Diminished Platelet Adherence to Type V Collagen

Thomas J. Parsons, Donna L. Haycraft, John C. Hoak and Helene Sage

Different types of collagen vary in their influence on platelet reactivity. Collagen Types III, IV, and V were obtained from human placental tissue, and Type I collagen was prepared from rat skin. Each collagen type was coated onto a plastic surface. Each collagen-coated surface or appropriate plastic surface control was studied using citrated human $^{51}$Cr-labeled platelet-rich plasma in both the presence and absence of 10 $\mu$M adenosine 5'-diphosphate (ADP). Both unstimulated and ADP-induced platelet adherence were: 1) reduced by Type V collagen coating in comparison to uncoated wells; and 2) increased by Types III and IV collagen coating in comparison to Type V coated or plastic surfaces. Addition of the fast-acting thrombin inhibitor dansylarginine (DAPA) had no significant effect on unstimulated and ADP-induced platelet adherence to Type III, IV or V collagen-coated surfaces. Type I collagen-coated surfaces, studied only in the presence of DAPA, caused greater platelet adherence than those coated with Types III, IV, or V collagen. We conclude that Type V collagen may be less thrombogenic than Types I, III, or IV.

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Interactions between platelets and collagen as a component of the early events in hemostasis and thrombosis were investigated. Damage to the non-thrombogenic endothelial surface of vessel walls exposes the circulating blood to a variety of matrix macromolecules that are integral to both the structure and function of vascular tissue. Among these components are specific types of collagen. Collagen Types I, III, IV, and V have been demonstrated in the subendothelium of the vessel wall by chemical and immunohistological methods.\(^5\)\(^\text{-8}\)\(^,\)\(^\text{12,13}\) In vitro culture of vascular endothelial cells has provided evidence for the synthesis of Types III, IV, and V collagen.\(^5\)\(^,\)\(^\text{7,11}\)

Type V collagen may be present on the endothelial surface as well as in the basement membrane.\(^5\)\(^,\)\(^\text{8,12,13}\) The contributions of specific types of collagen to hemostasis and thrombosis have been studied by platelet aggregation and adhesion techniques.\(^14\)\(^-18\) It has been shown that collagen Types I, III, IV, and V activate platelets.\(^12\)\(^,\)\(^\text{19-26}\) In this report, a platelet adherence technique was used to quantify the interaction between platelets and each type of collagen found in vascular tissue. Platelet adherence to Types I, III, and IV collagen was demonstrated, adding data from a different technique to a literature that contains conflicting reports.\(^19,20,25,27\) It was shown that Type V collagen promotes little platelet adherence, which distinguishes it from other types of collagen.

Methods

Reagents

Hanks’ balanced salt solution (10X) and Dulbecco’s phosphate-buffered saline (PBS) were purchased from Grand Island Biological Company, Grand Island, New York. Hepes buffer, Trizma base (Tris), and adenosine diphosphate (ADP) were obtained from the Sigma Chemical Company, St. Louis, Missouri. Pentex bovine serum albumin, fraction V, fatty-acid poor, was purchased from Miles.
Laboratories, Incorporated, Kankakee, Illinois. The fast acting thrombin inhibitor dansylarginine N-(3-ethyl-1, 5-pentanediyl) amide (DAPA) was kindly provided by K.G. Mann, Mayo Clinic and Foundation, Rochester, Minnesota. Sodium chromate $^{51}$Cr was obtained from E.R. Squibb & Sons, Princeton, New Jersey. All other chemicals were of reagent grade.

**Solutions**

Modified Hanks' balanced salt solution (MHBSS) was composed of Hanks' balanced salt solution without NaHCO$_3$, 15 mM Hepes buffer, pH 7.4. The incubation mixture (IM) contained 8.6 g/liter bovine serum albumin, 140.3 mM NaCl, 5.8 mM KCl, 2.7 mM CaCl$_2$, 16.3 mM Tris, pH 7.4.

**Supplies**

Linbro plates containing 12 flat bottom, 4.5 cm$^2$ wells were purchased from Flow Laboratories, Incorporated, McLean, Virginia. Plastic centrifuge tubes were obtained from Falcon Plastics, Oxnard, California.

