The Inhibitory Effect of Plasma Fibronectin on Collagen-Induced Platelet Aggregation

By D.G. Moon, J.E. Kaplan, and J.E. Mazurkewicz

Plasma fibronectin (Fn) has been proposed to have an antithrombotic effect, protecting against platelet and fibrinogen consumption after injury. The current study was designed to determine the effect of plasma fibronectin on collagen-induced platelet aggregation. In vitro aggregometry using an isolated homologous rat system, demonstrated a significant \( P < 0.05 \) inhibitory effect of 120 \( \mu g/mL \) Fn on platelet aggregation as induced by 60 \( \mu g/mL \) fibrilar collagen (type I). The inhibition was evidenced by a threefold increase in lag time and a significant decrease in the rate and extent of aggregation. The hypothesis was also tested using an in vivo model of collagen-induced platelet aggregation. The model used was intravenous injection of 2 mg/kg of homologous type I collagen into anesthetized Sprague-Dawley rats. Injection of collagen preincubated with 4 mg/kg Fn resulted in significantly less thrombocytopenia and fibrinogen consumption as compared with injection of collagen alone. The results of both the in vitro and in vivo studies are consistent with the proposed antithrombotic effect of plasma fibronectin.

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MATERIALS AND METHODS

Male Sprague-Dawley rats (Taconic Farms, Germantown, NY) weighing 300 to 350 g were used for platelet and protein isolations. Animals were housed with veterinary supervision under controlled conditions, with food (Purina Rat Chow) and tap water ad libitum. All animals were anesthetized with diethyl ether or sodium phenoobarbital before any surgical manipulation.

Adenosine diphosphate (ADP) and rat albumin were purchased from Sigma Chemical Co (St Louis). Purified \( \alpha \)-thrombin was the generous gift of Dr John Fenton (NY State Health Laboratories, Albany). Stractan II was purchased in powder form from St Regis Co., Tacoma, Wash.) Buffers and solutions were prepared using distilled deionized water and sterilized by filtration using a 0.45-\( \mu m \) filter (Nalge Co, Rochester, NY).

Platelets were isolated from whole rat blood using the method of Corash et al.17 Whole blood was drawn by venipuncture with an 18-gauge needle into a plastic syringe containing two parts 19% sodium citrate for every 100 parts blood. Platelet-rich plasma (PRP) was then prepared by adding 1 mL of a buffered saline glucose-citrate solution (BSG-citrate; 0.117 mol/L NaCl, 0.0136 mol/L sodium citrate, 0.011 mol/L glucose, 0.0086 mol/L \( Na_2HPO_4 \), and 0.0016 mol/L \( KH_2PO_4 \), pH 7.4) to 9 mL blood and centrifuging at 1,000 g for three minutes. This procedure was repeated three to five times until >95% of all platelets were obtained. The BSG-citrate-diluted PRP was layered over a discontinuous gradient consisting of 4 mL 20% Stractan II (isosmotic arabinogalactan solution) and 3 mL 10% Stractan II in a 15-mL conical centrifuge tube. The tube was centrifuged for 15 minutes at 3,500 g, after which the plasma/BSG and 10% Stractan layers were aspirated. The platelet layer was then removed and washed free of Stractan by centrifugation for eight minutes at 1,200 g in excess BSG-citrate. The platelet pellet was resuspended in modified (calcium/magnesium-free) Tyrode's solution (0.137 mol/L NaCl, 0.0030 mol/L KCl, 0.012 mol/L \( NaHCO_3 \), 0.006 mol/L glucose, and 0.004 mol/L \( Na_2HPO_4 \), pH 7.4) or appropriate medium. This method of isolation removes all plasma proteins while causing minimal damage to the platelets. All procedures were carried out using plastic or siliconized glassware at room temperature unless otherwise specified. Platelet counts were performed using a hemocytometer (American Optical, Buffalo, NY)

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and Nikon phase contrast microscope (Nikon, Garden City, NY) after the method of Bjorkman.8

