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Stimulation of Monocyte Tissue Factor Expression in an In Vitro Model of Bacterial Endocarditis

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The coagulation system plays a major role in the formation of the infected endocardial vegetation in bacterial endocarditis. Since monocytes can express tissue factor (TF) on their surfaces, they are thought to be responsible for the extrinsic activation of the coagulation cascade during this disease. The present study used an in vitro model in which fibrin plates, isolated adherent monocytes, and *Streptococcus sanguis* were used as an analog for endocardial vegetations. Adherence to fibrin by itself was found to stimulate TF expression on the monocytes, but stimulation by *S. sanguis* significantly increased TF expression, which was found to be maximal at a bacterium-to-monocyte ratio of 9 or more.

In bacterial endocarditis, the coagulation system plays a central role in the formation of a fibrin-platelet matrix, the so-called endocardial vegetation. Infecting bacteria are easily covered by fibrin and thus become inaccessible to the host defense systems (12). After partial fibrinolytic breakdown of the vegetation, the bacteria can reenter the bloodstream and cause a continuous bacteremia (4). For the formation of an endocardial vegetation, the coagulation system has to be activated. Earlier studies have shown that the extrinsic coagulation pathway is involved (6). Factor VII (FVII) has to bind to cell-associated tissue factor (TF) to efficiently initiate coagulation (10). Three cell types—endothelial cells, fibroblasts, and monocytes—are candidates for the cellular origin of endocardial TF activity because all these cells can express TF on their surfaces. Since endothelial cells are not present on a carefully removed endocardial vegetation (5) and since in the early stages of the disease fibroblasts are not present in a vegetation, monocytes are the most probable source of TF.

Since events on the vegetational surface can not be studied directly, we developed an in vitro model of bacterial endocarditis to study the activation of the coagulation system by monocytes. In this model, fibrin plates are used as a matrix for adherence of monocytes and bacteria (13). TF expression was determined in an amidolytic assay using purified FVII and FX and a chromogenic substrate for FXa. The amount of FXa measured in this assay reflects the amount of TF expressed on the surface of the monocytes (2). *Streptococcus sanguis* (strain NCTC7864), already extensively used in our previous studies (6, 9, 14), was used for stimulation. Peripheral blood mononuclear cells were isolated as described earlier (6). A 10-ml portion of the cell suspension was injected into Teflon culture bags (15) and cultured overnight at 37°C, with 5% CO_2 and a relative humidity of 95%. The next day, the cells were recovered and used in the experiments. In a 12-well tissue culture plate (Costar, Cambridge, England), 500 μl of soluble fibrinogen (5 mg/ml) dissolved in buffer D (50 mM triethanolamine [Fluka BioChemika, Buchs, Switzerland], 100 mM NaCl [Merck, Darmstadt, Germany] [pH 7.45]) was mixed with 100 μl of 100 mM $CaCl_2$ (Merck) before 10 μl of 0.5-U/ml thrombin (Central Laboratory of the Blood Transfusion Service, Amsterdam, The Netherlands) was added to induce fibrin formation. The mixture was allowed to polymerize overnight at 4°C. The next day, the plates were used in experiments. A 1-ml portion of the overnight culture of *S. sanguis* was layered on fibrin plates and incubated for 1 h at 37°C and 5% CO_2. Control plates were incubated only with RPMI 1640. After 1 h, the plates were washed with phosphate-buffered saline (PBS). To determine the amount of adherent bacteria, the plates were homogenized in 0.5 ml of PBS in a Potter Elvyhem homogenizer. Numbers of bacteria were determined in 0.1-ml samples of serial dilutions which were plated on blood agar plates after overnight incubation at 37°C. The experimental procedure for determining TF activity on the surfaces of adherent monocytes was adapted from that of Bom et al. (3). Approximately 1.5×10^6 monocytes in 2.5 ml of RPMI 1640 tissue culture medium were layered on fibrin plates and incubated at 37°C and 5% of CO_2 for adherence and stimulation. After the stimulation, the TF activity was measured as follows. The plates, with adherent cells in the presence or absence of adherent bacteria, were washed with buffer A (10 mM HEPES [*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid] [Sigma], 137 mM NaCl, 4mM KCl [Merck], 11 mM α-D-glucose [BDH Chemicals Ltd., Poole, England], 0.5 mM EDTA [pH 7.45]). Next, 600 μl of buffer B (10 mM HEPES, 137 mM NaCl, 4 mM KCl, 11 mM α-D-glucose, 5 mg of bovine serum albumin [BSA] [Sigma] per ml, 25 mM $CaCl_2$ [pH 7.45]) and 75 μl of 10.5 nM FVII (final concentration = 1.05 nM) in buffer B were added and incubated for 15 min at 37°C. A 75-μl sample of 1 μM FX (final concentration = 0.1 μM) in buffer B was then added, and 30-μl subsamples were taken at various times and mixed with buffer C (125 mM triethanolamine, 250 mM NaCl, 50 mM EDTA, 25 μM BSA [pH 8.2]) at 0°C to stop further activation of FX. To this mixture 200 μl of buffer D was added, and the mixture was then warmed to 37°C in a water bath. After 3 min, 50 μl of the chromogenic substrate S 2337 (2 mM) (Kabivitrum, Stockholm, Sweden) was added and incubated for 20 min. Finally 200 μl of 50% acetic acid (Merck) was added to stop the enzymatic conversion. The A_{405} was measured and converted to FXa concentration by using a calibration curve made with 0 to 10 nM purified FXa. Results are expressed as the nanomolar concentration of FXa per 10^6 monocytes. Under the experimental conditions used (excess FVII), the rate of FXa generation is a reliable measure for the amount of TF expressed on