**Collagens**

Native Types IV and V collagen, prepared from human placenta as previously described, was prepared together with Type III collagen (prepared from human placenta) and Type I collagen (prepared from rat skin) were provided by co-author, Helene Sage. Lyophilized collagen, previously stored at −20°C, was dissolved in 0.1 M acetic acid at a concentration of 0.5 mg/ml. Each collagen sample was stirred overnight at 4°C. It was then centrifuged at 12,000 rpm for 2 minutes to remove undissolved material. The supernatant was removed and dialyzed against PBS at 4°C over 24 hours (four changes, 1 ml collagen-containing solution: 1000 ml PBS). Each collagen sample was centrifuged at 12,000 rpm for 2 minutes to remove particulate material. The plastic surfaces of 12 well Linbro tissue culture plates were washed twice with 1.0 ml of either MHBSS or PBS. One ml of either MHBSS or PBS was placed in control wells; 0.9 ml of either solution was added to the experimental wells which were to receive collagen. The results were not different when experiments using PBS were compared to those using MHBSS. The data shown is from experiments where MHBSS was used. We added 0.1 ml (50 µg collagen) of each solution containing collagen to the appropriate experimental wells. The multiwell plates, containing the appropriate control or collagen-containing solutions, were placed in a 37°C metabolic incubator on a rocker platform with a 30° tilt (Bellco Glass Incorporated, Vineland, New Jersey) and rocked at a rate of ten updown cycles per minute. After 150-180 minutes of incubation, the plates were removed and each well was aspirated. Each well was washed twice with 1.0 ml MHBSS. Collagen-coated surfaces and appropriate uncoated controls were used immediately. No drying occurred.

**Preparation of Labeled Platelet Suspensions**

Venous blood was drawn from consenting, informed normal human donors. Donors had not taken aspirin or other antiplatelet drugs during the preceding 10 days. Five parts of blood were added to one part of acid citrate dextrose anticoagulant and mixed in 40 ml siliconized glass tubes. Nine parts of blood were mixed with one part sodium citrate (13 mM Na citrate) in different siliconized glass tubes. A mixture of whole blood and ACD (120 ml) was centrifuged at 325 g for 15 minutes and the resulting platelet-rich plasma (PRP-ACD) was separated. The PRP-ACD was centrifuged at 1000 g for 15 minutes and the resulting platelet-poor plasma (PPP-ACD) was saved. The platelet pellet was resuspended in 10 ml of PPP-ACD. For each 10 ml of resulting platelet suspension, 250 U Na heparin (to inactivate residual thrombin) and 300 µCi Na$_2$$^{51}$CrO$_4$ were added and incubated at 37°C for 20 minutes. The labeled platelet suspension was centrifuged at 3000 g for 10 minutes. The resulting platelet pellet was resuspended in 10 ml PPP-ACD and centrifuged again at 1000 g for 10 minutes. This platelet pellet was resuspended in 10 ml PPP-ACD for a second washing. Earlier the whole blood-Na citrate mixture was centrifuged at 1000 g for 15 min and the resulting PPP-Na citrate mixture was saved. The labeled platelet pellet obtained after centrifugation of the second washing was resuspended in 10 ml PPP-Na citrate. Of this labeled platelet suspension, 0.3 ml contained approximately 40,000 cpm of $^{51}$Cr radioactivity and 5–7 × 10$^8$ platelets. Platelet suspensions were incubated over the experimental surfaces not later than 4 hours after the blood was taken. Platelets were kept at 37°C before they were used. Aggregation studies using a Payton aggregometer (Payton Associates, Buffalo, New York) were performed at the time that the platelet suspensions were incubated over plastic or collagen-coated surfaces. ADP at a final concentration of 10 µM was used to aggregate the platelets suspended in PPP-Na Citrate. Aggregation studies showed appropriate aggregation with ADP and no spontaneous aggregation.

