

Effects of Interleukin-1, Lipopolysaccharide, and Streptococci on Procoagulant Activity of Cultured Human Cardiac Valve Endothelial and Stromal Cells

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Fibrin is the primary constituent of the vegetation in infective endocarditis, and tissue factor expression is a major mechanism of coagulation activation on infected valves. To determine which cells may participate in coagulation activation in this setting, expression of procoagulant activity (PCA; shown to be tissue factor) was studied in cultured endothelial and stromal cells derived from human cardiac valves. Endothelial cells had negligible PCA (99 ± 50 mU/ 10^5 cells, mean ± 1 standard deviation) unless stimulated by lipopolysaccharide or interleukin-1, which increased PCA to $5,592 \pm 1,482$ and $5,901 \pm 1,497$ mU/ 10^5 cells, respectively, in 6 h. Incubation of cells with viable enterococci or viridans streptococci or with an enterococcal cell wall preparation did not induce PCA. Cultured valve stromal cells constitutively expressed high levels of PCA ($14,276 \pm 8,738$ mU/ 10^5 cells) which was not changed with exposure to interleukin-1. PCAs of stromal or stimulated endothelial cells from valves of both right and left sides of the heart were comparable. The results suggest that endothelial cells may contribute to fibrin deposition during infection if stimulated, but PCA is not directly induced by bacteria. Stromal cells could contribute PCA if exposed to blood in the course of valve injury.

The central lesion of infective endocarditis is the vegetation, an infected fibrin-platelet thrombus which forms on the surface of a cardiac valve colonized by circulating microorganisms. Investigations in this laboratory have sought to elucidate the mechanisms of vegetation development, focusing on the thrombotic process, since fibrin is the primary and essential structural component of the lesion (10, 11). Previous studies, using an animal model of enterococcal endocarditis, identified tissue factor (TF) expression as the primary mechanism of local coagulation activation on infected valves, but the cell types expressing TF were not identified (11).

TF is the cell-associated activator of the coagulation protease cascade, being a cell surface membrane protein which functions as the receptor and essential cofactor for factor VII (25). It is not expressed by bacteria or platelets, the primary cellular constituents of the vegetation. Some cells in culture, such as smooth-muscle cells and fibroblasts, are reported to express TF constitutively (22, 29). Human umbilical vein endothelium in culture expresses TF only following induction by certain mediators such as lipopolysaccharide (LPS) and interleukin-1 (IL-1), suggesting that these normally nonthrombogenic cells may play an active role in thrombosis associated with inflammation (2, 6, 8). Constituent cells of the cardiac valve were therefore considered to be potential sources of procoagulant activity (PCA) expressed by infected valves.

In this study, both constituent cell types of human cardiac valves, endothelial cells and stromal cells, were isolated and propagated in culture. The procoagulant properties of each were investigated to determine under what conditions TF was expressed, whether TF expression varied among cells derived from different valves, and whether coincubation of cells with streptococci (enterococci and viridans group strains) influenced cellular TF expression.

MATERIALS AND METHODS

Materials. Tissue culture medium and certain supplements (pyruvate and antibiotics), collagenase (clostridopeptidase), and soybean trypsin inhibitor were from GIBCO Laboratories, Grand Island, N.Y. Fetal bovine serum was from Hyclone, Logan, Utah. Endothelial cell growth supplement and recombinant IL-1 α (rIL-1 α) were from Collaborative Research, Inc., Bedford, Mass. Low-molecular-weight heparin was from Calbiochem-Behring, La Jolla, Calif. Lipopolysaccharide (LPS; *Escherichia coli* O111:B4) was from List Biological Laboratories, Inc., Campbell, Calif. Other chemicals, rabbit brain cephalin, factor VII- and X-deficient bovine plasma, and Etoxate *Limulus* lysate detection kit for endotoxin were from Sigma Chemical Co., St. Louis, Mo. Tissue culture plastic ware was from Costar, Van Nuys, Calif. Bacterial media were from BBL Microbiology Systems, Cockeysville, Md. Fluorescein-conjugated antibody to factor VIII antigen (von Willebrand factor) was obtained from Atlantic Antibodies, Scarborough, Maine.

