Sheep, pig, and human platelet–material interactions with model cardiovascular biomaterials

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Abstract: The relationship between cardiovascular device performance in animals and humans is not straightforward. As the principal formed element in a thrombus, platelets play a major role in determining the hemocompatibility of mechanical heart valves and other high-shear-rate cardiovascular devices. Since larger animals are required to test many such devices, sheep and porcine platelet responses were compared to humans. Adhesion, spreading, and the formation of thrombuslike structures were examined in vitro on pyrolytic carbon mechanical heart valve leaflets, National Institutes of Health-reference polyethylene and silicone rubber, and Formvar. Principal findings were that platelet responses are strongly dependent upon the biomaterial and the species: Porcine and human platelets spread extensively on pyrolytic carbon, formed thrombuslike structures on Formvar, and were least active on silicone rubber. Human and porcine platelets responded differently to polyethylene: Human platelets spread extensively, while porcine platelets remained pseudopodial. In contrast, sheep platelets attached much less, never reached fully spread shapes, and were far less active overall. Since porcine responses were generally similar to humans, pigs may be a useful predictor of in vivo platelet–biomaterial interaction in humans. Conversely, as ovine platelets were much less active, this must be accounted for in the evaluation of cardiovascular devices tested in sheep. © 1999 John Wiley & Sons, Inc. J Biomed Mater Res, 45, 240–250, 1999.

Key words: platelets; in vitro; pyrolytic carbon; mechanical heart valves; thrombosis

INTRODUCTION

The evaluation of invasive cardiovascular prosthetics and devices requires testing of mechanical properties, hemodynamics, and material biocompatibility. While it is feasible if not desirable to evaluate these various parameters individually in the laboratory, eventually the entire device must be examined in vivo, as it is not possible to predict the complex interplay between these and other properties. By necessity, most in vitro testing is performed using animal subjects. However, this introduces experimental complications, since nonhuman species differ from humans in their responses to cardiovascular devices. While animal models provide considerable insight into the design of such devices, there are certainly gaps in our understanding of species differences which must be understood to fully evaluate their predictive ability.

The selection criteria for a suitable animal model include anatomical similarity and relative size compared to humans, comparative rheological and mechanical stresses, hematology, ease and expense of care, and other factors. When heart valve prosthetics and other larger devices are evaluated, larger animal species such as calves, pigs, sheep, and baboons are typically used. For evaluation of mechanical heart valves, the most extensively used animal is the sheep,1–4 while the use of pigs appears to be increasing owing to their apparently greater hematological and anatomical similarity to humans.5–9

The substantial literature comparing the hematolog and blood–material interaction of different species has been adequately reviewed elsewhere.10–17 While many such studies have used multiple measures of platelet responses to materials in their analyses, few have compared how platelets from different animal models attach, spread, and grow thrombi on biomaterials. Since platelet-mediated thrombosis may ultimately determine the success or failure of mechanical heart valves and other vascular prosthetics and devices used in high-shear-rate or arterial locations, this represents a significant gap in our ability to evaluate in vivo animal studies.

Recent investigations of the material properties and
biocompatibility of pyrolytic carbon (PYC) used in mechanical heart valves necessitates the need for a better understanding of animal models used in their evaluation. For example, the topography of PYC in clinical valves is much rougher at the submicron scale than previously described, and this has direct influences on platelet adhesion.\textsuperscript{18,19} Second, \textit{in vitro} studies have demonstrated that human platelets attach and become extremely well spread on PYC even in the presence of albumin,\textsuperscript{18,20} thereby indicating that PYC may not be as passive to platelets as previously described.\textsuperscript{21–24} Third, careful preparation of mechanical valves for scanning electron microscopy (SEM) subsequent to explantation from nonanticoagulated sheep indicates significant levels of platelet attachment and aggregation.\textsuperscript{1,25} To evaluate the relationship of these \textit{in vivo} studies with sheep to responses in humans, it is necessary to understand more fully how sheep platelet responses to PYC may differ from human platelets. Hence, the overall objective of the current study was to investigate platelet responses of the two most often used large animals models in the testing of cardiac valvular prosthetics: sheep and pigs. Such studies “to correlate \textit{in vitro} results with \textit{in vivo}’ were previously recommended in a National Institutes of Health (NIH) guidelines document.\textsuperscript{17} In the present work, a well-established \textit{in vitro} platelet-spreading assay was employed.\textsuperscript{18,20,26–29} This \textit{in vitro} experimental assay has been previously demonstrated to be predictive of \textit{in vivo} platelet responses in canines and macaques.\textsuperscript{30,31}