Rat platelet aggregation was measured in vitro according to the method of Born,9 using a Payton Single Channel 300-B Aggregation Module (Payton Assoc, Inc, Buffalo, NY) and recorded with an Omniscribe B-5000 chart recorder (Houston Instruments, Austin, Tex). Aggregometry was performed at a block temperature of 37°C and a stir bar speed of 900 rpm using a platelet concentration of 3 x 10^7 cells per milliliter. A typical aggregation assay consisted of 100 μL of isolated platelets in Ca^2+/-Mg^2+ free modified Tyrode's solution, 10 μL CaCl_2 (0.001 mol/L final concentration), appropriate volumes of aggregating agents and plasma fibronectin in phosphate-buffered normal saline (PBS; 0.01 mol/L NaH_2PO_4/ Na_2HPO_4, 0.15 mol/L NaCl, pH 7.4), or PBS vehicle alone. The assay mixture was brought up to 0.50 mL final volume using modified Tyrode's solution. For most experiments, the platelet suspension in Tyrode's with calcium added was allowed to equilibrate with fibronectin or PBS while stirring for two minutes before the addition of collagen or other aggregating agent. When fibrillar (preformed) collagen was desired, the appropriate amount of acid-soluble collagen was added to the Tyrode's solution in an aggregation cuvette (with calcium and Tyrode's solution but without platelets and fibronectin) and allowed to form fibrils overnight at room temperature. Rat plasma fibrogenin (200 μg) was added to the cuvette for ADP-induced aggregations. To characterize the aggregation response, the following parameters were measured: the slope of the recorder tracing, which is an indicator of the rate of aggregation; the length of lag time from addition of the aggregating agent (collagen) to the onset of upward pen deflection; and the maximal scale deflection attained by the recorder tracing, which is an indicator of the completeness of the aggregation. Aggregation rate was measured by determining the rate of rise of the pen tracing for the first 30 seconds after leaving baseline (ie, after completion of the lag phase) and expressed as micrometers per minute. Lag time was expressed in minutes (chart speed equalled 25 mm/min). Maximum pen deflection was determined five minutes after the completion of the lag phase and expressed in millimeters. In almost all cases, aggregation was complete within four to five minutes and did not change when observed for a subsequent ten minutes.

The observations made with the Payton aggregometer were confirmed and extended using a Model 500 Whole Blood Aggregometer (Chrono-Log Corp, Haverton, Pa). Aggregation assays in this device were also performed at 37°C with 3 x 10^7 platelets per milliliter and a stir bar speed of 900 rpm. A typical assay mixture contained 200 μL platelets, modified Tyrode's solution, 20 μL CaCl_2 (0.001 mol/L final concentration), 60 μL collagen (1 mg/mL stock concentration in 0.01 mol/L acetic acid), plasma fibronectin, and 40 μL Chrono-Lume Reagent (luciferase-luciferin reagent, Chrono-Log Corp) in a 1 mL final volume. An important feature of the Chrono-Log aggregometer is the ability to simultaneously measure changes in light transmittance and secretion of adenosine triphosphate (ATP), one of the constituents of the dense granule. Thus the kinetics of dense granule secretion could be followed continuously and compared with light transmittance data.

To determine the effect of fibronectin on in vivo collagen-induced platelet aggregation, male Sprague-Dawley rats weighing 200 to 300 g were anesthetized by intraperitoneal injection of 35 mg/kg sodium pentobarbital (Nembutal, Abbott, N Chicago). After the induction of surgical anesthesia, the tip of the tail was amputated to allow blood sampling without the use of indwelling cannulas or repeated venipuncture. This “tail sampling” method provides samples that have been shown previously to be reproducible.20

In vivo platelet aggregation was induced by injection of microfibrillar collagen (2 mg/kg body weight) suspended in PBS, pH 7.4, over approximately a one-minute time period. Injections were performed by way of the dorsal penis vein. Blood samples were taken just before injection (0 time) and at 5, 15, and 60 minutes after collagen injection. After the 60-minute sample, animals were sacrificed. Plasma fibronectin, fibrinogen, and fibrin(ogen) degradation products (FDPs) were also determined for each sample using anaglynoimmunological methods (see later). Because the first microcirculatory bed in which collagen could lodge would be the pulmonary circulation, lung wet-dry weight ratios were measured as an index of lung injury. For determination of wet-dry weight ratios, wet weights were measured, the lungs were then dried to constant weight, and the ratio of wet to dry weights calculated.

There were five treatment groups in the experiment, each group consisting of 12 animals. The injection protocol is shown in Table 1.