Fresh frozen plasma pooled from 10 donors was prepared as described previously (18) and frozen at -70°C in 1-ml portions. Fibronectin was purified from single-donor fresh frozen plasma obtained from the UCLA Transfusion Service by affinity chromatography, using gelatin-Sepharose 4B columns purchased from Pharmacia, Piscataway, N.J., as described before (13). Purified factors VIIa and X were kindly provided by Daryl Fair of the University of Texas Health Sciences Center in Tyler. Monoclonal antibody to human TF was kindly provided by James Morrissey of the Research Institute of Scripps Clinic, La Jolla, Calif.

Cell culture. Human cardiac valve endothelial cells (HCVE) were derived from cardiac valves of hearts removed during cardiac transplantation, using techniques described for large-vessel endothelium (21, 36, 39). Cells were grown on fibronectin-coated ($5 \mu\text{g}/\text{cm}^2$) tissue culture plastic ware in medium 199 supplemented with 25 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid), 20% fetal bovine serum, glutamine (292 $\mu\text{g}/\text{ml}$), pyruvate (110

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$\mu\text{g/ml}$), penicillin (100 U/ml), streptomycin (100 $\mu\text{g/ml}$), endothelial cell growth supplement (50 $\mu\text{g/ml}$), and heparin (90 $\mu\text{g/ml}$). Cells were passed at 1:2 split ratios and were used for experiments in passages 4 to 6.

Cardiac valve stromal cells were obtained by using a technique described for large-vessel smooth-muscle cells, with minor modifications (7). Following scraping of the valve surfaces to remove endothelium, the valves were finely minced and incubated in a mixture of elastase (0.24 mg/ml), soybean trypsin inhibitor (0.75 mg/ml), collagenase (2.0 mg/ml), and bovine serum albumin (1.0 mg/ml) in medium 199 at 37°C for 90 min with gentle shaking. The digest was passed through a 100- μm stainless-steel mesh, and dispersed cells were obtained by pelleting the filtrate. They were suspended in medium 199 with L-glutamine, antibiotics, and 20% heat-inactivated fetal calf serum, plated in 35-mm tissue culture dishes, and grown to confluence in an atmosphere of 5% CO_2 at 37°C. Cultures were passed at 1:2 split ratios and used for studies in passages 3 to 6.

For von Willebrand factor antigen detection, cells were grown on 15-mm-diameter glass cover slips and examined while subconfluent by an immunofluorescence method (19, 20). Cover slips were washed three times with phosphate-buffered saline (PBS), fixed in acetone for 15 min, air dried, and stored at -70°C. Monolayers were incubated with fluorescein-conjugated goat antiserum to human antihemophilic factor (diluted 1:25 in PBS) for 60 min and then washed three times in PBS and examined directly in a Zeiss fluorescence microscope.

Clinical strains of *Streptococcus faecalis* and viridans group streptococci used had originally been cultured from blood of patients with infective endocarditis; *S. faecalis* strain cor, had been used in previous studies of experimental endocarditis in rabbits (11) and had been passaged in rabbits prior to use for these in vitro studies. Bacterial stocks were maintained on glass beads at -70°C. Inocula were prepared from overnight growth in Trypticase soy broth (BBL); bacteria were washed twice in PBS (Dulbecco PBS, pH 7.2) and once in medium 199 and then were suspended in medium 199 prior to final dilution in test medium. Bacteria were quantitated by serial dilution in PBS and quantitative plating to sheep blood agar.

A cell well fraction of *S. faecalis* strain cor was prepared by the method of Peterson et al., with minor modifications (26). Briefly, organisms in logarithmic growth were harvested by centrifugation, suspended in distilled H_2O , and placed in a boiling-water bath for 20 min. After cooling, pelleting, and suspension in distilled H_2O , the suspension was mechanically sheared in a Bead Beater apparatus (Bio-spec Products) with glass beads, cooled by an ice jacket around the chamber. Disruption was verified by Gram staining. Glass beads and intact cells were then removed by centrifugation and the supernatant was treated with 4% sodium dodecyl sulfate for 30 min in a boiling-water bath. Sodium dodecyl sulfate was removed by multiple washes with 1 M NaCl, cell wall fragments being pelleted at 12,000 rpm for 10 min in a Microfuge (Beckman Instruments, Inc., Fullerton, Calif.); the pellet was then incubated in 8 M LiCl for 10 min, washed once with water, and then suspended in 0.05 M Tris hydrochloride containing RNase and DNase with MgCl_2 (37°C, 60 min). Nucleases were digested by incubation with trypsin for 3 h at 37°C. The sodium dodecyl sulfate treatment and washes described above were repeated, and the final preparation was lyophilized.