**MATERIALS AND METHODS**

Materials

Platelet responses were assessed to four different model materials chosen for clinical applicability and known ability to induce a wide variation in platelet responses.\textsuperscript{20,26,32,33} These materials include low-temperature isotropic PYC valve leaflets, polyethylene, silicone rubber, and Formvar. PYC was supplied by St. Jude Medical, Inc. (St. Paul, MN) and cleaned using successive ultrasonic baths of detergents, water, and organic solvents following manufacturing protocols. (For ease of sample handling, valve leaflets were cut into approximately 5 × 10-mm rectangles as used in clinical devices. (For ease of sample handling, valve leaflets were cut into approximately 5 × 10-mm rectangles prior to cleaning.) Previous studies have documented the surface properties of PYC, including its surface energetics, surface chemistry, morphology, protein adsorption, and platelet adhesion properties.\textsuperscript{18,20,22–40} The next two materials were NIH-NHLBI reference polyethylene (PE) and silicone rubber (SIL),\textsuperscript{32,33,41,42} supplied by Abiomed R&D, Inc. (Danvers, MA) as thin sheets in sterile packaging. These were used as received except for cutting into 5 × 10-mm rectangles. The final material was Formvar, a commercial polynvinyl formal resin commonly used as a surface to culture and support platelets and cells for transmission electron microscopic (TEM) imaging.\textsuperscript{26,27,43,44} Formvar films were cast from ethylene dichloride onto glass substrates and subsequently placed onto TEM grids as per standard protocols. Contact angle analysis of this hydrophilic material has been previously reported.\textsuperscript{26}

**Experimental animal species**

Blood was obtained using standard venipuncture methods from normal healthy sheep and pigs, maintained for purposes other than this study. Two Suffolk and one Dorset–Suffolk cross adult sheep 2–3 years old were used. One sheep had minor arthroscopic surgery 3 days prior to obtaining blood. (Preoperative and operative treatment included xylazine 0.2 mg/kg, ketamine 0.5 mg/kg, and diazepam 0.1 mg/kg, and surgical anesthesia with halothane 1.5–2.0% and oxygen 2.0 L/min.) No drugs were used after surgery or prior to obtaining blood. No differences in platelet responses were observed with the postsurgical animal or with the different strains; hence, all were analyzed together.

Blood was also obtained from three normal healthy Yorkshire cross juvenile pigs (8–12 weeks old, 21–25 kg). The pigs received 25 mg/kg pentobarbital to facilitate handling prior to obtaining blood. NIH guidelines for the care and use of laboratory animals (NIH Publication No. 85-23 Rev. 1985) were observed.

Blood was obtained from healthy 30–50-year-old adult humans who had taken no medication for at least the previous 2 weeks. Data in this report are based on platelets from two individuals. This was deemed appropriate, since the observed responses were comparable to those previously observed in dozens of normal healthy humans under identical or similar experimental conditions.\textsuperscript{18,20,26,31} All subjects enrolled in this research responded to an informed written consent which has been approved by the Institutional Committees on Human Research at the University of Wisconsin–Wisconsin and the University of Connecticut Health Center.