<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>First Injection</th>
<th>Second Injection</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Collagen</td>
<td>Vehicle (PBS)</td>
</tr>
<tr>
<td>2</td>
<td>Collagen</td>
<td>Fibrinogen</td>
</tr>
<tr>
<td>3</td>
<td>Collagen/Fibronectin</td>
<td>PBS</td>
</tr>
<tr>
<td>4</td>
<td>PBS</td>
<td>PBS</td>
</tr>
<tr>
<td>5</td>
<td>Fibrinectin</td>
<td>PBS</td>
</tr>
</tbody>
</table>

The key experimental groups are group 1, collagen injection followed by injection of phosphate-buffered saline (PBS) vehicle, and group 3, injection of collagen preincubated with plasma fibronectin (two times the amount of collagen on a per weight basis) for 30 minutes at room temperature followed by injection of PBS vehicle. Group 2 acted as a control for group 3, in that the same amount of fibronectin was injected as in group 3 but one minute after collagen injection (ie, the collagen was not "precoated"). This control necessitated a two-injection sequence, as shown in Table 1. Each injection was 2 mL in volume and given over approximately one minute. Groups 4 and 5 were the vehicle and the fibronectin alone control groups. Because the data were dependent in nature (ie, several measurements were made on each animal over time), the results were first analyzed by a repeated measures analysis of variance (BMDP:2V program), testing both treatment group effect and time effect.

Acid-soluble collagen (type I) was isolated in a manner similar to that of Morin et al.21 Rat tail tendon was excised and rinsed in cold saline, finely minced, and homogenized for five minutes in 0.5 mol/L acetic acid at 4°C with a Brinkman homogenizer (Model PT10-35, Brinkman, Westbury, NY). All subsequent steps in the procedure were done at 4°C. After homogenization, the suspension was stirred overnight and then centrifuged at 10,000 g for one hour to remove nonsolubilized material. Solid NaCl was added to the clarified homogenate until a 1 mol/L NaCl concentration was achieved. The precipitate formed was resuspended in 0.01 mol/L acetic acid and dialyzed extensively against this same solution. Collagen concentration was determined gravimetrically after lyophilization. Collagen purity was assessed by sodium dodecyl sulfate-polyacrylamide gradient gel (5% to 15%) electrophoresis under reducing conditions. For in vivo injection, fibrillar collagen was prepared by diluting purified acid-soluble collagen with PBS, pH 7.4, and incubating at 26°C overnight. Before injection, fibrillar collagen was homogenized with a Brinkman Polytron.

Plasma fibronectin was isolated from rats by affinity chromatography on gelatin-Sepharose. The procedure is a modification22 of the preparative gelatin-Sepharose column method used by Engvall and Ruoslahti.4 Fibronectin concentration in plasma was measured by the electroimmunoassay or "rocket" immunoelectrophoresis method23 using monospecific antirat fibronectin antibody prepared and isolated from rabbits as previously described.24 Rat plasma fibrino- gen was isolated by glycine precipitation,25 and elutability was...
measured by the procedure of Laki.26 Fibrinogen concentrations were determined by radial immunodiffusion27 also using monospecific antirat antibody raised in rabbits. FDPs were measured according to the method of Kaplan et al.28 Concentrations of isolated proteins in solution were determined by the Lowry assay.29

Statistical analysis of data was performed with the assistance of the BMDP statistical software package20 on an IBM 3033 computer. Comparison of two treatment groups was performed using a one-tailed Student's t test with the significance level set at P < .05. Comparison of more than two treatment groups was performed using a one-way (one-factor) analysis of variance (ANOVA). If the ANOVA showed a significant F ratio (P < .05), then either a Fisher's protected F test or Newman-Keuls multiple comparison test was applied to identify specific group differences. Lung wet-dry weight ratio data are not normally distributed. Therefore, statistical analysis was performed on the arc sin transformation of the raw data. The mean ± standard error of the mean (SE) unless otherwise stated.

RESULTS

The effect of plasma fibronectin on in vitro collagen-induced platelet aggregation was investigated using an isolated homologous rat system. Typical aggregation response curves using acid-soluble collagen and fibrillar collagen are depicted in Fig 1. The quantitative results are shown in Table 2. The effect of fibronectin on acid-soluble collagen-induced aggregation is shown in Fig 1A and Table 2. Increasing concentrations of plasma fibronectin significantly increased lag time (> threefold at 60 µg Fn as compared with PBS control) but did not affect aggregation maximum. The observations on in vitro aggregation made with the Payton aggregometer were confirmed and extended in a Chrono-Log Whole Blood Aggregometer using both luminescence and optical channels. The luminescence channel, however, enabled us continuously to measure the release of ATP as an indicator of dense granule release. Release of ATP is first detectable at the beginning of the shape change portion of the optical (transmittance) curve. This is consistent with the findings of others using human platelets.31 In the presence of plasma fibronectin, ATP release occurs at the same relative position but is delayed in absolute time because of the increased lag time. The extent of ATP release (as compared with the standard) was the same for both control and fibronectin aggregations.