Experiments were performed with confluent cell monolayers in 24-well tissue culture dishes, containing 1×10^5 to $2 \times$

10^5 HCVE or 2×10^4 to 4×10^4 stromal cells per well. Each experimental condition was set up in duplicate or triplicate and was repeated on at least one separate occasion for all experiments. For each experiment monolayers were washed three times with medium 199. Unless otherwise stated, test medium (medium 199 with 10% heat-inactivated fetal bovine serum) with or without the appropriate agonist in 1 ml was then added. At the completion of the incubation period (6 h total unless otherwise noted), medium was withdrawn and monolayers were washed twice with PBS and once with TSA assay buffer (20 mM Tris, 130 mM NaCl, 0.1% bovine serum albumin, pH 7.4). Monolayers were then mechanically scraped into 200 μl of TSA assay buffer and frozen at -70°C. Prior to assay, cell suspensions were freeze-thawed three times in a dry ice-ethanol bath.

Incubation media and bacterial inocula were tested for endotoxin contamination by the *Limulus* amoebocyte lysate technique (12) and were uniformly nonreactive.

Endothelial cell viability was measured by trypan blue dye exclusion (27). Cell density in culture plates was determined by detaching cells with brief exposure to trypsin-EDTA and performing manual cell counts on the suspensions with a hemacytometer.

Coagulation assays. PCA was measured by using either a one-stage plasma recalcification clotting time (33) or a two-stage procedure that uses purified factors VIIa and X (11). In the one-stage assay, 60 μl of test suspension was added to 60 μl of rabbit brain cephalin suspension and 60 μl of freshly thawed plasma. This mixture was incubated for 30 s; then 60 μl of 25 mM CaCl_2 was added, and the clotting time was determined in a fibrometer. In the two-stage assay, 60 μl of cell suspension was added to 20 ng of factor VIIa and 10 μg of factor X in 390 μl of TSA assay buffer. CaCl_2 (28 μl of 100 mM) was then added. After 10 min, 60 μl of assay medium was withdrawn and factor Xa generated during the first stage was measured by a clotting time procedure with factor VII- and X-deficient bovine plasma. For determination of surface-expressed activity, factors VIIa and X were incubated in assay buffer directly over washed intact cell monolayers in the first stage, and the second stage was performed as described above. For all assays, the amount of PCA was calculated from standard curves prepared with rabbit brain thromboplastin. In the one-stage assay 1,000 mU of activity had a clotting time of 30 s; in the two-stage assay 1,000 mU had a clotting time of 20 s in the second stage. Demonstration of TF as the primary source of the PCA measured was made by adding 10 μg of a monoclonal antibody to human TF per ml to the test suspension and determining extent of inhibition of PCA in the above assay (23a).

Statistics. Comparisons between groups were analyzed by using Student's *t* test or chi-square analysis with Yates correction (16).

RESULTS

HCVE and stromal cells in culture. HCVE cells in culture grew as monolayers of polygonal to spindled cells, with confluent cell densities of 1×10^5 to $2 \times 10^5/\text{cm}^2$ (Fig. 1a). Von Willibrand factor was demonstrable in these cells by immunofluorescence, confirming them to be of endothelial origin (Fig. 1b). Stromal cells in culture appeared larger and more spread out, with overlapping growth (Fig. 1c). At confluence, cell densities were 2×10^4 to $4 \times 10^4/\text{cm}^2$. These cells did not contain von Willibrand factor by immunofluorescence (Fig. 1d).

