Alteration of Platelet Function in Dogs Mediated by Interleukin-6

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To determine if interleukin-6 (IL-6) administration influences platelet function, platelet activation was analyzed sequentially in IL-6–treated (80 μg/kg/d) and control dogs. Platelet activation was determined in whole blood by flow cytometry by quantitating the binding of a monoclonal antibody to platelet surface P-selectin after stimulation with graded doses of thrombin. Administration of IL-6 resulted in a twofold decrease in the thrombin concentration required for induction of half-maximal P-selectin expression (ED50) compared with control animals. The ED50 returned to normal after cessation of IL-6 administration. As measured by P-selectin expression, enhanced responsiveness to the strong agonist platelet activating factor (PAF) was also observed in the IL-6–treated dogs. IL-6 had no effect on the susceptibility of platelets to thrombin activation when incubated with anticoagulated dog blood. The data show that, in addition to augmenting the platelet count in normal dogs, IL-6 enhances the sensitivity of platelets to activation in response to thrombin and PAF.

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IL-6. Recombinant human IL-6 was expressed in Escherichia coli and purified as previously described. Concentration and purity were assessed by the IL-6–responsive B9-cell bioassay and sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Endotoxin levels were measured using the limulus amebocyte lysate test at Endosafe Inc (Charleston, SC). The specific activity of the purified product was 2 to 5 × 10^8 U/mg protein and had an endotoxin level of less than 2 EU/mg.

Monoclonal antibodies (MoAbs). To detect canine platelets in whole blood, antibody 2F9, recognizing a component of the glycoprotein IIb/IIIa complex was used as described. To detect activated canine platelets, antibody G5 (subtype IgG; cell line provided by R. McEver, Oklahoma Medical Research Foundation, Oklahoma City, OK) raised against human P-selectin and cross-reacting with the canine counterpart, was used. 2F9 was directly fluoresceinated with fluorescein isothiocyanate (FITC; Calbiochem, La Jolla, CA) and G5 was biotinylated with biotin N-hydroxysuccinimide ester (Pierce, Rockford, IL) using standard techniques. Biotinylated control antibody (Tab) (an IgG, MoAb recognizing human, but not dog platelets) was used as an isotype-specific control to set gates for resting versus activated platelets.

Assessment of platelet activation in response to thrombin. Blood (4.5 mL) was withdrawn from a jugular vein into a syringe containing 0.5 mL acid-citrate-dextrose and 2 μmol/L (final concentration) prostaglandin E1 (Sigma Chemical Co, St Louis, MO). Blood was mixed with 46 mL buffered saline-glucose-citrate solution (BSGC) pH 6.59 in a 50-mL conical tube and centrifuged at 1,250g for 18 minutes at room temperature to remove the plasma (at this dilution, this centrifugation speed will pellet all the cells, including all the platelets). The supernatant was discarded and the sedimented cells gently resuspended to original volume in BSGC pH 7.3. Using an electronic particle counter, the supernatant was virtually free of platelets (300,000 platelets/μL before centrifugation: <1,000 platelets/μL after centrifugation). To determine if the processing induced clumping or otherwise altered the original whole blood size distribution of the platelets, flow cytometric sizing analysis was performed using pulse processing on a FACStar plus flow cytometer (Becton Dickinson, Mountain View, CA). No difference in the size distribution between whole blood and the sample processed as described was observed (data not shown).

The plasma-depleted blood (10 μL) was then added to each of seven 15-mL polypropylene tubes containing 952 μL of BSGC pH 7.3 supplemented with 5 mL 2F9-FITC (1 mg/mL); 3 μL of G5-biotin (1 mg/mL); and 40 μL of various concentrations of diluted bovine thrombin (Sigma) in BSGC pH 7.3. The tubes were incubated in a 37°C water bath for 10 minutes, followed by the addition of 1 mL of 0.6% formaldehyde (Fisher Scientific, Fairlawn, NJ) in phosphate-buffered saline. The tubes were then set at room temperature for 10 minutes, followed by the addition of 10 mL BSGC pH 7.3 supplemented with 0.1% bovine serum albumin (BSGC-BSA; Sigma), and centrifuged at 1,250g for 15 minutes at room temperature.
tured to remove the fixative. The pelleted cells were resuspended in 1 mL of BSCG pH 7.3, followed by the addition of 5 µL streptavidin conjugated to phycoerythrin (streptavidin-PE; Calbiochem). After incubation at room temperature for 30 minutes, non-bound streptavidin-PE was depleted by washing with BSCG-BSA twice as described above. After resuspension in 1 mL BSCG-BSA, the samples were analyzed by flow cytometry.