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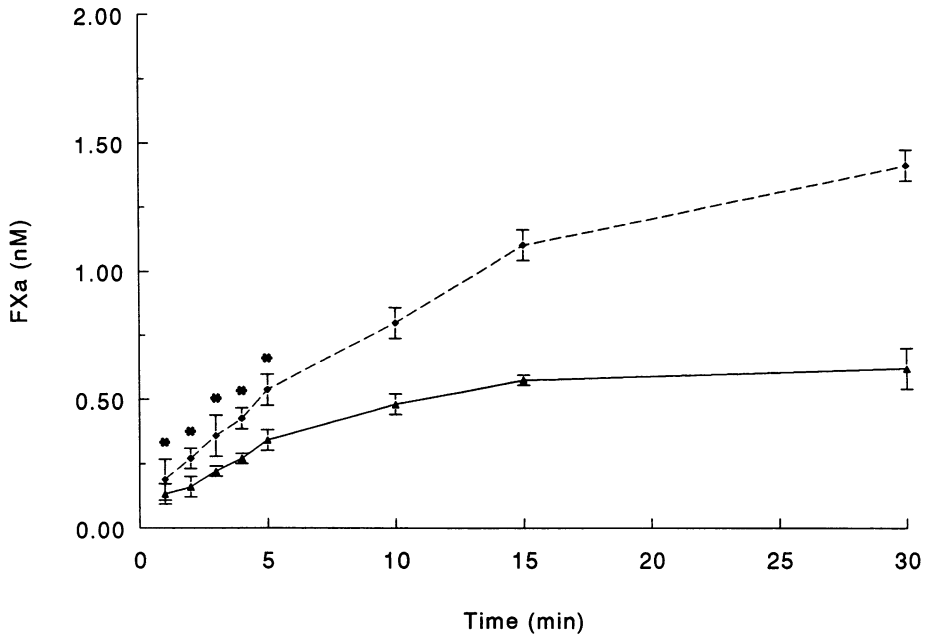

FIG. 1. Kinetics of TF expression of 10^6 monocytes, adhered to a fibrin plate in the absence (▲) or presence (◆) of 10^7 CFU of *S. sanguis*. Results are means ± standard deviations for three experiments performed in duplicate. Cells were stimulated for 4 h. Significant increases in TF expression due to streptococcal stimulation are indicated (*, $P < 0.001$).

the surface of the monocytes. In the reagents used, no lipopolysaccharide was detectable with a *Limulus* lysate gelation test (Haemachem, St. Louis, Mo.). For determination of significance of differences between control monocytes and stimulated cells, the paired Student *t* test and the Wilcoxon rank sum test for matched pairs were used. The significance level (α) was 5%.

