ (20 ng) and X (10 μg). The Factor X$_a$ generated was measured by clotting assay (10). Clotting times were compared with a standard curve generated by using rabbit brain thromboplastin, Factor VII$_a$, and Factor X. Stock thromboplastin (0.001 ml, 7 μg protein) was designated as having 1 U of tissue factor activity. Valve tissue factor activities were related to a standard curve constructed with thromboplastin dilutions and expressed in terms of milliunits thromboplastin standard.

Contact activation assay. Intrinsic coagulation pathway activation was assessed by measuring the conversion of prekallikrein to kallikrein, a Factor XII$_a$-dependent reaction, while plasma was incubated over a glass surface and over an albumin-coated petri dish containing either control or infected aortic valves obtained as described for the tissue factor assay. Details of this assay have been described previously (11).

Determination of bacterial procoagulant activity. The ability of bacteria to activate Factor X or prothrombin was studied using the Factor X$_a$ assay (10) or a prothrombinase assay, respectively. *S. fecalis* strain cor was grown overnight in trypticase soy broth, washed twice in saline, and resuspended in assay buffer. The final concentration of bacteria in each incubation was 2×10^8 colony-forming units per milliliter. In the prothrombinase assay, bacteria in 1 ml assay buffer were incubated with 100 μg purified prothrombin and 10 mM CaCl$_2$. Thrombin generation was measured by a clotting time (12). The tissue factor expression of bacteria was studied using the assay described above; for this assay, bacteria grown overnight in broth containing 50% rabbit serum were also examined.

Results

Tissue factor expression by excised heart valves. Aortic valves of rabbits infected for 4 d with either strain of *S. fecalis* expressed significantly greater tissue factor activity (110–600 mU, mean 231.1±17.2 SE) than did valves from control rabbits ($\leq$90 mU, mean 51.2±7.1). Valves of five normal rabbits and three rabbits injected intravenously with the bacterial inoculum but not catheterized had levels of tissue factor comparable to those of catheterized controls ($\leq$30 to 110 mU). Deletion of Factor VII$_a$, Factor X, or both from the incubation medium proved that the majority of activity measured represented tissue factor (Fig. 1). Factors VII$_a$ and X incubated in buffer without aortic valves generated no activity. The magnitude of activity of 4 d infected valves did not significantly correlate with the total number of bacteria in vegetations ($r = 0.52$, $P > 0.1$); nor with wet weight ($r_s = 0.67$, $P > 0.05$, <0.1). Expression of tissue factor by aortic valves at levels greater than in controls occurs within 24 h of infection in most animals (Fig. 2). Mean activities (±SEM) of valves infected for 1, 2, and 4 d (strain cor only) were not
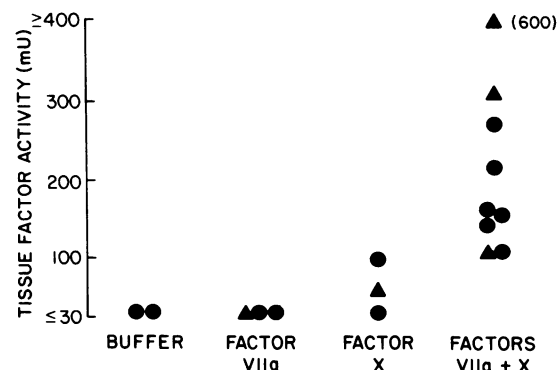

Figure 1. Tissue factor activity of 4-d infected valves. Tissue factor activity was assayed as described in Methods using buffer and Factors VII$_a$ and X, or each alone. Each point represents one animal infected with strain cor (●) or hol (▲).

different (169.8±20.6, 168.8±27.0, and 176.7±23.2 mU, respectively, $P > 0.5$). Overall, combining data for days 1 to 4, mean tissue factor expression in infected valves (169.1±12.3) was significantly greater than controls (63.6±12.4, $P < 0.001$). Total numbers of bacteria in vegetations (mean±SE, log 10 colony-forming units) increased from 3.7±0.1 at 20 min post-infection to 4.9±0.1 at 1 d, 6.0±0.2 at 2 d, and 6.9±0.2 at 4 d. Although mean (±SE) vegetation size after 24 h of catheterization alone was comparable to that after 24 or 48 h of infection (19.6±1.7 vs. 17.9±1.3 and 17.4±0.8 mg valve weight, respectively), tissue factor expression of the valves with sterile catheter-induced vegetations was lower in five of six rabbits (Fig. 2, 0 d) being in the same range as normal valves. Similar results were also obtained from valves of uninfected rabbits in which catheters had been left in place for 3 ($n = 6$) or 5 ($n = 4$) d (i.e., for total study durations equal to those of 2- and 4-d infections); valve weights were 15.8±4.1 and 26.1±7.3 mg (mean±SE), respectively. Tissue factor activity of valves from
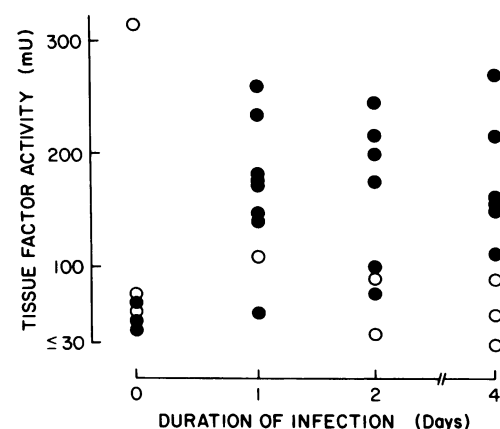