Table 2. Effect of Plasma Fibronectin on In Vitro Collagen-Induced Platelet Aggregation

<table>
<thead>
<tr>
<th>Group</th>
<th>Lag Time (min)</th>
<th>Aggregation Rate (mm/min)</th>
<th>Maximum Aggregation (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acid-soluble collagen</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>2.10 ± 0.08</td>
<td>100.8 ± 6.2</td>
<td>143.0 ± 6.4</td>
</tr>
<tr>
<td>30 µg Fn</td>
<td>2.51 ± 0.07*</td>
<td>108.6 ± 4.6</td>
<td>141.2 ± 4.6</td>
</tr>
<tr>
<td>45 µg Fn</td>
<td>0.81 ± 0.09</td>
<td>105.8 ± 3.6</td>
<td>140.2 ± 7.3</td>
</tr>
<tr>
<td>60 µg Fn</td>
<td>3.17 ± 0.14*</td>
<td>89.5 ± 17.0</td>
<td>147.0 ± 8.6</td>
</tr>
<tr>
<td>Fibrillar collagen</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>0.98 ± 0.05</td>
<td>96.6 ± 7.6</td>
<td>138.0 ± 3.7</td>
</tr>
<tr>
<td>30 µg Fn</td>
<td>3.18 ± 0.08*</td>
<td>93.5 ± 2.1</td>
<td>134.6 ± 6.1</td>
</tr>
</tbody>
</table>

Platelet aggregation was initiated by the addition of 30 µg of acid-soluble type I rat collagen to a suspension of rat platelet (1.5 x 10⁶) in Tyrode's solution with 1 mmol/L (final concentration) calcium added. Final cuvette volume was 0.5 mL. Plasma fibronectin in phosphate-buffered saline (PBS) in the amounts indicated was equilibrated with the platelets before collagen addition. Controls consisted of the appropriate volumes of PBS being added in lieu of fibronectin. Data are expressed as mean ± SE. Platelet aggregation also was initiated by the addition of 1.5 x 10⁹ rat platelets in 100 µL Tyrode's solution to 30 µg of preformed fibrillar rat collagen (type II). The indicated amounts of fibronectin (or equal volume of PBS vehicle) were added just before platelet addition. The fibrillar collagen was formed by overnight incubation at 26 °C in 250 µL Tyrode's solution with calcium added. Fn, fibronectin.

*Significantly (P < .05) different from control value (n = 5 to 7 in each group).

Fig 1. (A) Typical acid-soluble collagen-induced rat platelet aggregation curves. Type I collagen (30 µg) was added to platelets (1.5 x 10⁶ cells) suspended in Tyrode's solution with either fibronectin (Fn) in PBS or PBS vehicle alone (control). The final cuvette volume was 0.5 mL. (B) Typical fibrillar collagen-induced rat platelet aggregation curves. Aggregation was initiated by the addition of 100 µL of 1.5 x 10⁹ platelets in Tyrode's solution to fibrillar collagen that had been preformed in the cuvette. Plasma fibronectin or PBS control was added just before platelet addition. The final cuvette volume was 0.5 mL.

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consistent with plasma fibronectin having an inhibitory effect on the initial platelet–collagen interaction.

The effect of fibronectin on two other aggregating agents, ADP and purified thrombin, was also studied. Even using 200 μg Fn/mL, a concentration almost double those that inhibited in collagen-induced aggregation, fibronectin had no effect on the rate or extent of either ADP or thrombin-induced platelet aggregation (Table 3). Thus the effect of fibronectin, at the concentrations used, appears to be specific to the collagen–platelet interaction and not an inhibition of the platelet–platelet interaction, which has been reported to occur with much higher concentrations of fibronectin.33

As a model of collagen-induced platelet aggregation in vivo, anesthetized rats were injected IV with collagen (2 mg/kg body weight) suspended in PBS. Five treatment groups were established with a two-injection protocol, as shown in Table 1. The basic hypothesis was that if plasma fibronectin did inhibit collagen-induced platelet aggregation, then collagen preincubated (“precoated”) with fibronectin would cause less thrombocytopenia or platelet consumption than injection of collagen alone.

