Embolsitzation itself stimulates thrombus propagation in pulmonary embolism

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MOST PATIENTS with pulmonary emboli (PE) survive their illness when treated with anticoagulants at doses similar to those used to treat deep venous thrombi (DVT) (3). However, a subpopulation of patients with massive PE have a high mortality when treated with “standard doses” of anticoagulants, even when combined with thrombolytic medications (10). It is generally agreed that those who succumb from PE usually do so because of right ventricular dysfunction and ischemia due to elevated pulmonary artery resistance (17, 24). However, eventual right ventricular collapse is difficult to predict (5, 7), and the pathophysiological mechanisms leading to eventual right ventricular deterioration are not entirely clear. Other factors in addition to pure mechanical obstruction are likely to contribute to the increasing load on the right ventricle because the pulmonary vascular resistance measured in otherwise healthy patients with pulmonary embolism is higher than one would predict from the degree of pulmonary artery occlusion alone (15). For example, ongoing thrombosis on the surface of the emboli may increase pulmonary vascular resistance through mechanisms such as ongoing platelet accumulation and degranulation, as well as microembolization into small pulmonary arteries. The experiments in this report test the hypothesis that embolization itself causes preexisting thrombi to reactivate, possibly leading to clot enlargement, release of clot associated inflammatory mediators, and downstream microembolization. The results may have implications for the intensity of anticoagulant treatment necessary for treating hemodynamically significant pulmonary embolism.

An additional goal of these experiments is to validate a practical method for measuring thrombotic activity in an in vivo model of DVT and PE. Clinicians who treat patients with PE do not yet have a practical method of determining the degree to which anticoagulation has arrested the process of clotting. Without this information, the types and doses of anticoagulants recommended for treating PE mirror those that have been proven to be effective for DVT or clinically stable PE in large randomized trials. However, the measurements of “efficacy” used in these clinical trials, such as recurrence of the disease weeks or months after treatment, may not be directly applicable to the critical condition of patients with massive, hemodynamically significant emboli. In those patients, more intensive anticoagulant regimens may be necessary due to the increased clot burden, high levels of anticoagulant “sinks,” such as platelet factor 4 (25) and, perhaps most importantly, the precarious function of the right ventricle when already under strain (6).

These experiments employed a previously described immunoassay for fibrinopeptide B (FPB), a 14-amino acid peptide only released from the β-chain of fibrinogen during active thrombosis (18) and its primary metabolite, des-arginine FPB. Because of the specificity of the immunoassay for human FPB, all experiments were performed in dogs in which canine fibrinogen had been completely inactivated and replaced with purified human fibrinogen. Some of the results of these studies have been previously reported in abstract form (20).

EXPERIMENTAL PROCEDURES

Thromboembolism model using human fibrinogen. The University of California, San Diego Animal Subjects Committee approved this protocol. Care and handling of experimental animals conformed to the standards established by the University of California, San Diego Department of Veterinary Services, which comply with Federal recommendations and the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Thirteen healthy male mongrel dogs (20–24 kg) were used in the experiments. Each dog was anesthetized with intravenous propofol (6–10 mg/kg), intubated, and mechanically ventilated with the use of halothane (1–2%) for anesthesia. The dog’s functional fibrinogen was depleted by treatment with anncrd (2–3 U/kg dissolved in 40 ml of sterile 0.9% NaCl solution; Sigma; St. Louis, MO), infused intravenously over 4 h, which effectively inactivates autologous fibrinogen in the circulation (1). Blood samples (5 ml) were collected before and after anncrd treatment for

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determination of functional fibrinogen levels, and the animals were then allowed to recover. After 2 days, during which ancrod was cleared from the circulation but functional fibrinogen levels remained below the limits of detection, the animals were anesthetized, intubated, and mechanically ventilated as before. Fibrinogen levels were restored by transfusion with purified human fibrinogen (6.0 g dissolved in 250 ml of sterile 0.9% NaCl solution; Calbiochem, San Diego, CA), infused intravenously over 90 min. Tranexamic acid (Sigma) was administered intravenously (110 mg/kg dissolved in 40 ml of sterile 0.9% NaCl solution) every 6 h to completely inhibit fibrinolysis (13).

Bilateral femoral vein thrombi were then induced by injecting bovine thrombin (200 U) through a catheter between two inflated intravascular balloons as previously described (19). During a 4-h clot-aging period, the balloons were partially deflated to allow for restoration of blood flow and clot propagation.