After overnight culture in Teflon bags, 2.5 ml of the cell suspension containing 1.5×10^6 monocytes was incubated on the fibrin plates for 4 h at 37°C and 5% CO_2 to allow the monocytes to adhere. After two washes in buffer A, fixation with methanol, and staining with Giemsa (Merck), the adherent cells were counted. In these experiments, the average number of adherent cells was 8.7×10^5 (standard deviation = 0.6×10^5; $n = 3$). Adherence of monocytes in the presence of *S. sanguis* was similar (8.8×10^5; standard deviation = 0.4×10^5; $n = 3$). The streptococcal adherence to fibrin plates was determined as described above. The attachment rate, calculated as the ratio of the number of attached bacteria to the number of bacteria in the inoculum was found to be 4.5% for inocula ranging from 10^6 to 10^9 CFU. *S. sanguis* was then used to stimulate the monocytes. The bacteria were layered on the fibrin plates 60 min before the addition of the monocytes. Adherence of monocytes to the fibrin resulted in stimulation of TF expression. However, in the presence of streptococci the stimulation of the monocytes was significantly higher (Fig. 1). To check whether activation of FX indeed occurred through the TF pathway, we determined whether it was FVII dependent. No activation of FX occurred in the absence of FVII, indicating that the FXa generation is strictly dependent on the presence of FVII. After 5 min FXa accumulation started to decline in the presence of fibrin-stimulated or *S. sanguis*-stimulated monocytes. This could be caused either by a decrease in FXa generation due to a shortage of FVII or FX, by a decrease in levels of TF, or by disappearance of FXa through binding to FXa inhibitors. Addition of FVII or FX after 15 min of FXa generation did not result in an increase in FXa accumulation. Thus, concentrations of FVII and FX are not rate-limiting factors. When FXa was incubated on fibrin plates, there was no decrease in FXa regardless of the presence or absence of adhered *S. sanguis*. However, the FXa concentration decreased in a time-dependent, linear manner during incubation with adhered, fibrin-stimulated monocytes as well as with adhered *S. sanguis*-stimulated monocytes. This suggests that monocytes are involved in the decline in FXa accumulation. Activation of monocytes seems to be required for this mechanism, since nonadherent, nonstimulated monocytes suspended in Teflon bags were found to be unable to bind FXa, whereas adherent activated monocytes on fibrin plates did bind FXa. Production of an FXa inhibitor by monocytes is unlikely to account for this decline, since the TF pathway inhibitor, which is a well-known FXa and TF/FVIIa inhibitor produced by a variety of cells (including the monocyte-like cell line U937 [11]), could not be detected in culture supernatants of stimulated adherent monocytes (1). In a previous study, we found that a bacterium-to-monocyte ratio of 10 was the optimal ratio for activation of the coagulation cascade by monocytes in suspension (10, 16). In these experiments, *S. sanguis* as well as *Staphylococcus epidermidis* was added to whole blood, in which the procoagulant activity (PCA) of the monocytes was subsequently measured. To determine whether there was also such an optimal ratio for isolated, fibrin-adherent monocytes, cells were incubated in fibrin plates in various ratios to the bacteria. As shown in Fig. 2, with a bacterium-to-monocyte ratio of 9 to 1, maximum stimulation of the monocytes was attained. Thus, in this in vitro stimulation there is a maximum but no optimum for stimulation.

In this study, we investigated the activation of the coagulation system in an in vitro model of bacterial endocarditis. Although others doubted that monocytes played a role in initiation and perpetuation of TF activity during the onset and the course of endocarditis (7), we had several indications that