The changes in circulating platelet number after in vivo collagen injection are shown in Table 4. Injection of collagen alone in vehicle (group 1) caused a 29% drop in platelet count within five minutes. Over the next hour, however, platelet count returned to preinjection levels. Injection of collagen preincubated with two times its weight of plasma fibronectin (group 3) resulted in only a 17% decrease in platelet count at the five-minute time point and was significantly (P < .05) different from collagen alone (group 1). Group 2 (collagen followed by fibronectin injection) was not statistically different from group 1 at five minutes. In other words, at the first measurement time after injection, collagen preincubation with fibronectin (group 3) appeared to protect against the thrombocytopenia after injection of collagen alone (group 1), whereas injection of collagen followed by injection of fibronectin (group 2) was less protective. Injection of vehicle alone (PBS) resulted in a decreased platelet count due to “dilution effects.” The injection of fibronectin alone (group 5), which served as one of the controls, resulted in a rise in platelet counts by five minutes postinjection. At 15 and 60 minutes postinjection, only the fibronectin control group (group 5) was significantly different from the collagen group (group 1).

Measurement of fibronectin during the experiment (Table 5) revealed that the only significant changes that occurred were elevations in fibronectin concentration in groups 2 and 5. Group 2 (collagen followed by fibronectin) was significantly different from groups 1, 3, and 4 at five minutes postinjection, as was group 5 (injection of fibronectin alone). At this time point, groups 2 and 5 were not significantly different from each other. By 15 minutes postinjection, fibronectin levels in group 2 had returned to baseline values, while those of group 5 remained significantly elevated. The fact that there was no significant elevation in plasma fibronectin levels in group 3 (collagen preincubated with fibronectin) animals indicates that most of the injected fibronectin was associated with the injected collagen.

Measurement of plasma fibrinogen levels indicated that injection of collagen (group 1) resulted in a rapid consumption or depletion of fibrinogen. These data are presented in Table 6. Within five minutes, fibrinogen levels dropped 36% in the collagen group (group 1) and stayed depressed throughout the 60 minutes of the experiment. In contrast, collagen preincubated with fibronectin (group 3) caused only a 14% drop in fibrinogen concentration at the five-minute time point, which was significantly (P < .05) different from group 1. After a second drop at 15 minutes postinjection, fibrinogen levels in group 3 returned to baseline at 60 minutes. Injection of collagen followed by fibronectin (group 2) was not significantly different from injection of collagen alone (group 1) at any time. Thus, much like the observations made on platelet counts, collagen preincubated with fibronectin (group 3) appeared to protect against fibrinogen consumption when compared with injection of collagen alone (group 1), whereas collagen followed by fibronectin afforded no protection.

Although measurements of FDPs were made, the amount of FDPs generated was below the detection limits of the assay. In our hands, this assay for FDPs has shown significant changes in FDP levels in other rat models of thrombosis, such as intraperitoneal thrombin injection.16,28 Therefore, it appeared that during the one hour after collagen injection, there was little or no detectable fibrinolysis.

Lung wet-dry weight ratios were measured as an index of lung injury as induced by the collagen injection (Table 7). Injection of collagen alone (group 1) caused a significant (P < .05) increase in lung wet-dry weight ratios when compared with injection of vehicle (group 4). Injection of collagen preincubated with fibronectin (group 3) and injection of collagen followed by injection of fibronectin (group 2) resulted in lung wet-dry weight ratios that were significantly lower than those of group 1. Groups 2, 3, and 5 were not statistically different from vehicle injection (group 4). The vehicle group had a wet-dry ratio of 4.89 ± 0.15.

**DISCUSSION**

Plasma fibronectin significantly (P < .05) inhibits collagen-induced platelet aggregation in the isolated rat platelet system. Using homologous cells and proteins, fibronectin increased the lag time using acid-soluble collagen to induce
Data are expressed as mean ± collagen (group 1), collagen followed one minute later by injection of 4 mg/kg fibronectin (group 2), collagen preincubated with 4 mg/kg fibronectin (group 3), PBS vehicle control (group 4), or 4 mg/kg fibronectin control (group 5) into anesthetized rats. Data are expressed as the mean ± SE and each group consisted of 12 animals. Fn, fibronectin.

