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◆ EXPERIMENTAL INVESTIGATION ◆

A Model System to Assess Key Vascular Responses to Biomaterials

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Purpose: To establish a reproducible laboratory test to evaluate prospective vascular biomaterials with respect to their thromboinflammatory properties by examining fibrinogen, platelet, and monocyte binding. Endothelial migration onto these surfaces was used as an index of vascular healing.
Methods: To evaluate biomaterials for potential thrombogenicity and inflammation, binding assays of radiolabeled human fibrinogen, platelets, and monocytes were performed on standard pieces of vascular biomaterials, including metals and polymeric and ceramic-coated materials. Using an established in vitro endothelial cell migration model, the relative migration rate of cultured human aortic endothelial cells onto these vascular biomaterials was measured and compared. The fibrinogen, platelet, and monocyte binding results were combined along with the migration results to create an overall score of biocompatibility.
Results: A significant direct relation of platelet and monocyte binding to the amount of adsorbed fibrinogen was observed. In contrast, migration rates of cultured human aortic endothelial cells onto the same biomaterial surfaces were found to be inversely related the amount of bound fibrinogen. Among the materials tested, stainless steel received the highest score of biocompatibility, while turbostratic carbon scored the lowest.
Conclusions: Fibrinogen, platelet, and monocyte binding levels, as well as endothelial migration rates onto vascular material surfaces, provide a basis for evaluating thrombogenicity, inflammatory potential, and endothelialization in the laboratory prior to in vivo testing.

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Procedures involving angioplasty with stenting have become a dominant approach to restore patency in arteries with occlusive vascular disease. Though these procedures have become established and well accepted, in-stent restenosis related to intimal hyperplasia remains a significant potential long-term problem. A continual quest to find a biomaterial surface that optimally interfaces with the biology of the vascular environment is ongoing. Material-related in-stent restenosis may be due to events occurring within minutes or months following implantation. Although delayed responses, such as the direct toxic effect of biomaterials and/or hypersensitivity reactions, may be quite relevant to the restenosis process, the acute events following implantation are felt to be determinants of

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biocompatibility. The test method proposed here addresses these short-term postimplantation interactions.

Thrombosis, inflammation, and vascular healing directed toward re-establishing a viable endothelium at sites of angioplasty-induced injury are key elements of the vascular environment that determine the restenotic response. An in vitro model system that reproducibly predicts these key responses to a novel or currently available biomaterial could be advantageous in screening biomaterials prior to costly animal and clinical studies. As hallmarks of these three key response elements, we have selected fibrinogen and platelet binding (thrombosis), monocyte binding (inflammation), and endothelial cell migration (vascular healing) to study in a proposed biomaterial evaluation model system.

Though accumulation of fibrinogen at sites of injury associated with arterial revascularization procedures, including stent implantation, is an important element in normal vascular wound healing, it is also a key factor determining possible restenosis due to acute thrombosis or occlusive intimal thickening. Accordingly, elevated plasma fibrinogen levels have been demonstrated to be an independent predictor of restenosis in patients treated with balloon angioplasty[1] and stents.[2] Therefore, there seems to be a cause-and-effect interplay with fibrinogen that gives this molecule an important role in the mechanisms leading to thrombosis, inflammation, and ensuing restenosis. Adsorbed fibrinogen provides the ligands for platelets and monocytes, which are central elements in the response to vascular materials. For this reason, we used fibrinogen adsorption on material surfaces as one of the tests of vascular material biocompatibility.

Fibrinogen is a complex molecule strategically equipped to promote interaction with key cellular elements involved in wound healing, hemostasis, and inflammation. Accordingly, high-affinity binding sites exist within the fibrinogen molecule, which provide ligand sites for receptors on platelets, leukocytes, and endothelial cells. The specific platelet binding site within the fibrinogen molecule is a dodecapeptide (400–411) at the carboxy terminus of each of the two gamma chains that serves as a specific ligand for the glycoprotein (Gp) IIb/IIIa receptor on the surface of activated platelets.[3,4] Though there are RGD (arginine, glycine, aspartic acid) peptide adhesive sequences within fibrinogen that can also act as binding sites for the activated platelet receptor, these appear to remain inaccessible until a conformational change has occurred within the fibrinogen molecule associated with an initial platelet-binding event at the gamma site.[5]

The monocyte/macrophage, now recognized as an important contributor to arterial restenosis associated with intimal hyperplasia,[6] binds to adsorbed fibrinogen through specific binding sites for the Mac-1 receptor expressed on the monocyte/macrophage. These intramolecular Mac-1 binding sites, designated P1 and P2,[7] are protected deep within the primary structure of the fibrinogen gamma chain and the tertiary structure of the D domain of the circulating molecule. These internal Mac-1 binding sites are known to become accessible as fibrinogen changes its conformation upon binding to vascular injury sites or artificial surfaces.