Assessment of platelet activation in response to PAF. Dog blood was drawn into 0.38% Na citrate. Five microliters of the whole blood was added to 995 µL of HEPES buffer (10 mmol/L HEPES, 145 mmol/L NaCl, 5 mmol/L KCl, 1 mmol/L MgSO4, pH 7.4) containing 2 µg of G5-biotin and varying concentrations of PAF (Sigma; 10^-7 to 10^-10 mol/L in ethanol). After incubation at 37°C for 10 minutes, the samples were fixed in formaldehyde and processed as described above for the thrombin experiments, except that the pelleted cells were resuspended in 0.5 mL of BSCG.

Flow cytometric analysis. The percentage of platelets activated at the various concentrations of thrombin was determined with a FACSScan flow cytometer (Becton Dickinson) with settings (all log scale) as follows: forward scatter (FSC), E00; side scatter, 332; fluorescence detector 1 (FL1), 520; fluorescence detector 2 (FL2), 520; FSC threshold, 176; compensation: FL1-%FL2, 0.8; FL2-%FL1, 22.5. Ten thousand events were collected for each specimen. The percentage of platelets (identified by 2F3-FTTC on FL1) that were activated (identified by streptavidin-PE bound to G5-biotin on FL2) was determined for each dose of thrombin. Activated platelets were distinguished from resting platelets by analysis of the tube having no added thrombin and by use of an isotypic control antibody. The dose-response curve was plotted and the concentration of thrombin inducing half-maximal activation (ED50) was calculated with an enzyme-linked immunosorbent assay program (Beckman Biomek; Fullerton, CA).

In vivo protocol. Samples for baseline determinations were drawn, followed by administration of IL-6 at 80 µg/kg/d in two divided subcutaneous doses for 10 days. Control animals received the concurrent controls before IL-6 administration; progressively decreased to 47.8% (±6.17%; range, 38.3% to 55%; P < .001) of the concurrent controls by day 8 to 9; and returned to baseline levels (93.8% ± 16.2%; range, 77.5% to 109.9%) after cessation of IL-6. The platelet counts of these dogs are shown in Table 1.

To determine if the changes in P-selectin expression were limited to changes in thrombin reactivity, the response of canine platelets to the strong agonist PAF was evaluated. Figure 4 shows that the response to PAF was also altered by IL-6 administration. Two IL-6-treated dogs were compared with one control animal. In these experiments, the maximal effect was observed on day 6, when the ED50 of the IL-6-treated animals was 1.96 × 10^-9 mol/L and 1.29 × 10^-9 mol/L, respectively, whereas the ED50 of the control animal was 3.52 × 10^-9 mol/L.

In vitro response of platelets to IL-6. To determine if exposure of canine blood to IL-6 in vitro altered the capacity of the platelets to activate in response to thrombin, blood was exposed to a large concentration of IL-6 (100 ng/mL) for 30 minutes. No alteration in the thrombin dose-response curve was observed (Fig 5). The maximum concentration of IL-6 achieved after administration of 80 µg/kg IL-6 was 1 ng/mL (data not shown), thus 100 ng/mL represents a 100-fold increase in IL-6 concentration compared with that observed in vivo.

DISCUSSION

Although platelet aggregation studies have been used to evaluate platelet function in response to cytokines, the disparate platelet counts and fibrinogen levels in IL-6–treated compared with control dogs requires normalization of these parameters to accurately interpret aggregation studies. In our hands, the physical manipulations (gel filtration and dilution with combinations of platelet-rich plasma [PRP] and platelet-poor plasma) required to normalize these levels altered the aggregation curves, compromising the interpretation of standard platelet-aggregation studies.

Translocation of the α-granule membrane protein P-selectin to the platelet surface has been shown to be a sensitive and precise marker of platelet activation, and use of flow cytometric detection of this protein permits measurements of platelet activation in whole blood. Thus, not only can the entire platelet population be evaluated, eliminating con-
cern over selection of platelet subpopulations as might occur with the preparation of PRP, but platelets can also be analyzed individually.