The animals were then randomly assigned to one of two groups. In the first group (n = 9), the animals received no heparin during the experiment, to determine the magnitude and consistency of FPB release during both thrombosis and embolization. The second group (n = 4) received a bolus of intravenous heparin (300 U/kg) 1 h before embolization, followed by a continuous heparin infusion (90 U/kg min<sup>-1</sup>) throughout the remainder of the experiment. The purpose of the smaller group was to determine whether the pattern of FPB release observed during embolization was due to ongoing thrombosis or merely to the diffusion of preformed FPB from within the clot as it is exposed to the pulmonary circulation. Embolization was achieved by passive motion of one of the thrombus-containing legs and removal of the double-balloon catheter from that side. The animals were followed for an additional 5 h after embolization, during which time they remained mechanically ventilated and anesthetized. Blood samples (5 ml) were collected at various times before and after thrombosis/embolization for functional fibrinogen and FPB assay.

At the conclusion of the experiment, each animal was given a bolus intravenous injection of heparin (3,000 U) to prevent postmortem blood coagulation, followed by pentobarbital sodium (120 mg/kg iv) to induce cardiac arrest. All femoral vein thrombi and pulmonary emboli were collected on postmortem examination and weighed.

Fibrinogen and FPB assay. Functional fibrinogen levels in citrated plasma were determined using a commercially available kit (Sigma). Plasma FPB levels were determined by competitive ELISA as previously described (18). The specificity of the antiserum used in the assay for various fibrinopeptides of human and canine origin was also evaluated by competitive ELISA.

Statistics. We used limited dependent variables regression analysis (11, 14) to assess group differences in average plasma FPB levels. Limited dependent variables regression was used to account for (left) censoring of values below the 3 ng/ml limit of detection of the FPB assay; results were confirmed using a standard (log) linear model (14).

Subject-specific intercept terms were included to adjust for between-dog differences in baseline FPB level, and permutation testing was used to account for serial correlation in the (within dog) FPB time series. A P value <0.05 was accepted as demonstrating a significant difference. All other data are presented as means ± SE, unless otherwise noted.

RESULTS

Specificity of FPB assay. The specificity of the antiserum used in the FPB assay for various fibrinopeptides of human and canine origin was assessed by competitive ELISA. As shown in Fig. 1A, native human FPB was an effective inhibitor of antibody binding to surface-bound synthetic human FPB. In contrast, native canine FPB did not compete for antibody binding to surface-bound synthetic human FPB. Thus the assay is specific for human FPB. As shown in Fig. 1B, the competition curves for human FPB and des-arginine FPB were nearly identical. The cross-reactivity of des-arginine FPB, defined as IC<sub>50</sub> of FPB (6.7 nM) divided by IC<sub>50</sub> of des-arginine FPB (8.9 nM), was 75%. Thus the FPB assay measures both FPB and its primary metabolite, des-arginine FPB. Although fibrinopeptide A, a small peptide released from the α-chain of fibrinogen during thrombosis, exhibited essentially no cross-reactivity (<0.1%), fibrinogen showed significant cross-reaction. The IC<sub>50</sub> of fibrinogen (2.3 nM) was about one-half the IC<sub>50</sub> of FPB, which was not unexpected because each fibrinogen molecule harbors two potentially cross-reacting FPB sequences. Centrifugal ultrafiltration with Biomax-100 (100,000 Da cutoff) membranes effectively removed cross-reacting fibrinogen and fibrin monomer (both ~340,000 Da) from samples before FPB assay (data not shown).

Depletion of canine fibrinogen. In each animal, functional canine fibrinogen was depleted from the circulation to <20 mg/dl after ancrod treatment (Table 1). During the 2-day rest period in between ancrod treatment and human fibrinogen...
transfusion, ancrod itself was cleared from the circulation (data not shown) and functional fibrinogen levels remained below the limit of detection. After transfusion with purified human fibrinogen, the plasma fibrinogen level was restored to ~400 mg/dl and remained within normal limits (200–400 mg/dl) throughout the remainder of the study.

**Clot characteristics.** One (unilateral) femoral DVT and at least one PE were recovered from each animal postmortem. No residual thrombi were found within the femoral veins of the leg that had undergone the embolization procedure. PEs were sometimes lodged in multiple lobar and/or segmental pulmonary arteries. The nonheparinized group had a (statistically insignificant) trend toward larger clots than the heparinized group. The DVT masses were 0.89 ± 0.26 and 0.64 ± 0.18 g, respectively (P = 0.44). The PE masses were 0.51 ± 0.06 and 0.47 ± 0.09 g, respectively (P = 0.72).