*Platelet count (× 10^6/L).
†Significantly (P < .05) different from collagen group (group 1). See Materials and Methods for details of statistical analysis.

### Table 5. Changes in Plasma Fibronectin Concentration (μg/mL) During In Vivo Collagen-Induced Platelet Aggregation

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>1 Collagen</th>
<th>2 Collagen Followed by Fn</th>
<th>3 Collagen Preincubated With Fn</th>
<th>4 PBS Vehicle</th>
<th>5 Fibronectin</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>344.7 ± 22.4</td>
<td>375.5 ± 40.4</td>
<td>402.4 ± 26.9</td>
<td>382.6 ± 29.8</td>
<td>369.4 ± 12.9</td>
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<tr>
<td>5</td>
<td>311.3 ± 21.2</td>
<td>430.4 ± 34.6</td>
<td>384.1 ± 32.6</td>
<td>362.9 ± 27.8</td>
<td>398.6 ± 10.9</td>
</tr>
<tr>
<td>15</td>
<td>333.1 ± 28.2</td>
<td>373.7 ± 34.7</td>
<td>386.5 ± 31.3</td>
<td>351.2 ± 31.6</td>
<td>398.6 ± 18.8</td>
</tr>
<tr>
<td>60</td>
<td>319.6 ± 33.1</td>
<td>397.7 ± 34.2</td>
<td>361.2 ± 32.9</td>
<td>338.9 ± 34.6</td>
<td>399.3 ± 9.4</td>
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</tbody>
</table>

Plasma fibronectin levels were measured by rocket electroimmunoassay as previously described, using monospecific antirat antibody raised in rabbits. Data are examined as mean ± SE. Each treatment group consisted of 12 animals. Fn, fibronectin.

*Significantly (P < .05) different from collagen group (group 1) at that time.

### Table 6. Changes in Fibrinogen Concentration (mg/mL) During In Vivo Collagen-Induced Platelet Aggregation

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>1 Collagen</th>
<th>2 Collagen Followed by Fn</th>
<th>3 Collagen Preincubated With Fn</th>
<th>4 PBS Vehicle</th>
<th>5 Fibronectin</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>2.32 ± 0.46</td>
<td>2.88 ± 0.38</td>
<td>2.44 ± 0.38</td>
<td>3.21 ± 0.35</td>
<td>2.69 ± 0.39</td>
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<tr>
<td>5</td>
<td>1.49 ± 0.09</td>
<td>2.12 ± 0.37</td>
<td>2.09* ± 0.24</td>
<td>2.47* ± 0.36</td>
<td>2.45* ± 0.36</td>
</tr>
<tr>
<td>15</td>
<td>1.46 ± 0.23</td>
<td>1.99 ± 0.37</td>
<td>1.75 ± 0.16</td>
<td>2.70 ± 0.46</td>
<td>2.61* ± 0.41</td>
</tr>
<tr>
<td>60</td>
<td>1.53 ± 0.30</td>
<td>2.06 ± 0.33</td>
<td>2.88* ± 0.35</td>
<td>1.82 ± 0.42</td>
<td>2.88* ± 0.53</td>
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</table>

Fibrinogen concentrations were measured by radial immunodiffusion as previously described, using monospecific antirat antibody raised in rabbits. Data are expressed as mean ± SE. Each treatment group consisted of 12 animals. Fn, fibronectin.

*Significantly (P < .05) different from collagen group (group 1).

### Table 7. Lung Wet-Dry Weight Ratios

<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>1 Collagen</th>
<th>2 Collagen Followed by Fn</th>
<th>3 Collagen Preincubated With Fn</th>
<th>4 PBS Vehicle</th>
<th>5 Fibronectin</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>5.64 ± 0.15</td>
<td>5.24 ± 0.11</td>
<td>5.22 ± 0.10</td>
<td>4.89 ± 0.15</td>
<td>4.69 ± 0.10</td>
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</tbody>
</table>

After the 60-minute blood sample, animals were sacrificed and the lungs rapidly excised, rinsed in cold saline, and weighed. Dry weight was measured after oven drying to a constant weight. Data are expressed as mean ± SE. Each group consisted of 12 animals. Values for groups 2 through 5 were significantly (P < .05) different from the collagen group (group 1) as determined by one-way analysis of variance and a Newman-Keuls multiple comparison test performed on an arc sin transformation of the raw ratio data. Fn, fibronectin.
aggregation. In part, this may be attributable to an inhibition of collagen fibrillogenesis as reported by Kleinman et al.32 However, when fibrillar collagen was used to induce aggregation, plasma fibronectin still increased lag time. Furthermore, use of higher concentrations of fibronectin (1.5 x and 2.0 x collagen, wt/wt) resulted in a significant decrease in aggregation rate and the maximum extent of aggregation, as if fibronectin had blocked platelet binding sites on collagen, limiting the stimulus for aggregation.