Several platelet agonists were tested initially with the requirements that the agonist produce a broad range dose-response curve so that small changes in sensitivity to agonist induced by IL-6 would be detectable, and the agonist dose-response curve be reproducible. Phorbol ester produced too steep a dose-response curve, whereas ionophore A23187 showed marked day-to-day variability. Thrombin receptor agonist peptide (Ser-Phe-Leu-Leu-Arg-Asn)

activated human, but not canine platelets (data not shown). Thus, thrombin itself was chosen. Although clotting caused by thrombin addition could be avoided by dilution and with the fibrin monomer polymerization inhibitor tetrapeptide Gly-Pro-Arg-Pro,

grossly disparate amounts of fibrinogen in IL-6–treated versus control dogs (fibrinogen levels are routinely 8 to 10 g/L in IL-6–treated dogs, compared with approximately 2 g/L in controls) might compete differentially for the added thrombin and confound the analysis. Thus, it was important to remove the fibrinogen without activating the platelets or losing subpopulations of platelets. Gel filtration of the whole blood was compromised by variable depletion of fibrinogen and the complexity of this procedure for multiple samples. Although it was initially thought that centrifugation of the whole blood would result in excessive platelet activation, this was not the case, with less than 8% of platelets exhibiting surface P-selectin expression after centrifugation.

The pattern of activation detected by flow cytometry is of interest. If each incremental concentration of thrombin merely augmented the number of molecules of P-selectin

Fig 1. Flow cytometric analysis of platelet activation in whole blood. Anticoagulated dog blood treated with various concentrations of thrombin, followed by labeling with FITC-2F9 and GS65-biotin/streptavidin-PE. The abscissa represents log fluorescence intensity of PE, whereas the ordinate represents numbers of platelets. Note the progressive increase in the number of platelets activated as the concentration of thrombin is increased. Bar 1, resting platelets, determined using platelets not exposed to thrombin; bar 2, activated platelets. (A) no thrombin; (B) 0.03 U/mL thrombin; (C) 0.06 U/mL thrombin; (D) 0.25 U/mL thrombin.

Fig 2. Dose-response characteristics of platelets exposed to thrombin in one pair of dogs 9 days after commencement of IL-6 (e—e) or IL-6 diluent buffer (O—O). The ordinate represents the percentage of activated platelets, whereas the abscissa represents the log concentration of thrombin (Units per milliliter). The dose-response curve is shifted to the left in the IL-6–treated animal. A representative experiment of five similar experiments.
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Fig 3. Sequential alterations in the activation capacity of platelets derived from IL-6-treated (n = 5) and control (n = 3) dogs. For each time period, the dose of thrombin inducing half-maximal activation was determined (ED$_{50}$), and calculated as a percentage (± 1 SD) of the ED$_{50}$ of the concurrent control (ordinate). Time after initiation of IL-6 administration is on the abscissa. IL-6 was administered from days 1 through 10. A significant difference was observed on days 5 through 6 (P < .05); 8 through 9 (P < .001); and 12 through 13 (P < .005). The absolute ED$_{50}$ of the control dogs at each time point were (in units of thrombin per milliliter): pre, 0.047 ± 0.008; days 2 through 3, 0.046 ± 0.014; days 5 through 6, 0.046 ± 0.002; days 8 through 9, 0.049 ± 0.009; days 12 through 13, 0.048 ± 0.021; days 15 through 16, 0.053 ± 0.023; days 19 through 20, 0.047 ± 0.010.

Exposed on the platelet surface, then one would expect to see a continuum of response across the abscissa of Fig 1. In contrast, the data show that although each incremental concentration of thrombin induces progressively more activated platelets, there are two clearly separable populations of activated and resting platelets (Fig 1, B and C). Thus, platelets appear to have a threshold for activation. This finding is in contrast to what has been observed with adenine diphosphate (ADP)-induced fibrinogen binding, where it appears that in addition to augmentation of the number of cells capable of binding fibrinogen, progressively more fibrinogen binding per platelet is observed with increasing concentrations of ADP.

Previous studies have suggested that cytokines are capable of altering platelet aggregation ex vivo. Thus, it is conceivable that the plasma IL-6 in the treated dogs was affecting platelet function directly. However, incubation of blood with 100 ng/mL of IL-6 did not alter the thrombin dose-response curve, suggesting that the changes observed in the IL-6-treated dogs were mediated via an effect on megakaryocytes, ensuing in the production of platelets with altered function. It is possible that the conditions of ex vivo incubation were inadequate to permit the detection of platelet activation changes, because there are technical limitations as to how long platelets can be maintained in whole blood at 37°C without inducing activation. Because the concentration of IL-6 used exceeded the ambient IL-6 concentration (measured in previous studies; data not shown) by 2-logs, and previous data suggested that immature but not mature human megakaryocytes express IL-6 receptor, it