**Plasma FPB levels during thromboembolism.** FPB levels in plasma were measured during the control period (before thrombin administration), during induction and propagation of femoral vein thrombosis, and after embolization (Fig. 2). The baseline FPB levels (just before thrombin administration) for the nonheparinized and heparinized groups were 4.7 ± 1.1 and 9.2 ± 2.7 ng/ml, respectively. Subject-specific baseline FPB levels did not differ significantly between the two groups (P = 0.330). Plasma FPB levels were significantly elevated over baseline for both groups in the hour after thrombosis (18.3 ± 2.0 ng/ml, nonheparinized group; 19.1 ± 3.4 ng/ml, heparinized group; P < 0.001), but there was no significant difference in FPB levels between the two groups over this time period (P = 0.138). The FPB levels gradually decreased in both groups and were 8.8 ± 2.5 and 7.7 ± 2.9 ng/ml in the nonheparinized and heparinized groups, respectively, by the third hour after thrombus induction, before any of the subjects receiving heparin (Fig. 2).

The two groups did differ significantly, however, in the hour following embolization, with FPB levels in the heparinized group significantly suppressed relative to those in the nonheparinized group (7.8 ± 1.8 vs. 15.1 ± 1.6 ng/ml, respectively; P = 0.038).

**DISCUSSION**

These experiments demonstrate that embolization of deep vein thrombi intensifies the thrombotic process. In fact, embolization caused rapid increases in plasma FPB levels that were comparable to those associated with the formation of the thrombi themselves, despite the fact that the thrombi had been nearly instantly formed by high-dose thrombin. The fact that premedication with large doses of heparin completely blocked the FPB increase after embolization confirms that the phenomenon is due to thrombosis, and not to diffusion of FPB from the previously unexposed surfaces of the emboli. Furthermore, the embolized clots demonstrated further release of FPB during the hours subsequent to embolization. This phenomenon could also be blocked by large doses of heparin, confirming the role of thrombosis.

The group that did not receive heparin displayed elevations of FPB during the later half of the experiment but did not have thrombi or emboli of greater masses than those found in the other groups. Visual inspection of the lung tissue down to the third subsegmental division did not disclose evidence for fragmentation of the emboli into smaller clots, although microscopic thrombosis may have occurred undetected. This finding suggests that the products of coagulation did not adhere to the emboli in the pulmonary arteries but were washed downstream through the pulmonary microvasculature, perhaps contributing to the pathophysiological effects of the emboli.

Some variability in response was observed within the groups. Most notably, one animal manifested a significant (albeit dampened) postembolization spike in plasma FPB levels despite pretreatment with heparin. One possible explanation is that, in this animal, the plasma FPB spike was due to nonthrombotic processes, such as diffusion of preformed FPB within the thrombus. The other is that, in this animal, even large doses of heparin were insufficient to completely extinguish postembolization reactivation of thrombosis. It is apparent that this animal was an exception; yet even with this subject included in the analysis, the postembolization FPB level of the heparinized group was still significantly decreased compared with the nonheparinized group.

These experiments are limited by the fact that only relatively fresh thrombi were embolized whereas clinically apparent pulmonary emboli are likely to come from deep vein thrombi that are days older. Longer periods of clot incubation (i.e., days) were not practical in this model, due to the specificity of the assay for human FPB and problems of maintaining a

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**Table 1. Functional fibrinogen plasma levels**

<table>
<thead>
<tr>
<th>Time since Ancrod, h</th>
<th>Event</th>
<th>No heparin group</th>
<th>Heparin group</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>Before ancrod</td>
<td>139±12</td>
<td>135±17</td>
</tr>
<tr>
<td>4</td>
<td>After ancrod</td>
<td>&lt;20 in all subjects</td>
<td>&lt;20 in all subjects</td>
</tr>
<tr>
<td>48</td>
<td>Before fibrinogen transfusion</td>
<td>&lt;20 in all subjects</td>
<td>&lt;20 in all subjects</td>
</tr>
<tr>
<td>50</td>
<td>After fibrinogen transfusion</td>
<td>371±20</td>
<td>371±22</td>
</tr>
<tr>
<td>54</td>
<td>Before clot formation</td>
<td>338±11</td>
<td>357±23</td>
</tr>
<tr>
<td>58</td>
<td>Before embolization</td>
<td>261±30</td>
<td>271±15</td>
</tr>
</tbody>
</table>

Values are means ± SE.
constant level of human fibrinogen in the dogs for that amount of time. However, we feel that the study does offer insights into the clinical response of thrombi to embolization. The response of clinical thrombi to anticoagulants (8) suggests that they, like the thrombi in the current experiments, are actively propagating, at least at a low level. The phenomenon of pulmonary embolization increasing the amount of clot propagation on preexisting thrombi, and the affect of anticoagulation in blocking this phenomenon, are therefore likely to occur in clinical situations as well.