The inhibitory effect of plasma fibronectin, at the concentrations used, appeared to be specific for collagen in that neither ADP- nor thrombin-induced aggregation was affected even in the presence of 200 μg Fn/mL. This concentration was almost twice that at which inhibition of collagen-induced aggregation could be detected. Santoro33 has reported that plasma fibronectin can inhibit in vitro human platelet aggregation as induced by thrombin or ionophore A23187. In that system, the threshold for fibronectin’s inhibitory effect was above levels used in this study, with maximal inhibition occurring at 500 μg Fn/mL. These concentrations are significantly greater than those needed to inhibit collagen-induced aggregation in our homologous rat system. Thus, while at first glance our observations might appear contrary to those of Santoro, this is not the case at all. We have demonstrated a specific inhibitory effect of fibronectin on collagen-induced aggregations at concentrations lower than those Santoro needed to show any inhibitory effect of fibronectin on thrombin- or A23187-induced aggregation. These observations suggest that fibronectin can inhibit platelet aggregation through two mechanisms. Low concentrations of fibronectin may inhibit the platelet–collagen interaction, whereas high concentrations may directly influence the platelet–platelet interactions.

Results of the in vivo collagen-induced platelet aggregation experiment were consistent with fibronectin’s inhibitory effect in the in vitro experiments. Preincubation (“precoating”) of collagen with fibronectin (group 3) before IV injection into anesthetized rats resulted in significant protection of the thrombocytopenia, fibrinogen consumption, and increase in lung wet-dry weights when compared with injection of fibrillar collagen alone (group 1). Injection of the same amount of fibronectin one minute after the collagen (group 2) was not as effective in preventing the thrombocytopenia and fibrinogen consumption. Injection of fibronectin alone (group 5) caused a significant rise in circulating platelet count, presumably by triggering release of a temporarily sequestered platelet pool. The argument might be raised that the protective effect of platelet consumption seen with group 3 (collagen precoated with fibronectin) was simply release of a new pool of platelets due to increased circulating fibronectin. However, plasma fibronectin was not elevated in group 3. Group 2, which did not show a significant increase in plasma fibronectin at five minutes, was not as effective in preventing the thrombocytopenia as treatment group 3. Furthermore, injection of fibronectin one minute after collagen injection (group 2) did not prevent fibrinogen consumption at any time. Therefore, the protective effect seen with treatment group 3 is not attributable to elevated fibronectin levels in those animals. Rather, the data suggest that for fibronectin to exert its protective (antithrombotic) effect, it must be intimately associated with the collagen.

The influence of plasma fibronectin on the collagen-induced changes in lung wet-dry weight ratio appears more complex. Here both group 2 and group 3 treatments had significantly lower ratios than that of group 1 (collagen alone). Because an increase in wet-dry weight ratio could reflect both thrombosis and edema formation, different fibronectin effects may come into play with group 2 v group 3. Whereas the collagen precoated with fibronectin (group 3) may have resulted in less pulmonary platelet aggregation and thrombosis, the elevated plasma fibronectin levels of group 2 may have been acting to limit pulmonary edema formation by influencing vascular permeability.34 Obviously, caution must be used in interpreting the results of the in vivo collagen injection study because of the complexities of the whole animal model. Nevertheless, the results of the in vivo collagen-induced platelet aggregation study are consistent with the more defined in vitro platelet aggregometry. This suggests that plasma fibronectin may indeed play a physiologically significant antithrombotic role by inhibiting or limiting platelet–collagen interactions.

The mechanism of plasma fibronectin’s inhibitory effect on collagen-induced platelet aggregation is not yet clear. It is apparent that inhibition occurs in the early phases of platelet aggregation (ie, the lag time). The timing of this inhibition suggests that fibronectin may be interfering with platelet–collagen binding (adhesion) or perhaps platelet activation. Santoro35 reported inhibition of thrombin-induced aggregation with 500 μg Fn/mL but found no difference in serotonin release in the presence of fibronectin. Therefore, he concludes that under his conditions, fibronectin inhibited some process after primary platelet activation. However, the possibility still exists that fibronectin does inhibit platelet activation induced by collagen. This question is currently under study.