PCA of HCVE in culture. Unstimulated HCVE in culture expressed relatively little PCA (generally <100 mU/ 10^5

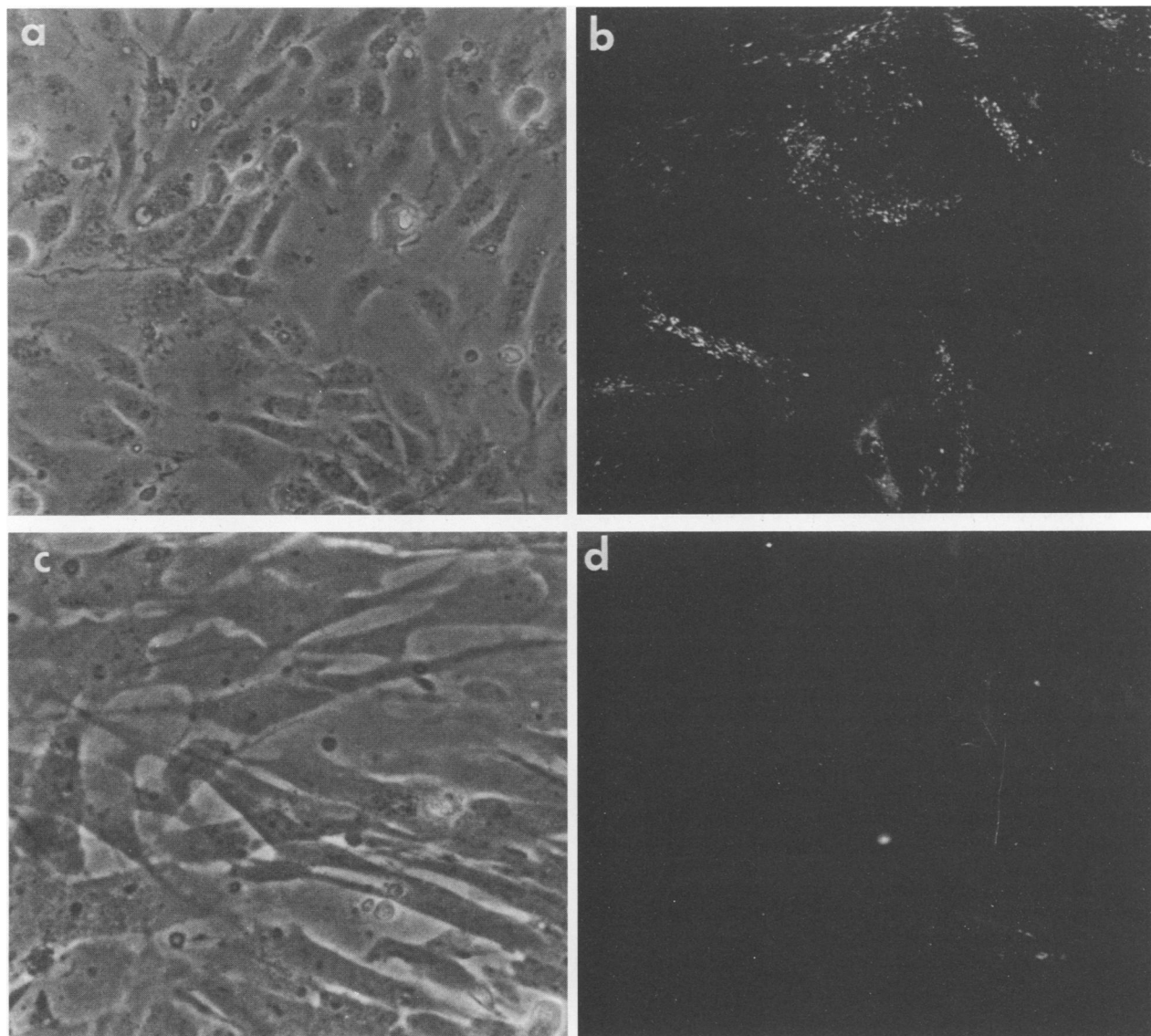


FIG. 1. Appearance of cultured HCVE (a) and stromal cells (c) with phase-contrast microscopy and demonstration of von Willibrand factor antigen by immunofluorescence in endothelium (b) but not stromal (d) cells. Magnification, $\times 400$.

cells). Exposure to LPS resulted in significant increases in activity, peaking at 6 to 8 h and declining substantially by 24 h (Fig. 2). Over the course of this striking functional response, HCVE did not undergo morphologic changes by light microscopy, nor did they detach from the culture dish. Cell viability assessed by trypan blue dye exclusion was $>90\%$. Dose-response determinations demonstrated LPS effects at 0.1 ng/ml and maximal response at ≥ 10 ng/ml (Fig. 3). PCA of LPS-stimulated HCVE was substantially inhibited ($83.5 \pm 6.6\%$) by preincubating cell lysates with a monoclonal antibody to TF prior to PCA assay.