The current experiments demonstrate that embolization stimulates thrombus propagation in pulmonary embolism. However, the emboli created from femoral vein thrombi during the experiments were not large enough to lead to right ventricular pressure or cardiac output changes in the canine model. While hemodynamically significant emboli can be generated from repeated embolization of inferior vena cava thrombi (21a), we chose not to do so for these experiments because the high mortality rate and variability in clot burden over time would have made it problematic to establish a clear association between embolization and thrombus propagation. For this reason, the hemodynamic effects of embolism-associated thrombus propagation, and the benefits of pharmacologically suppressing it, must be determined by further research.

The humoral effects of pulmonary embolism on pulmonary vascular resistance and right heart function have been reviewed comprehensively elsewhere (12, 22). Pulmonary embolism induces the release of putative mediators of pulmonary artery vasoconstriction from many sources, including platelets, neutrophils, endothelial cells, and autonomic afferent nerve endings (12). Recent attention has been focused on the effects of thromboxane (22), serotonin (22), and endothelin (4, 22, 23) on pulmonary artery vasoconstriction. Although the specific role of these mediators is still under investigation, they all may be released in response to stimulation by thrombin or other by-products of active thrombosis (12). It follows that progressive pulmonary artery vasoconstriction would occur in the presence of intensified thrombosis during pulmonary embolization.

An alternative hypothesis is that clinical deterioration is mediated by factors generated during formation of the thrombus before embolization and that anticoagulation will have little direct effect once the thrombus has embolized. For example, release from the embolus of vasoactive substances (such as serotonin) discharged by previously trapped platelets could mediate pulmonary vasoconstriction and right ventricular failure. However, platelet degranulation begins immediately on activation by the thrombus, and plasma serotonin levels generally peak by 1 h (2). By contrast, deterioration after pulmonary embolism is often delayed for hours (6) or even days (16) after the initial event, supporting the argument that active thrombosis induces ongoing mediator production and release.

Another possibility is that it is the initial embolic event, causing an acute rise in pulmonary vascular resistance that results in progressive right ventricular failure, even in the absence of subsequent thrombosis or vasoconstriction. Clinical experiments to correlate FPB measurements to pulmonary embolism outcome are currently being designed in our laboratory, which should help determine which of these explanations are more likely to be true in humans.

In the current experiments, embolism-induced thrombosis propagation was suppressed with larger doses of heparin than are commonly used for the treatment of DVT or PE (9). The heparin dose corresponded to a dose that had previously been demonstrated to virtually eliminate ongoing thrombosis in previous models of DVT and PE (21). It is unknown whether lower doses of heparin or other anticoagulants, such as low molecular weight heparins, hirudin-like medications, and other glucose-amino-glycans are also capable of suppressing embolism-associated clot propagation.

The current experiments suggest that aggressive anticoagulation of some type may have a therapeutic role for hemodynamically significant PE that is distinct from the treatment of DVT. In the case of massive pulmonary embolism, the suppression of thrombotic activity may lessen the effect of the emboli on pulmonary vascular resistance and improve right heart function. However, it must be noted that our experiments utilized a complex animal model and the relevance of the results to human disease (and to clinical treatment in particular) remains speculative. Further basic and clinical research is needed to determine whether higher doses of anticoagulation are necessary during acute pulmonary embolism, especially in patients who may be hemodynamically unstable.

These experiments support measurements of FPB as a research tool to indicate ongoing thrombosis. In all of the animals studied, plasma FPB levels significantly rose during DVT formation and propagation. Furthermore, consecutive plasma FPB levels could distinguish between animals with untreated pulmonary emboli and those with completely inactivated emboli. As the FPB assay is further refined to enable a turnaround time appropriate for clinical use, it may in fact have a role in diagnosing and guiding the treatment of pulmonary embolism.

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REFERENCES