Table 1. Platelet Counts in IL-6–Treated and Control Dogs

<table>
<thead>
<tr>
<th>Time (d)</th>
<th>Control (x10$^7$/μL)</th>
<th>IL-6 (x10$^7$/μL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pre</td>
<td>371 ± 105</td>
<td>330 ± 26</td>
</tr>
<tr>
<td>2-3</td>
<td>397 ± 118</td>
<td>342 ± 38</td>
</tr>
<tr>
<td>5-6</td>
<td>364 ± 60</td>
<td>434 ± 55</td>
</tr>
<tr>
<td>8-9</td>
<td>399 ± 115</td>
<td>569 ± 89</td>
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<tr>
<td>12-13</td>
<td>405 ± 68</td>
<td>666 ± 105</td>
</tr>
<tr>
<td>15-16</td>
<td>384 ± 107</td>
<td>640 ± 194</td>
</tr>
<tr>
<td>19-20</td>
<td>407 ± 157</td>
<td>578 ± 198</td>
</tr>
</tbody>
</table>

Fig 4. Dose-response characteristics of platelets exposed to PAF in two dogs 6 days after commencement of IL-6 (closed and open circles) or in one dog given diluent buffer (open triangles). IL-6 was administered for 10 days. The maximal change was noted at day 6. The ordinate represents the percentage of activated platelets, whereas the abscissa represents the concentration of PAF (mol/L). The dose-response curve is shifted to the left in the IL-6–treated animals. The ED$_{50}$ of the control animal was 3.52 X 10$^{-5}$ mol/L, whereas the ED$_{50}$ of the treated animals were 1.96 X 10$^{-4}$ mol/L and 1.29 X 10$^{-4}$ mol/L, respectively.

Fig 5. Exposure of canine platelets to IL-6 ex vivo does not alter their responsiveness to thrombin (expressed as percentage of activated platelets on the ordinate). Anticoagulated whole blood was incubated with 100 ng/mL of IL-6 for 30 minutes, followed by exposure to 0 to 0.25 U/mL of thrombin (abscissa). No differences were observed between blood incubated with IL-6 (O — — O) or the IL-6 diluent buffer (O — — O). One of two identical experiments.
seems unlikely that the cytokine directly alters platelet activation capacity.\(^{27}\)

In contrast to the lack of influence of IL-6 on platelets in vitro, in vivo administration of IL-6 at 80 \(\mu\)g/kg/d does alter the capacity of platelets to respond to thrombin, with more than a twofold reduction in the concentration of thrombin required to half-maximally activate platelets. The maximal decrease was observed at days 8 to 9 in the IL-6-treatment course, suggesting the progressive accumulation of platelets with enhanced activation capacity. Because normal platelet survival in our hands has been 5.5 to 7.5 days,\(^{28}\) the maximal effect observed at day 8 is occurring at a point when all circulating platelets have been synthesized after the initiation of IL-6. Subsequent normalization of platelet activation was noted after the discontinuation of IL-6.

The mechanism by which this alteration in platelet responsiveness to thrombin stimulation occurs is unknown. Conceivably, IL-6 administration results in the production of platelets with specific augmentation of the thrombin-signaling pathway, perhaps by increasing synthesis of thrombin receptor or altering subsequent steps in the thrombin-signaling pathway. The similar changes observed using the strong agonist PAF, operative via a unique receptor,\(^{29}\) suggests that the effects of IL-6 are not solely upon the thrombin-signaling mechanism. IL-6 may induce accelerated production of new platelets, which may exhibit a more global augmentation of function.\(^{30,31}\) Although a number of studies have suggested that young platelets are hyperfunctional, the available methodology to distinguish young from old platelets is not precise.\(^{30,32-37}\) Despite the present data suggesting that the new platelets produced in response to IL-6 are hyperfunctional, there is no definitive evidence that they are hyperfunctional because they are young. Further studies will be required to determine if young platelets and IL-6-driven platelets show similar responsiveness to agonists.

The physiologic significance of enhanced platelet responsiveness to thrombin and PAF is unclear. Such platelets may be so dysfunctional that they are prematurely removed from the circulation and serve no useful purpose. Conversely, these platelets may not exhibit shortened survival under normal circumstances, but may possess a greater propensity to be used at sites of injury than normal platelets. Direct functional analysis and survival of platelets in IL-6-treated animals with and without hemostatic stress may distinguish between these possibilities.

Alteration of platelet function by IL-6 administration represents another example of the influence of cytokines on differentiated cell functions,\(^{38,39}\) although the effects of IL-6 on platelet function appear to be initiated at the level of the megakaryocyte, rather than directly upon the mature platelet. In addition to augmenting the platelet count, the potentially prohemostatic effect of IL-6 may prove to be useful clinically.

REFERENCES


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