Exposure of HCVE to rIL-1 α at concentrations of 20 U/ml induced procoagulant responses of comparable magnitude and time course to those induced maximally by LPS. PCAs of HCVE derived from one heart and tested at the same passage were $5,592 \pm 1,482$ and $5,901 \pm 1,497$ mU/ 10^5 cells when stimulated by LPS and rIL-1 α , respectively, compared with 99 ± 50 mU for unstimulated HCVE ($n = 4$; each determined in duplicate). Heat inactivation (15 min, 80°C)

completely abolished the procoagulant-inducing activity of rIL-1 α preparations, indicating that possible contamination by LPS did not cause these responses. Dose-response determinations showed consistent induction of PCA at ≥ 0.312 U/ml (Table 1). Maximal induction occurred at 20 U/ml (40 U/ml was the highest concentration tested).

Examination of procoagulant response to rIL-1 α for HCVE from each of the cardiac valves did not demonstrate significant differences for either low or high concentrations of the cytokine (Table 2). In separate experiments, no differences according to valve source were observed for LPS-induced PCA either; results for HCVE from aortic, mitral, pulmonic, and tricuspid valves exposed to 1 μg of LPS per ml for 6 h were $4,237 \pm 1,602$, $3,250 \pm 2,280$, $3,883 \pm 2,357$, and $3,779 \pm 966$ mU/ 10^5 cells, respectively (± 1 standard deviation; $P > 0.2$). More limited studies of PCA expressed by intact cell monolayers also showed no significant differences. HCVE from aortic and tricuspid valves stimulated with 20 U of rIL-1 α per ml expressed 40.0 ± 15.5

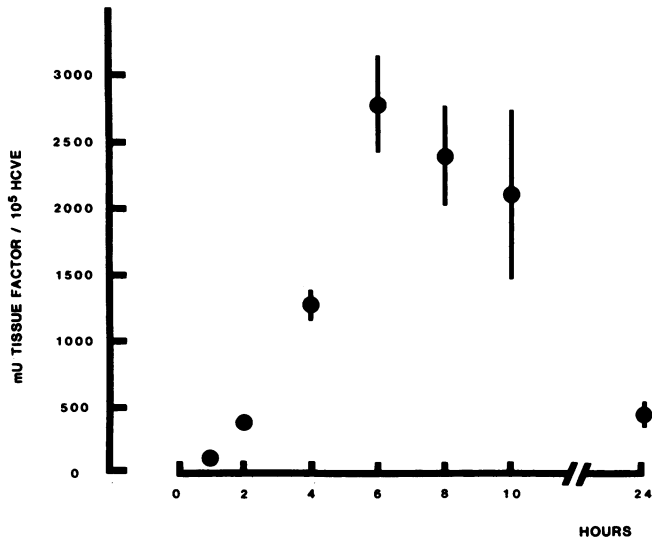


FIG. 2. Time course of PCA response in HCVE exposed to 1 μ g LPS per ml.

and 73 ± 45 mU/10⁵ cells, respectively; levels for unstimulated HCVE were 4.5 ± 1.0 and 6.0 ± 3.6 mU/10⁵ cells.