**Platelet studies**

Platelets from all three species were purified from acid-citrate-dextrose (1:9) or sodium citrate anticoagulated whole blood by centrifugation at 120 × \(g\) for 15 min, followed by gel filtration.\textsuperscript{45} HEPES–Tyrodes buffer containing 1 mg/mL bovine serum albumin (BSA) (Sigma A-7888; St. Louis, MO) was used as the platelet suspension buffer and as the column-washing eluent.\textsuperscript{46} Column-washed platelets were collected early in their elution, since these are free of detectable fibrinogen, fibronecrotin, thrombospondin, and von Willebrand factor.

Test materials were initially hydrated in HEPES-Tyrodes platelet buffer minus BSA for at least 1 h. Platelet adhesion was then evaluated by applying column-washed platelet suspensions to the test materials for 45 min at the appropriate physiological temperature for the specie. The concentrations of column-washed platelet suspensions were 222,000–288,000/\(\mu\)L for pigs, 114,000–360,000/\(\mu\)L for sheep, and approximately 200,000–300,000/\(\mu\)L for humans. Platelet
suspensions were incubated on surfaces for 45 min, since this leads to extensive surface coverage and permits surface aggregates to form, as previously observed with human platelets.

Adherent platelets were prepared for electron microscopy by fixation in 1% glutaraldehyde in 0.1M HEPES buffer at pH 7.3 for 30 min, rinsing with HEPES buffer, followed by 15 min postfixation in 0.05% HEPES-buffered osmium tetroxide. Samples were then dehydrated with graded ethanols and dried by the critical-point procedure with carbon dioxide. Samples were then ion beam–coated with 2–4 nm Pt or sputter-coated with 5 nm of AuPd and imaged at 1.5 keV with a Hitachi S-900 FE-SEM, or at 2.5–4 keV with a Jeol 6320 FEV-SEM.

Analysis of platelet responses

Scanning electron microscopic images were obtained from randomly selected areas at ×1000 or ×2000 magnification for quantitative analysis of platelet–material interaction. The extent of platelet spreading was examined by categorizing platelet shapes into five morphological forms describing increasing activation. These are discoid or round (R), dendritic (D) or early pseudopodial, spread dendritic (SD) or intermediate pseudopodial, spreading (S), and fully spread (FS). The spreading stages are shown in Figure 1 and defined in the legend. Additional measured parameters included the number of adherent platelets per area (platelet deposition) and the percentage of the material surface covered by platelets (platelet coverage %). Two measures were also made of the tendency to form thrombuslike structures: (a) the ratio of platelet–platelet cohesion to platelet–material adhesion, defined as the number of platelets adherent only to other platelets relative to the number of platelets in direct material contact; and (b) the number of focal centers, defined as microthrombi-like tight aggregates of three or more platelets.

The number of platelets analyzed per material varied from about 200 to as many as 1000, as this was dependent on the extent of platelet deposition. Analysis of variance of platelet responses was calculated using SPSS Version 6.1, with all tests performed at \( p \leq 0.05 \). Levene tests for homogeneity of variances were checked before analysis of variance, and the Tukey-HSD test was used to determine significant differences.

RESULTS

Human platelets

The response of human platelets varied greatly within the four test materials. PYC and PE were almost completely covered by spread platelets, platelets on FVR were extensively clustered into aggregates, and minimally shape-changed (pseudopodial) platelets were scattered over the SIL surface (Fig. 2). Numerical shape analysis confirmed mostly fully spread platelets on both PYC and PE, with a substantial percentage of dendritic or early pseudopodial forms on top of the material adherent platelets (Fig. 3). Platelets in direct material contact with PYC were spread to a greater extent (greater spread area) than those in contact with PE. Comparative numerical analysis suggests slightly greater deposition levels on PYC than PE, and a slightly greater extent of cohesion on PE, although group means were not significantly different at \( p < 0.05 \) (Fig. 4). Platelets on both PYC and PE produced similar low levels of focal centers (Figs. 2 and 3).