Another possible mechanism for fibronectin’s inhibitory action on platelet aggregation is that fibronectin might decrease the platelet adhesion response to collagen. Sochynsky et al35 reported that preincubation of fibrillar collagen inhibited human platelet adhesion. Most other studies, however, have reported that fibronectin enhances platelet adhesion or attachment and spreading to collagen.3637 Our own experiments on the effect of fibronectin on platelet adhesion to collagen in a homologous rat model (Moon and Kaplan, unpublished observations, 1983) have yielded results virtually the same as those reported by Kotelsiansky et al37 for human platelets. A major limitation of most platelet adhesion/attachment studies is that they were done under nonflow, unstirred conditions. As has been well demonstrated, changes in shear stress and shear rate can be important parameters in the function of proteins involved in platelet attachment to collagen.38 A recent report by Houdijk et al39 describes the influence of fibronectin and von Willebrand factor (vWF) on the interaction of platelet with acid-soluble and fibrillar collagens types 1 and 3 under defined flow conditions. Optimal platelet adhesion to acid-soluble (monomeric) collagen was dependent on both vWF and fibronectin. vWF was required only at relatively high
shear rates, whereas platelet deposition on acid-soluble collagen was depressed at all shear rates when fibronectin-free plasma was used as a perfusate. Similar results were obtained with fibrillar collagen. However, when preincubation of collagen with vWF and fibronectin was performed, fibrillar collagen showed a requirement for only vWF and not fibronectin. These results are in contrast to preincubation studies with acid-soluble collagen, which demonstrated a requirement for both vWF and fibronectin. These experiments point out the differences that can arise in using acid-soluble vs fibrillar collagen. While these reports suggest a role for fibronectin in mediating platelet attachment to and spreading on collagen, they do not explain the mechanism of fibronectin's inhibitory action.

The study reported here was designed to address the potential antithrombotic role of plasma fibronectin in collagen-induced platelet aggregation. The influence of neither the cell surface (tissue) fibronectin nor the platelet's own fibronectin has not been delineated. Houdijk and Sixma\(^{40}\) suggest that fibronectin in the subendothelium is important for platelet adhesion. Using human umbilical artery subendothelium in an annular perfusion chamber, they found that preincubation of the vessel with antifibronectin IgG reduced indium-111 platelet deposition. This effect was significant at a shear rate of 800 S\(^{-1}\) or greater, and we suggest that tissue fibronectin enhances platelet attachment. However, despite the apparent similarities of the in vitro actions of the fibronectins, they may play very different roles physiologically in the platelet–collagen interaction.

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REFERENCES

31. Feinman RD, Lubowsky J, Charo I, Zabinski M: The lumia-
ggregometer: A new instrument for simultaneous measurement of
secretion and aggregation. J Lab Clin Med 90:125, 1977
32. Kleinman HK, Wilkes CM, Martin GR: Interaction of
33. Santoro SA: Inhibition of platelet aggregation by fibronectin.
34. Niehaus GD, Schumacker PT, Saba TM: Influence of
opsonic fibronectin deficiency on lung fluid balance during bacterial
35. Sochynsky RA, Boughton BJ, Burns J, Sykes BC, McGee
36. Hoffmann RP, Hynes RO: Hemostasis: The role of fibronec-
37. Koteliansky VE, Leytin VL, Sviridov DD, Repin VS,
Smirnov VN: Human plasma fibronectin promotes the adhesion and
spreading of platelets on surfaces coated with fibrillar collagen.
38. Baumgartner HR, Muggli R, Tschopp TB: Interaction of
platelets with subendothelium in flowing blood, in Rotman A, Meyer
FA, Gitler FA, Gitler C, Silverberg A (eds): Platelets: Cellular
Response Mechanisms and Their Biological Significance. John
Wiley & Sons, 1980, p 17
39. Houdijk WPM, Sakariassen KS, Nievelstein PFEM, Sixma
JJ: Role of factor VIII–von Willebrand factor and fibronectin in the
interaction of platelets in flowing blood with monomeric and fibrillar
human collagen types I and III. J Clin Invest 75:531, 1985
40. Houdijk WPM, Sixma JJ: Fibronectin in artery subendothe-
lium is important for platelet adhesion. Blood 65:598, 1985
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