**Adherence of Platelets to Collagen-Coated Surfaces**

After the second 1.0 ml wash with MHBSS, the solution was aspirated from the collagen-coated or empty well surfaces. Each well received a combination of the following solutions: 0.3 ml IM (control); 0.3 ml IM with DAPA, at a final concentration of 10 µM; 0.3 ml IM with ADP, at a final concentration of 10 µM; 0.3 ml $^{51}$Cr-labeled platelets in PPP-Na Citrate. DAPA was included in these experiments in order that the interactions between collagen and platelets would not be altered by thrombin which might be generated in the platelet-rich plasma. In those wells that received 10 µM ADP, visible platelet aggregation occurred in the platelet suspension which was rocked over the collagen-coated surfaces. The
multiwell plates were again placed in the 37°C metabolic incubator on the rocker platform and rocked as described above. After a 30-minute incubation, the supernatant suspension containing unattached labeled platelets was placed in a plastic centrifuge tube. MHBSS (0.9 ml) was added to each well and the plates were returned to the rocker for 5 minutes. This 0.9 ml MHBSS and an additional wash of 0.6 ml MHBSS were added to the supernatant-labeled platelet suspension in the plastic centrifuge tube. After centrifugation at 1000 g for 10 minutes, the 51Cr radioactivity contained in the tube was quantitated with a Beckman 4000 gamma counter (Beckman Instruments, Incorporated, Irvine, California).

The amount of 51Cr radioactivity remaining on the collagen-coated plastic surfaces was determined by solubilizing attached material with 1.5 ml of 1 M acetic acid. After one hour of exposure, this solution and a 1.0 ml wash with 1 M acetic acid were combined in a second plastic centrifuge tube and the 51Cr radioactivity was measured. The percentage of platelet adherence was derived by dividing the adherent counts per minute by the total (adherent plus supernatant with washes) counts per minute, × 100.

**Demonstration of Collagen Coating by Slab Gel Electrophoresis**

The plastic surfaces of 12-well Linbro plates were coated with collagen as described above. After incubation for 150–180 minutes on the rocking platform at 37°C, the coated wells were aspirated and washed twice in the described fashion. The surfaces were then washed with 0.2 ml of sample buffer and were analyzed on Na Dod so-polyacrylamide slab gels according to methods modified by Laemmli[30] and summarized by Takacs.[31]

**Further Characterization of Surfaces Coated with Type V Collagen**

Individual lanes of Na Dod SO4-polyacrylamide slab gels containing equal volumes of equivalent sample buffer washings of wells coated with separate generic collagens were compared using a scanning densitometer (model RF2, Transidyne Incorporated, Ann Arbor, Michigan). Indirect immunofluorescence of wells coated with Type V collagen was performed using an antibody and techniques previously reported.[7]

**Scanning Electron Microscopy**

The plastic surfaces of 12-well Linbro plates were coated with Types I, III, IV, and V collagen as described above. After 150–180 minutes on the rocking platform at 37°C, the coated surfaces were aspirated and washed twice as described. One ml of 1% glutaraldehyde in cacodylate buffer was added to each well, and the plates were prepared for scanning electron microscopy. Electron micrographs were taken with a JEOL-JSM-35C scanning electron microscope at 13 Kv with 100 µamps of current.

**Platelet Aggregation Characteristics of Collagen Types I, III, IV, and V**

To determine whether the collagens prepared for this experiment were capable of inducing platelet aggregation, three experiments were performed using each purified collagen as the aggregating agent.[21, 27] Platelet-rich plasma was prepared in exactly the same manner as above. 150 µl of PRP-Na citrate and 300 µl of DAPA in IM, final concentration 10 µM DAPA, were mixed with 50 µl of each collagen in PBS, or a PBS control. The final concentrations of collagen which were tested by this method were 200 µg/ml and 20 µg/ml. The solutions were added to the aggregation chamber in the following order: PRP, IM with DAPA, and lastly the collagen-containing solution or appropriate PBS control. This design resulted in concentrations of platelets and DAPA in the aggregation chamber that were identical to those in the platelet-adherence experiments. DAPA was included to inactivate thrombin that might be generated in the PRP preparation so that aggregation would reflect the interaction between collagen and platelets. To test whether DAPA was masking the interaction between the purified collagen and platelet-rich plasma, two platelet aggregation studies were also performed in a system that lacked DAPA but was otherwise identical to the above design. Aggregation was recorded over 30 minutes in the Payton module.