Platelet responses to SIL and FVR were quite different from those on PYC and PE. SIL induced minimal spreading with mostly dendritic forms and almost no fully spread, spreading, or spread dendritic shapes (Figs. 2 and 3). The level of platelet cohesion on SIL was essentially zero (Fig. 4). (The SIL surface was often extensively wrinkled, and in some cases platelets appeared to be partially buried in the material. This appearance is likely an artifact due to the effects of

Figure 1. Diagrammatic depiction of platelet spreading divided into five shape categories for analysis. From left to right, these stages of spreading are defined as follows: (R) or discoid: no pseudopodia present; dendritic (D) or early pseudopodial: one or more pseudopodia with no evident flattening; spread dendritic (SD) or intermediate pseudopodial: one or more pseudopodia flattened, hyaloplasm not spread between pseudopodia; spreading (S): hyaloplasm spread between pseudopodia; and fully spread (FS): hyaloplasm extensively spread, no distinct pseudopodia.
high-pressure CO$_2$ on the silica-free NIH-reference silicone during critical-point drying.) In contrast, FVR induced a high level of platelet cohesion and the highest level of focal centers for all materials (Figs. 2 and 4). The extent of clustering (surface aggregation) on FVR was so great as to obscure virtually all of the spread platelets in material contact [Fig. 2(d)]. (From other studies, it is known that a base layer of spreading and/or fully spread platelets underlies such thrombuslike surface aggregates.$^{26,46}$) The level of platelet deposition and surface coverage levels on SIL and FVR were lower than on PYC and PE. Rank orders of the extent of the platelet responses are summarized in Table I.

**Sheep platelets**

In all respects, the level of response of sheep platelets was considerably less compared to those from humans. Sheep platelets on PYC were mostly round and dendritic with 20 ± 13% in the spreading shape category. No fully spread platelets were observed. The shape distribution on PE was similar to PYC, although fewer (10 ± 11%) were of spreading shapes. SIL induced the greatest percentage of dendritic forms as well as significant numbers of spread dendritic and dendritic forms. In contrast, no spread dendritic and dendritic forms were observed on FVR (Figs. 3 and 5).

On all materials, the number of adherent platelets...
was much lower than that observed with human platelets. Figures 3–5 illustrate the minimal response of sheep platelets compared to human ones by all measures: minimal numbers of adherent platelets, almost no cohesion, very low surface coverage, and no focal centers. The extent of spreading was also much lower, with no fully spread sheep platelets on any material (Figs. 3 and 5).

Porcine platelets

The response of porcine platelets to the various materials was much more extensive than sheep. Platelets in direct contact with PYC reached fully spread and spreading morphologies. These, in turn, formed a base layer for large numbers of pseudopodial platelets on top (Figs. 3 and 6). Platelets on PE were evenly distributed over the material surface and were almost entirely of dendritic and spread-dendritic shapes. Formvar induced extensive clustering of pseudopodial platelets on top of mostly spreading-type morphologies. Platelets on SIL were of round or dendritic shapes.

The extent of most porcine platelet responses was comparable to that of human platelets (Fig. 4). The number of adherent platelets per area was quite similar, including greater platelet deposition on PYC and PE and lower deposition on SIL and FVR ($p < .05$). Surface coverage was also roughly similar between porcine and human platelets, although the lower level of coverage on PE by porcine platelets ($p < .05$) was unlike the human platelet response. There were some species differences between the levels of cohesion and

Figure 3. Platelet shape distributions for each material and species. Each histogram shows the relative percent of platelets (mean ± SD) in each of five morphological forms as defined.
focal centers. The porcine platelet level of cohesion was much higher on PYC than on other materials, although there were few focal centers. Thus, while there were many platelets in contact with others on PYC, few were tightly aggregated. The cohesion level for PE was much lower for porcine than for human platelets. Few focal centers were observed on the four materials (Fig. 4), but the highest level was observed on FVR, although this was statistically significant only in comparison to PE.

DISCUSSION

The objective of the present study was to examine how platelets from different species respond to model biomaterial surfaces. There were significant differences in the response of platelets from sheep, pigs, and humans. Since the principal reason for this investigation was to use this as a basis for evaluating sheep and pig responses, the following discussion will emphasize this species comparison.