Figure 2. Tissue factor activity of infected (●) and uninfected (○) valves (cor strain only), at 0 (20 min), 1, 2, and 4 d.

two leukopenic rabbits (500 and 600 total leukocytes/mm^3 with 0 and 9 total granulocytes, and 12 and 33 total monocytes/mm^3) infected for 1 d were 290 and 600 mU compared with 65 and 110 mU in two leukopenic controls. Neither granulocytes and monocytes were observed histologically in these same valves examined after the tissue factor assay.

Assay for contact activation by excised heart valves. Kallikrein generation during incubation of fresh-frozen plasma is dependent on Factor XII activation. The presence of 4-d infected ($n = 3$) or control ($n = 2$) aortic valves did not increase the amount of kallikrein detected above the low level generated in albumin-coated plastic petri dishes alone, while incubation of plasma alone in glass tubes generated significant kallikrein activity (data not shown). Vegetation size and bacterial titer of the valves tested were comparable to those shown to express significant tissue factor activity.

Assays for procoagulant activity of S. fecalis in culture. Suspensions of *S. fecalis* in numbers equal to or greater than those found in infected valves had no demonstrable prothrombinase, Factor X-activating, or tissue factor activity.

Discussion

This study of experimental enterococcal endocarditis demonstrates that activation of coagulation, ultimately leading to vegetation formation, occurs via enhanced expression of tissue factor by infected valves. Tissue factor is a membrane-associated lipoprotein which initiates coagulation by specifically binding Factor VII$_a$, forming a complex capable of activating Factor X (5); Factor X$_a$ in turn activates prothrombin to thrombin, resulting in deposition of fibrin, an essential structural component of the vegetation. Alternate mechanisms of coagulation activation (Factor XII activation as measured by the kallikrein assay and direct bacterial activation of Factor X or prothrombin) were not detected in this model. Under the experimental conditions used, bacterial infection of susceptible cardiac valves was by itself sufficient to significantly increase tissue factor expression, while catheter trauma in the absence of infection was not. The levels obtained for infected valves were comparable to those expressed by 6×10^5 nonvascular cells (human fibroblast or bovine corneal endothelial cells) described in previous studies (10). Cultured bovine aortic endothelial cells (unstimulated) express negligible activity by comparison.

The use of purified Factors VII$_a$ and X in the first stage of the tissue factor assay make this assay specific for tissue factor, since Factor X activation is directly related to the quantity of tissue factor expressed (13). Platelets, monocytes, and endothelial cells undoubtedly contribute to the local coagulation process by amplifying tissue factor-generated Factor X$_a$ activity via enhanced prothrombinase complex assembly on their surfaces (14, 15). However, this activity would not be recognized by the tissue factor assay used here, and by itself could not initiate coagulation.

Several specific components of the infected valve may be responsible for tissue factor expression. Cellular elements of the lesion are bacteria, platelets, and occasional leukocytes intermixed with fibrin in the substance of the vegetation, stromal cells in the valve connective tissue core, and endothelial cells lining the valve surfaces. Neither platelets nor bacteria express tissue factor, but each has been shown to stimulate its activity in other cells (16–18). Vascular endothelial cells, although normally lacking tissue factor activity, will express significant activity in response to a variety of stimuli (5, 16, 17). Fibroblasts inherently express tissue factor (10), and those in the valve core may be exposed as valve destruction occurs. Monocytes have been proposed as the source of procoagulant activity in vegetations (19). They have also been shown in vitro to express tissue factor in response to platelets, endotoxin, and in collaborative function with lymphocytes that have been exposed to bacterial products (5). Granulocytes and lymphocytes, in contrast, do not express tissue factor in vitro (5, 13). Monocytes are usually not major constituents of the vegetation and in our study, infected valves of leukopenic (monocytopenic and granulocytopenic) rabbits expressed amounts of tissue factor equivalent to those of nonleukopenic animals. It is thus likely that the local host cells (stimulated or damaged endothelial cells, fibroblasts, and, when present, monocytes) all express tissue factor in response to the continued presence of bacteria, thereby causing progressive fibrin deposition and vegetation enlargement.

Acknowledgments

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