**Statistical Method**

We fit the parameters of this study into an analysis of variance model. All calculations were done using the Statistical Analysis System (SAS Users’ Guide 1982), Publisher: SAS Institute (Program General Linear Model (GLM) Univariate).

**Results**

Collagen-coated and uncoated wells were rocked with the control solution, IM, with or without 10 µM ADP, and 51Cr-platelets for 30 minutes at 37°C. Over uncoated surfaces, 4.3% of the added radioactivity remained adherent to the wells with IM alone (Table 1). When ADP was present in the IM, 13.9% of the radioactivity became adherent to the uncoated surface. When the surface was coated with Type III collagen, the values for platelet adherence were 11.1% with IM alone and 53.9% with IM containing ADP. When the surface was coated with Type IV collagen, the values for platelet adherence were 9.3% with IM alone and 49.3% with IM containing ADP. However, when platelet adherence was quantitated over surfaces coated with Type V collagen, the values were 1.7% with IM alone and 2.6% with IM containing ADP.

**Comparison of Platelet Adherence in the Absence and Presence of DAPA**

The potential role of thrombin formation in this system was studied by adding the fast acting thrombin...
Table 1. Percentage of Adherence of Human Blood Platelets to Surfaces Coated with Specific Types of Human Collagen

<table>
<thead>
<tr>
<th>Coating</th>
<th>Unstimulated (%)</th>
<th>ADP-induced (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>(n = 14/14)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>4.3 ± 1.9</td>
<td>13.9 ± 14.3</td>
</tr>
<tr>
<td>Type III</td>
<td>(n = 7/6)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>11.1 ± 3.2</td>
<td>53.9 ± 13.6</td>
</tr>
<tr>
<td>Type IV</td>
<td>(n = 13/10)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>9.3 ± 4.3</td>
<td>49.3 ± 13.2</td>
</tr>
<tr>
<td>Type V</td>
<td>(n = 11/8)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.7 ± 0.9</td>
<td>2.6 ± 1.6</td>
</tr>
</tbody>
</table>

The percentage of adherence was calculated as the number of platelets adhering to the surface divided by the total number of platelets in the incubation mixture, expressed as a percentage. The results are means ± SD.

Comparison of Platelet Adherence among Uncoated and Collagen Surfaces in the Presence of DAPA

In the presence of DAPA, the amount of radioactivity that adhered to the surfaces coated with either Type III or Type IV collagen remained increased in comparison to the uncoated surfaces that were studied under identical conditions (Table 2). In addition, the presence of DAPA, the platelet adherence to surfaces coated with either Type III or Type IV collagen remained increased in comparison to Type V collagen-coated surfaces that were also studied in the presence of DAPA. There was little difference between platelet adherence values over uncoated surfaces and Type V coated surfaces using IM alone or IM containing ADP.

Platelet Adherence to Surfaces Coated with Solutions of Type V Collagen in PBS

Since phosphate concentration is important in the formation of fibrils by Type V collagen, the coating of plastic surfaces was performed in PBS rather than in MHBSS.21 Surfaces coated in the presence of the higher phosphate concentrations of PBS were studied in the presence of DAPA. Platelet adherence to surfaces coated with Type V collagen in this manner was quantitated at 1 ± 0.2% without ADP (n = 11) and 3.7 ± 0.9% with ADP (n = 12).

Platelet Adherence to Surfaces Coated with Type I Collagen

Surfaces coated with Type I collagen were studied in the presence of DAPA. Platelet adherence with Type I collagen (n = 8) was quantitated at 5.5% ± 1.6% without ADP and 56.5% ± 20.7% with ADP.