Incubation of HCVE with viable *S. faecalis* strain cor (the strain used previously in animal model studies) or with a cell wall preparation of that organism did not induce PCA. Incubation of HCVE for 8 h with viable *S. faecalis* (10^6 /ml; $n = 16$), cell walls (10μ g/ml; $n = 4$), or diluent alone ($n = 16$) yielded PCAs of 75 ± 22 , 80 ± 16 , and 60 ± 19 mU/10⁵ cells (± 1 standard deviation), respectively, while incubation with 1 μ g of LPS per ml induced $1,762 \pm 342$ mU/10⁵ cells ($n = 4$). Viable organisms were tested over a range of concentrations from 10^6 to 10^8 per ml; cell wall preparations were tested from 0.1 to 100 μ g (dry weight)/ml. Two additional enterococcal strains and two viridans group streptococcal strains tested similarly (viable organisms only) were also without effect. (In previous studies we have documented a moderate effect [two- to fourfold increase] on PCA of *Staphylococcus aureus*-infected HCVE, while several *S. epidermidis* strains caused no increase in PCA.)

PCA of cardiac valve stromal cells in culture. Stromal cells in culture constitutively expressed high levels of PCA which varied considerably in magnitude. The mean PCA of cells derived from aortic valves (five different hearts) was $14,276 \pm 8,738$ mU/10⁵ cells, while that of cells from tricuspid valves (two hearts) was $17,365 \pm 3,017$. Exposure to rIL-1 α (20 U/ml) for 6 h before assay did not affect PCAs. PCA of intact cell monolayers was 7 to 12% that of disrupted cells. The cellular PCA measured was primarily TF, as was shown for stimulated HCVE; a monoclonal antibody to human TF blocked PCA by $77.8 \pm 6.5\%$.

DISCUSSION

These experiments have demonstrated that both endothelium and stromal cells cultured from human cardiac valves are capable of expressing TF, thus potentially contributing to vegetation formation in infective endocarditis. However, the conditions under which TF was expressed by each differed.

Endothelium is now recognized to play a potentially active role in thrombosis (28, 35). Although normally maintaining a nonthrombogenic surface at the vascular lumen, it may

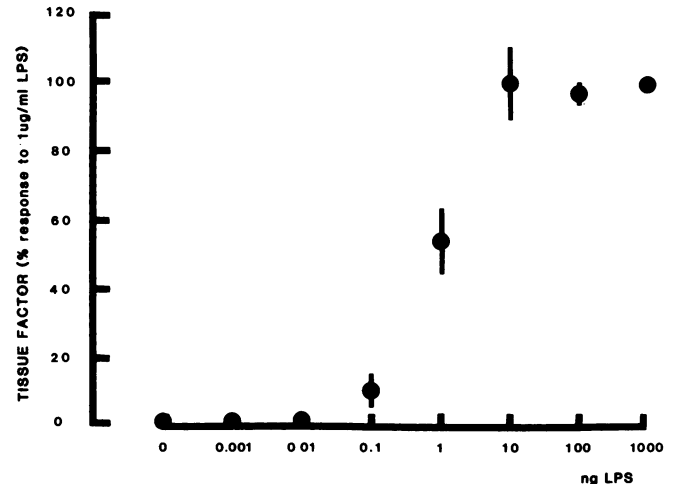


FIG. 3. Dose dependence of PCA response in cultured HCVE exposed to LPS for 6 h.

initiate coagulation by expression of TF following stimulation, as demonstrated here for cardiac valve endothelium. In addition, other coagulant-related properties such as thrombomodulin expression and fibrinolytic activity have been shown to be modulated in concert in a manner which promotes thrombosis (4, 23, 24). The kinetics and magnitude of the TF response in HCVE to IL-1 and LPS stimulation was similar to endothelium derived from umbilical vein, suggesting that other coagulant properties in HCVE may be similarly influenced. The very low levels of PCA detectable in unstimulated endothelium are assumed to be negligible, although it must be acknowledged that there is no information available concerning the amount of TF that is pathogenetically relevant.

Additional physiologic stimuli shown to induce TF expression in human umbilical vein endothelium include tumor necrosis factor, thrombin, and immune complexes (3, 15, 38). Although previous studies from this laboratory have demonstrated modest TF expression by cultured cardiac valve endothelium infected with *S. aureus*, exposure of HCVE to viable enterococci, viridans group streptococci, or a concentrated enterococcal cell wall preparation did not induce TF expression (10). Thus, although cardiac valve endothelium may contribute to local PCA in endocarditis if induced to express TF, direct induction by bacterial elements does not appear to occur, except with *S. aureus*. Infection could lead to TF expression by valve endothelium by indirect generation of active mediators such as IL-1, thrombin, or immune complexes. Monocytes, which can also express TF, are a potent source of IL-1 and may be present in vegetations (9, 40). Thrombin is obviously generated locally, and immune complexes are detectable in the circulation of most patients with infective endocarditis (1).