Sheep

The most widely used animal model for the evaluation of prosthetic heart valves and many other implanted cardiovascular devices is the sheep. In the present study, large differences were observed be-
thrombi on biomaterial surfaces, this may significantly alter the biological response to PYC in vivo. Although it is clear that platelets do attach and form microthrombi on carbon heart valves prosthetics in vivo in unanticoagulated sheep, the mechanisms for surface activation and thrombus growth on biomaterial surfaces are clearly different in extent and may also be different in some aspects of mechanism. This should be kept in mind when evaluating explanted PYC devices from sheep.

**Pigs**

The use of pigs as a model species for the evaluation of cardiovascular devices appears to be increasing. Hence, it is becoming increasingly important to understand how porcine platelets act at biomaterial interfaces. In the current investigation, porcine platelets behaved similar but not identically to human platelets. On PYC, porcine platelets spread more than on any of the other three surfaces, with 25 ± 15% [mean ± standard deviation (SD)] reaching fully spread. In contrast, 59 ± 16% of human platelets reached the fully spread stage on PYC (Fig. 3). The level of spreading of porcine platelets on SIL and FVR was roughly similar to the human platelet responses, with the maximum extent of both species reaching the spreading shape, and with dendritic platelets most common. However, the absence of fully spread porcine platelets on PE was markedly different from the 65 ± 15% fully spread human platelets on this material (Fig. 3).

The levels for porcine platelet deposition and surface coverage were quite similar to humans, except that surface coverage was apparently less on PE owing to the lower surface area of dendritic versus fully spread and spreading platelets (Fig. 4). Differences in platelet cohesion were also apparent between pig and human platelets on PYC and PE, with greater cohesion on PYC, and much lower cohesion on PE. (Again, this is likely due to porcine platelets not spreading on PE.) On FVR, the extent of cohesion was similar for human and pig platelets. The number of focal centers or thrombus-like structures was greatest on FVR for both species (Fig. 4). The rank orders of the extent of these various responses are similar for both pig and human platelets, except for the lower response of porcine platelets to PE (Table I).

**TABLE I**

<table>
<thead>
<tr>
<th>Specie</th>
<th>Measure of Platelet Response</th>
<th>Order of Response to Materials</th>
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<tbody>
<tr>
<td>Human</td>
<td>Spreading</td>
<td>PYC, PE, FVR, SIL</td>
</tr>
<tr>
<td></td>
<td>Deposition</td>
<td>FVR, PE, PYC, SIL</td>
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<tr>
<td></td>
<td>Cohesion/adhesion</td>
<td>FVR, PYC, PE, SIL</td>
</tr>
<tr>
<td></td>
<td>Surface coverage</td>
<td>PE, PYC, PYC, FVR, SIL</td>
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<tr>
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<td></td>
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<td>PYC, FVR, PE, SIL</td>
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<tr>
<td></td>
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*Responses of sheep platelets were much lower than other species, as noted: Spreading = no fully spread morphologies; deposition = much lower levels than other species; cohesion/adhesion = very low, not material differences; focal centers = extremely few observed; surface coverage = very low.

The observation that sheep platelets did not attach or spread to nearly the same extent. Major differences between sheep and human platelets were observed in all evaluation categories: No fully spread forms were observed on any material, the level of deposition and surface coverage was much lower, and there was much less interaction between platelets, as evidenced by the low cohesion/adhesion ratio and the number of focal centers. Sheep platelets responded with the greatest extent of spreading on PYC, somewhat less on PE and SIL, and least on FVR. This was quite different from human platelets, as shown in Figure 3 and Table I. Levels of human platelet deposition and platelet coverage were greatest on PYC and less on the other materials (Figs 3 and 4 and Table I).