Table 2. Percentage of Adherence of Human Blood Platelets to Surfaces Coated with Specific Types of Human Collagen in the Presence of DAPA

<table>
<thead>
<tr>
<th>Coating</th>
<th>Unstimulated (%)</th>
<th>ADP-induced (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>(n = 10/10)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.1 ± 0.7</td>
<td>6.9 ± 2.0</td>
</tr>
<tr>
<td>Type III</td>
<td>(n = 3/4)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>6.9 ± 2.0</td>
<td>7.0 ± 3.9</td>
</tr>
<tr>
<td>Type IV</td>
<td>(n = 6/6)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>41.0 ± 20.7</td>
<td>4.6 ± 5.2</td>
</tr>
<tr>
<td>Type V</td>
<td>(n = 8/8)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.7 ± 0.2</td>
<td></td>
</tr>
</tbody>
</table>

The following volumes and concentrations were added to each uncoated or collagen-coated well: 0.3 ml incubation mixture (IM) or IM with 30 μM dansylarginine amide (DAPA) (final DAPA concentration 10 μM), 0.3 ml IM containing 30 μM adenosine 5'-diphosphate (ADP) (final ADP concentration 10 μM) and 0.3 ml of 51Chromium-labeled human platelets. Wells were rocked on a platform with a 30° tilt at a rate of 10 updown cycles per minute for 30 minutes at 37°C. Results are means ± SD.
type of collagen, the stimulation with ADP, and the use of DAPA, we performed an analysis of variance (ANOVA). The ANOVA showed that the interactions between stimulation with ADP and the use of DAPA, between the use of DAPA and the generic type of collagen, and among stimulation with ADP, the use of DAPA, and the type of collagen were not different from zero. The interaction between the absence or presence of stimulation with ADP and the generic type of collagen was different from zero \( p < 0.0001 \). We now estimate the percentage of adherence in each stimulation-type combination by using the estimates of the parameters in the model which were different from zero (Table 3). These estimated percentage responses will not be equal to the mean responses within the stimulation-type combination unless equal numbers of observations are obtained. This estimate (Table 3) will be more precise in the ANOVA.

Our data also find significance in the following observations. Types III and IV collagen present surfaces that cause platelets to adhere to them. In the absence of ADP stimulation, platelet adherence to surfaces coated with Type III collagen \( p < 0.01 \) or Type IV collagen \( p < 0.01 \) is greater than platelet adherence to uncoated surfaces. In the presence of ADP stimulation, platelet adherence to surfaces coated with Type III collagen \( p < 0.001 \) or Type IV collagen \( p < 0.001 \) is greater than platelet adherence to uncoated surfaces. Platelet adherence to Type III collagen is not significantly different from Type IV collagen at a 1% level of confidence in the absence or presence of ADP stimulation.

Type V collagen has poor platelet adherence characteristics that distinguish it from other types of collagen found in the blood vessel wall. Type V collagen presents a surface to which fewer platelets adhere than a surface coated with Type III collagen in the absence \( p < 0.01 \) or presence \( p < 0.001 \) of ADP stimulation. Surfaces coated with Type V collagen cause fewer platelets to adhere than those coated with Type IV collagen in the absence \( p < 0.01 \) or presence \( p < 0.001 \) of ADP stimulation.

### Scanning Electron Microscopy

Scanning electron micrographs of platelet adherence to uncoated and collagen-coated surfaces were obtained. In the absence of ADP stimulation, platelets do not adhere to uncoated surfaces or to surfaces coated with Types IV or V collagen. Individual platelets adhere to surfaces coated with Types I and III collagen when these surfaces are exposed to platelet-rich plasma in the absence of added ADP. Occasional platelet aggregates are present on surfaces coated with Types I and III collagen that are exposed to platelet-rich plasma in the absence of ADP stimulation (Figure 1).

ADP-induced platelet adherence was associated with the presence of individual platelets and platelet aggregates on surfaces coated with Types I, III, and IV collagen. In this part of the system where ADP stimulation has induced platelet aggregation, uncoated surfaces and surfaces coated with Type V collagen again do not adhere either individual platelets or platelet aggregates.