Cultured human cardiac valve stromal cells constitutively expressed high levels of TF. These cells have been shown to have characteristics of smooth-muscle cells rather than of fibroblasts, which they morphologically resemble by light microscopy (14). Both of these cell types in culture have been shown to express relatively high levels of TF constitutively (22, 29). Valve stromal cells are normally embedded in the internal valve stromal matrix, being separated from the blood by overlying endothelium. However, these cells may become exposed to the vascular lumen in the course of progressive valve damage following bacterial colonization of

TABLE 1. PCA of HCVE exposed to rIL-1 α for 6 h

Valve no.	PCA (mU/10 ⁵ cells \pm SD) after given rIL-1 α ^a					
	20 U/ml	5 U/ml	1.25 U/ml	0.31 U/ml	0.08 U/ml	Control
20	5,950 \pm 1,230	3,680 \pm 1,622	1,066 \pm 489	176 \pm 109	66 \pm 28	40 \pm 15
31	4,843 \pm 1,457	3,423 \pm 1,045	1,426 \pm 486	274 \pm 205	72 \pm 22	45 \pm 9

^a n = 4; determinations were made in duplicate.

the valve surface. This process alone could thus cause expression of significant PCA by infected valves if the phenotype observed in culture is representative of that in vivo.

Nonbacterial thrombotic endocarditis lesions (sterile thrombi on cardiac valves) have been shown to play a critical role in the pathogenesis of most cases of infective endocarditis by serving as the site to which circulating microorganisms adhere, thus initiating the infection (31, 37). The pathogenesis of these lesions, which occur in a wide range of clinical settings, including intracardiac catheter placement, primary cardiac valve disorders, malignancy, infection, disseminated intravascular coagulation (DIC), and connective-tissue disorders, is not well understood (5, 30). These studies suggest two potential common mechanisms for local coagulation activation which may be active among these diverse conditions. By analogy with the mechanisms discussed above in infective endocarditis, in cases of nonbacterial thrombotic endocarditis, TF-initiated thrombosis could occur either by valve injury leading to stromal cell exposure or by mediator-induced TF expression in endothelial cells.

The frequency of valve involvement in both nonbacterial thrombotic endocarditis and infective endocarditis is nonuniform among the four cardiac valves (5, 32). Except in particular settings, the mitral and aortic valves are far more commonly affected than the tricuspid or pulmonic valves. Since endothelium derived from different vascular sites has been shown to vary in expression of some cell properties, we hypothesized that either qualitative or quantitative differences in TF expression by valve cells might account for the differences in valve involvement observed clinically (17, 34). However, relative levels of TF expressed by stimulated HCVE and stromal cells were comparable among cells derived from different valves. Although phenotypic changes in cells may occur during passage, thus limiting the reliance one places on such results, these findings indirectly support the contention, long espoused, that hemodynamic factors play a central role in the localization of endocarditis lesions (41).

These experiments document the potential ability of both constituent cells of human cardiac valve to express TF. HCVE and stromal cells may thus each play an important role in vegetation formation in infective endocarditis and nonbacterial thrombotic endocarditis. Although in vivo studies are needed to confirm investigations with cultured cells, the availability of cardiac valve cells in culture will further our ability to dissect the complex and dynamic aspects of the pathogenesis of endocarditis.

TABLE 2. rIL-1 α -induced PCA of HCVE from different valves

rIL-1 α (U/ml)	PCA (mU/10 ⁵ cells)			
	Aortic	Mitral	Pulmonic	Tricuspid
Control	76 \pm 66	56 \pm 20	60 \pm 32	60 \pm 27
0.31	268 \pm 217	299 \pm 99	325 \pm 86	250 \pm 158
20	5,175 \pm 801	6,195 \pm 2,069	5,620 \pm 630	4,080 \pm 856

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