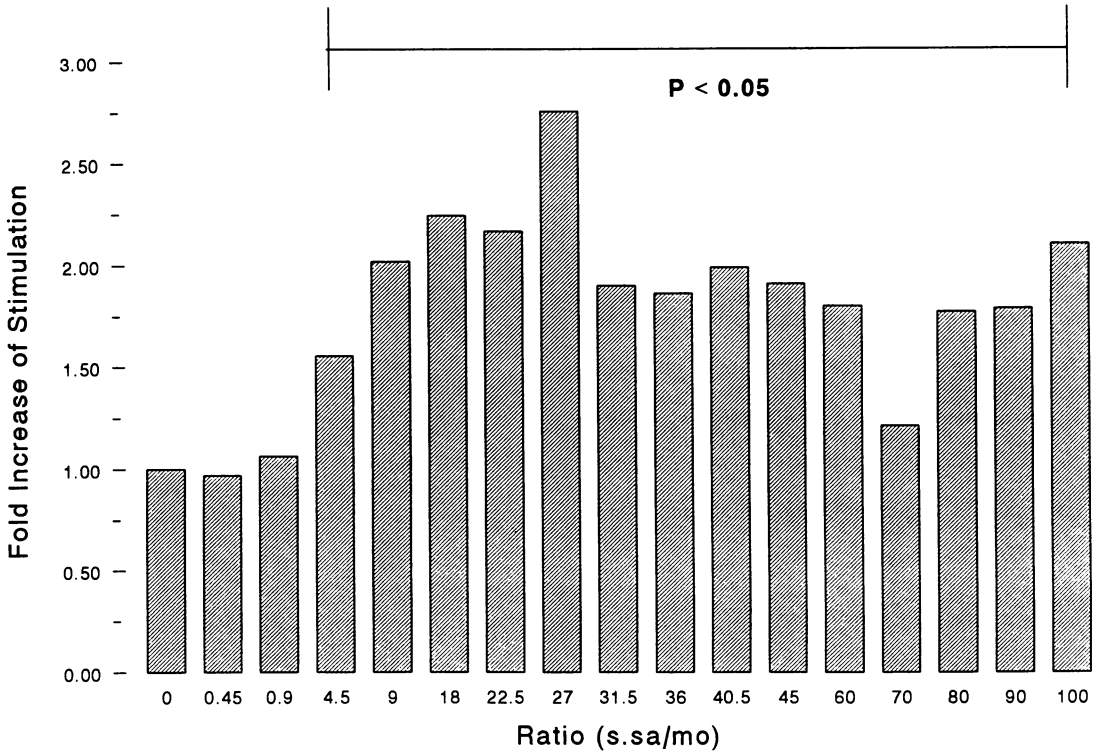

FIG. 2. Influence of bacterium-to-monocyte ratio (s.sa/mo) on stimulation of TF activity, expressed as fold increase of TF expression compared with that of non-streptococcus-stimulated fibrin-adhered monocytes. The figure shows results of one representative experiment. Cells were stimulated for 4 h. Significant increases in TF expression over that of fibrin-adhered monocytes are indicated.

these cells could have such a role (6, 14). Therefore, we used isolated monocytes as a TF source in our model. In an earlier study, we had shown that monocytes in suspension which were cultured overnight in Teflon bags do not generate PCA (6). This is important, since the present study shows that adherence of monocytes to fibrin can induce TF expression on the surfaces of these cells, which is the first step in the activation of the coagulation pathway. This is in agreement with the findings of van Ginkel et al. (17) that adherence of monocytes to a surface such as glass or cuprophane also stimulated PCA in these cells. Thus, since monocytes in suspension do not express TF, it can be concluded that adherence of monocytes to fibrin in itself is a sufficient stimulus to induce TF expression. However, stimulation of these adherent monocytes to *S. sanguis* leads to a significantly higher level of expression of TF. Experiments were performed in the absence of FVII to study the possibility that FVII-independent factors, such as the complement receptor 3 (or Mac-1 [8]) or the intrinsic coagulation pathway, contributed to the FX activation. These experiments showed that neither fibrin-stimulated monocytes nor *S. sanguis*-stimulated monocytes can activate FX in the absence of FVII. It appeared that during the initial 5 min, FXa accumulation was linear. During this initial period, the rate of FXa generation is a reliable measure for TF, expressed on the surfaces of the monocytes.

From the results of this study in an in vitro model of endocarditis we conclude that under our experimental conditions, generation of FXa is a reliable measure of the PCA of monocytes. Results indicate that mere attachment of the monocytes to fibrin already is a sufficient stimulus to induce TF expression on the cell surface. Moreover, TF expression increased significantly as a result of stimulation by streptococci with an increasing bacterium-to-cell ratio, reaching a maximum at a ratio of 9 to 1. The results of the present study indicate that monocytes indeed can play a role in the activation of the clotting cascade in bacterial endocarditis. It remains possible that at the onset of the endocardial infection, there are other sources of cell-bound PCA, such as valvular endothelial cells. However, since these cells are not present on the vegetational surface, they cannot contribute to the activation of the clotting system leading to the continuous fibrin formation which in turn results in the growth of the vegetations during the course of the disease.

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