### Further Characterization of the Surfaces Coated with Type V Collagen

Two methods were used to demonstrate that Type V collagen was present and evenly distributed on the appropriate plastic surfaces. To demonstrate that Type V collagen was present on the appropriate

<table>
<thead>
<tr>
<th>Collagen</th>
<th>Concentration (µg/ml)</th>
<th>37°C exposure</th>
<th>Aggregation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Type I</td>
<td>200</td>
<td>yes</td>
<td>all positive</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>no</td>
<td>negative</td>
</tr>
<tr>
<td>Type III</td>
<td>200</td>
<td>yes</td>
<td>one positive</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>no</td>
<td>negative</td>
</tr>
<tr>
<td>Type IV</td>
<td>200</td>
<td>yes</td>
<td>all positive</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>no</td>
<td>incomplete</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>yes</td>
<td>aggregation X1</td>
</tr>
<tr>
<td>Type V</td>
<td>200</td>
<td>yes</td>
<td>one positive</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>no</td>
<td>negative</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>yes</td>
<td>negative</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>no</td>
<td>negative</td>
</tr>
</tbody>
</table>

Each aggregation chamber contained 150 µl of platelet-rich plasma and 300 µl of dansylarginine amide (DAPA) in incubation mixture (IM); final concentration was 10 µM DAPA. 50 µl of each collagen in solution in phosphate-buffered saline (PBS) or a PBS control were added to the cuvette. Aggregation was recorded over 30 minutes in the Payton module.
coated surfaces, five coated wells were washed and a sample taken for slab gel electrophoresis. This procedure was repeated with Types I, III, and IV collagen. Identical volumes of buffer containing collagen were subjected to electrophoresis. The resulting gels were run in a reduced and unreduced manner and showed typical collagen bands and were quantitated by use of a scanning densitometer. The amount of Type V collagen removed from the wells was approximately equal to that of Type I collagen. The amounts of Type V or Type I collagen removed by washing were in excess of quantities of Type III and Type IV collagen obtained in this way: Type V > Type I > Type III, Type IV. In addition, six wells coated with Type V collagen were washed as above and then incubated for 30 minutes with platelet-poor plasma under conditions which duplicated the adherence experiments. The characteristic chains of Type V collagen were demonstrated on the coated surfaces by unreduced gel electrophoresis after procedures identical to those used in the platelet adherence experiments (Figure 2). To show that the coated surface presented an even distribution of Type V collagen, indirect immunofluorescence was performed with an antibody to Type V collagen. Well surfaces coated with Type V collagen were photographed and distinguished by bright green uniform fluorescence. The presence and uniform distribution of Types I, III, and IV collagen were demonstrated by the even adherence of platelets over the entire coated surfaces in excess of the rare adherence to an uncoated surface.

Platelet aggregation was performed in the presence of DAPA with each of the purified collagens at a final concentration of 200 µg/ml or 20 µg/ml as the aggregating agent. The solutions which contained collagen were either brought from 4°C to room temperature before addition to the aggregation chamber or taken from 4°C and heated at 37°C for 120 minutes before use. The results are shown in Table 4. Type I collagen that had been exposed to 37°C was associated with platelet aggregation following the
Figure 1. B. The plastic surface was coated with 1.0 ml of a 50 \( \mu \)g/ml solution of Type IV collagen, washed, and exposed to platelet-rich plasma in the presence of 10 \( \mu \)M ADP. Platelet aggregates and individual platelets adhered to the entire surface. This representation is characteristic of the scanning electron micrographs showing platelet adherence to surfaces coated with Types IV, III, or I collagen in the presence of ADP stimulation. This technique showed no platelet adherence to uncoated surfaces or surfaces coated with Type V collagen in the presence of ADP stimulation. \( \times \) 1000.