The observation that sheep platelets did not reach fully spread morphological forms is not surprising, since not all species do. For example, adherent spreading bovine platelets typically exhibit a more stellate, rather than pancake, appearance. (R. M. Albrecht, University of Wisconsin, personal communication). Hence, this lack of full spreading may be common to platelets from ruminant species. Since human platelets spread extremely extensively on PYC in vitro, and as spreading is a part of how human platelets form large thrombi on biomaterial surfaces, this may significantly alter the biological response to PYC in vivo. Although it is clear that platelets do attach and form microthrombi on carbon heart valves prosthetics in vivo in unanticoagulated sheep, the mechanisms for surface activation and thrombus growth on biomaterial surfaces are clearly different in extent and may also be different in some aspects of mechanism. This should be kept in mind when evaluating explanted PYC devices from sheep.

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drawing blood. O’Rourke et al. reported that in vitro exposure of canine platelets to sodium Thiamylal (a barbiturate) reduced the number of fully spread platelets on glass by up to about one third of untreated platelets. Furthermore, this effect was also induced by transient in vitro exposure of human platelets to Thiamylal for only 10 min. Of particular relevance to the present study, these authors also reported inhibited shape change and platelet aggregation with non-thiol-containing barbiturates including pentobarbital (used in the present study) and barbital. Human platelet aggregation to several agonists is also inhibited by pentobarbital and other barbiturates, although this study did not examine platelet spreading. It is thus possible that the sedation of pigs with pentobarbital may have reduced the level of platelet spreading. If so, porcine platelet responses may be even more similar to the human platelet responses than reported here.

**Summary**

The rank order of response was fairly similar among all species: Spreading and deposition were greatest on PYC and slightly less on PE and FVR for all species, while minimal responses were generally elicited to SIL (Table I). Similar ordering of responses to materials has been previously reported for canines and rhesus macaques, depending upon the parameter measured indicated in Table I. Since no single measure was sufficient to describe the platelet response, this indicates the need for comprehensive measurements. Furthermore, multiple parameter in vitro models provide mechanistic insight into platelet–material interaction and thrombosis by separating different aspects of the response. For example, does a surface promote thrombi (measures of cohesion and focal centers), is it “sticky” to platelets (platelet deposition), or does it
induce spreading (shape categorization)? Using these measures provides insight into these thrombotic processes, which would be difficult or impossible to examine in vivo. These measures can then be used to guide the interpretation of in vivo results in animal models.

With respect to the comparison of the different species to that of humans, the major findings of the present study are that:

1. Sheep platelets are considerably attenuated in their response to biomaterials compared to human platelets. In particular, the observation that sheep platelets do not reach fully spread forms must be considered, since human platelets become extremely spread on PYC. Based upon in vitro studies with human platelets, full spreading and surface coverage may be important in the reasonable clinical biocompatibility of this material.\(^{18,20}\) (However, it should be noted that the extent of spreading of human platelets on PYC MHVs in vivo has not yet been examined.) This is not to state that the sheep is an inappropriate model, since nonanticoagulated sheep platelets do form thrombi in vivo on carbon heart valves. Sheep are also an appropriate model to evaluate other aspects of cardiovascular biocompatibility such as hemodynamics and calcification. Based upon the present in vitro study, it should simply be recognized that sheep platelets do not attach, spread, and grow thrombi to the same extent and the same manner as human platelets do. This has also been supported by observations of minimal platelet spreading on MHVs explanted from sheep.\(^{1,25,39,48}\)

2. Porcine platelet responses were generally similar to human platelet responses. The major differ-

**Figure 6.** Scanning electron micrographs of porcine platelets adherent to (a) PYC, (b) PE, (c) FVR, and (d) SIL.
ence was that porcine platelets did not spread quite as extensively on PYC, and the level of spreading on PE was much less extensive than human platelets. Since the pigs were treated with barbiturates which could have caused this inhibition, it is possible that porcine platelets in vivo will actually more closely model human responses than indicated by the present results. The spreading behavior of porcine platelets on PYC in vivo has not yet been examined using the methods outlined in the present study, and in conjunction with SEM sample preparation to enhance the imaging of adherent platelets on explanted valves. Nonetheless, the present findings do suggest that the pig is a suitable model to examine platelet attachment and thrombosis on cardiovascular biomaterials.

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