Figure 2. Human Type V collagen. Lane 1. Sample of stock solution 500 \( \mu \)g/ml in PBS (Type V collagen standard). Lane 2. Sample of coated material removed from wells after incubation with 50 \( \mu \)g stock solution in 1 ml MHBSS for 150 to 180 minutes and two washes. Lane 3. Sample of coated material removed from wells after incubation with 50 \( \mu \)g stock solution in 1 ml MHBSS for 150 to 180 minutes, two washes, and 30 minutes of incubation with platelet-poor plasma at 37°C on rocking platform.

addition of a 200 \( \mu \)g/ml or (variably) 20 \( \mu \)g/ml concentration to an aggregation chamber containing PRP and IM with 10 \( \mu \)M DAPA. Type I collagen that was not exposed to the 37°C temperature did not yield platelet aggregation in this system. Type III collagen was not associated with platelet aggregation when 200 \( \mu \)g/ml or 20 \( \mu \)g/ml concentrations of either unheated or 37°C-exposed solutions were added to the aggregation chambers containing PRP and IM with 10 \( \mu \)M DAPA. Type IV collagen that had been exposed to 37°C for 120 minutes was associated with platelet aggregation following the addition of a 200 \( \mu \)g/ml or 20 \( \mu \)g/ml concentration to the aggregation chamber containing PRP and IM with 10 \( \mu \)M DAPA. Type IV collagen that was not exposed to the 37°C temperature variably gave partial platelet aggregation. Solutions containing Type V collagen that had not been exposed to the 37°C temperature did not result in platelet aggregation when added to the chambers containing PRP and IM with 10 \( \mu \)M DAPA. Solutions containing Type V collagen were warmed
to 37°C for 120 minutes before being added to the chamber containing PRP and IM with 10 μM DAPA and were variably associated with the aggregation of platelets.

Platelet aggregation was performed in the absence of DAPA with each of the purified collagens that were not associated with platelet aggregation when added as the aggregating agent at a final concentration of 200 μg/ml. The results are shown in Table 5. In the absence of DAPA Types I, III, and V collagen that were unexposed to 37°C were not associated with platelet aggregation when each was added at a final concentration of 200 μg/ml to the aggregation chamber containing PRP. Type III collagen that had been exposed to 37°C was not associated with platelet aggregation following the addition of a 200 μg/ml final concentration. Type IV collagen that was not exposed to the 37°C temperature variably gave incomplete platelet aggregation.

### Table 5. Collagen-Induced Aggregation of Platelet-Rich Plasma In the Absence of DAPA (n = 2)

<table>
<thead>
<tr>
<th>Collagen</th>
<th>Concentration (μg/ml)</th>
<th>37°C exposure</th>
<th>Aggregation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Type I</td>
<td>200</td>
<td>no</td>
<td>negative</td>
</tr>
<tr>
<td>Type III</td>
<td>200</td>
<td>no</td>
<td>negative</td>
</tr>
<tr>
<td>Type IV</td>
<td>200</td>
<td>yes</td>
<td>negative</td>
</tr>
<tr>
<td>Type V</td>
<td>200</td>
<td>no</td>
<td>aggregation X1</td>
</tr>
</tbody>
</table>

Each aggregation chamber contained 150 μl of platelet-rich plasma and 300 μl of incubation mixture. 50 μl of each collagen in solution in PBS or a PBS control were added to the cuvette. Aggregation was recorded over 30 minutes in the Payton module.

### Discussion

Human vascular tissue contains numerous structural macromolecular components. These materials are located in proximity to the endothelium and its basement membrane as well as at deeper subendothelial loci. Among them are collagens which have been further characterized according to genetic type. Types I, III, IV and V collagen have been associated with vascular tissue.1-11 Previous investigators12-27 have reported the interactions between collagen and platelets. Types I, III, IV, and V collagen have been shown to interact with platelets under defined experimental conditions.5,19-26 These same studies, however, provide conflicting evidence that supports a conclusion of no interaction between specific collagen types and platelets.19-26

Our results using high concentrations of purified collagens as the aggregating agent in a platelet aggregation method were different from the results with techniques that measured platelet adherence to collagen-coated surfaces, scanning electron microscopy, and the 51 Chromium-labelled platelet adherence system. When we studied the interaction between collagen and platelets using the aggregation method, adding Types I and IV collagen to platelet preparations identical to those used throughout these experiments resulted in platelet aggregation. Under the same conditions, the addition of Type III collagen to the platelet-rich plasma did not result in any platelet aggregation. The use of Type V collagen as the potential aggregating collagen was variably associated with platelet aggregation using this platelet-rich plasma system. In this experiment, the adherence of platelets was measured on surfaces coated with these highly purified collagen types and the results differed from those suggested by platelet aggregation studies under similar conditions. Platelet adherence was demonstrated with Types I, III, and IV collagen. Platelet adherence exceeded control (uncoated surface) values with Types I, III, and IV collagen, without other stimulation. ADP-induced platelet adherence similarly exceeded control situations with Types I, III, and IV collagens. The results using Types I, III, and IV collagen in the presence of DAPA suggested that platelet adherence in this system does not reflect an effect due to thrombin that might be generated in the platelet-rich plasma technique.

Platelet adherence studies with Type V collagen produced results quite different from those with Types III and IV collagen. Platelet adherence to Type V collagen was significantly decreased in comparison to Types I, III, or IV collagen in unstimulated or ADP-induced stimulation. ADP was used rather than thrombin-induced platelet adherence because Type V collagen is known to be sensitive to the protease activity of thrombin.32,33

The similar results with unstimulated and ADP-induced platelet adherence between uncoated and Type V collagen-coated wells, particularly in the presence of DAPA, led to questions concerning the structure and quantitative levels of the Type V collagen relative to the other collagen types, which was present on the dish.

It has been suggested21,27 that Type V collagen exists in at least two forms: one that is amorphous and not associated with platelet aggregation, and the other that is fibrillar and produces positive aggregation results. Dialysis against solutions of inorganic phosphate, as used in the preparation of Type V collagen in this study, is associated with the fibrillar state.21 Type V collagen prepared for these experiments produced positive platelet aggregation results.

Quantitation of the Type V collagen present on the experimental surface by slab gel electrophoresis of equivalent washings confirmed that Type V collagen was present in amounts similar to Type I collagen, and in excess of the amounts of Types III and IV collagen. The presence of Type V collagen on the
plastic surface under conditions identical to those used during the experiment was documented also by slab gel electrophoresis. The uniformity of the surface coating, assuring exposure of platelets to Type V collagen over the entire well surface, was demonstrated by immunofluorescence.

The relationship between the specific form which any generic collagen assumes when it adheres to a surface and the native form of that collagen is not established, although work with other proteins suggests that proteins that adhere are not irreversibly bound to the surface and may assume a number of configurations. The native form of Type V collagen, fibrillar, amorphous, or other, has not been established by available techniques. Our results suggest that a form of Type V collagen, which produces platelet aggregation, is not associated with platelet activation when coated onto a plastic surface and quantitated by an adherence technique. Further, our results demonstrate that this poorly adherent surface presented by Type V collagen differs in the degree of platelet adherence from other types of generic collagen that were tested.

Type V collagen has been associated with the endothelium and the immediate subendothelial basement membrane. Platelet adhesion to the subendothelium has been studied on damaged vessel walls by a morphometric technique. The rate of platelet adhesion to an exposed basement membrane surface is slow and the subsequent platelet thrombi that form reversibly detach and then disappear. In unstimulated platelet adherence experiments, we have found that quantitative platelet adherence is less at 30 minutes on surfaces coated with Type V collagen than to surfaces coated with Types III and IV collagen. In experiments with ADP-induced platelet adherence to Type V collagen, we have shown that platelet adherence is virtually absent in comparison with Types III and IV collagen. The poor platelet adherence characteristics found with Type V collagen in these experiments suggest an explanation for the reduced thrombogenic nature of the basement membrane of the intact subendothelium in comparison to deeper vessel wall components, as described by Baumgartner et al. Synthesis of Type V collagen has been demonstrated in vascular smooth muscle cells. It is possible that Type V collagen may have a role in promoting a less thrombogenic surface when smooth muscle cells replace endothelium as the interface with flowing blood following vascular injury and removal of the endothelium. Testing of this hypothesis is a logical extension of our observations.

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References
Diminished platelet adherence to type V collagen.
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