Kipshidze et al.[8] demonstrated that re-endothelialization at arterial interventional sites is an important factor in limiting the amount of smooth muscle cell (SMC) activity involved in the chronic restenotic disease process. Previous studies in animals indicated that the more rapid the rate of re-endothelialization in areas of balloon-induced arterial injury, the lower the amount of SMC-associated intimal hyperplasia.[9,10] The beneficial influence of restoring the endothelium is associated not only with re-establishing a barrier to plasma protein, platelet, and monocyte adherence to the injury site, but also with the endothelial production of factors that limit SMC migration and proliferation (e.g., nitric oxide, heparan sulfate, tissue plasminogen activator, and thrombomodulin). Thus, stent biomaterial surfaces that encourage more rapid endothelial cell coverage should be advantageous.

Since determining the accumulation of fibrinogen at sites of arterial stent placement reflects the relative affinity of circulating fibrinogen to foreign surfaces, the objective of the present study was to examine the relative binding and function of fibrinogen on a wide

range of potential intravascular materials from metals to polymers and ceramic materials. Predictable substrate-specific fibrinogen function should provide a means of objectively evaluating currently employed intravascular materials, as well as a means of evaluating proposed novel surfaces for intravascular use.

METHODS

Substrate Materials

Test materials were standardized to a uniform size of 1×1-cm flat squares of 600-μm thickness. The metals evaluated were obtained as high purity standards from Goodfellow Corporation (Berwyn, PA, USA) with the exception of electropolished 316L stainless steel (Cordis Corporation, Nutley, NJ, USA) and electropolished nitinol (NDC, Santa Clara, CA, USA). Nonmetallic materials evaluated included silicone (Dow Chemical, Dearborn, MI, USA), silicon (*www.UniversityWafer.com*), silicon carbide (gift from Dr. Dave Nieman at Hewlett-Packard, Inc., Corvallis, OR, USA), Teflon (polytetrafluoroethylene [PTFE]; DuPont, Inc., Wilmington, DE, USA), polyester film (3M, Minneapolis, MN, USA), and Corethane (polycarbonate urethane; gift from The Polymer Technology Group, Berkeley, CA, USA). Diamond-like carbon (DLC) was a generous gift from Dr. John Woodford at Argonne National Laboratories (Argonne, IL, USA). Turbostratic carbon was obtained from the McMaster-Carr Company (Atlanta, GA, USA).

Fibrinogen Binding Studies

Fibrinogen binding studies were performed using human fibrinogen I 125 (Amersham Biosciences, Amersham, UK) in human plasma standardized to a total fibrinogen concentration of 250 mg/dL. The amount of labeled fibrinogen added was equivalent to 0.1% of the total plasma fibrinogen. Test specimens were incubated with radiolabeled fibrinogen for 60 minutes at 37°C, rinsed with phosphate buffered saline, and counted in a Packard Bell radiospectrometer. Total bound fibrinogen (ng/cm^2) was calculated using the known specific activity of the radiolabeled protein, normalizing the final amount of labeled bound concentration based on its proportion of the total fibrinogen.

Monocyte and Platelet Isolation and Binding Assays

Blood was collected into tubes containing acid-citrate-dextrose (ACD; 1:10 ACD:blood) by venipuncture from healthy human volunteers and divided equally for platelet and monocyte isolation. Monocytes were isolated with a standard buoyant density centrifugation technique as described previously,[9] radiolabeled with indium 111 for 60 minutes at room temperature, and resuspended in autologous platelet-free plasma at 2×10^6 monocytes/mL for binding studies. Cell specific radioactivity was determined by cell (Coulter counter model Z1) and radioactive counting of a known aliquot of cells.

Platelets were isolated and radiolabeled with indium 111 using the method described by Thakur et al.[11] and resuspended in autologous platelet-free plasma at 10^8 platelets/mL. For platelet and monocyte binding assays, test material pieces were affixed to culture dish surfaces and incubated with either radiolabeled platelets or monocytes for 60 minutes at 37°C. After rinsing, each piece was counted for radioactivity, and surface-bound cell counts were calculated from the total counts per minute divided by the cell specific radioactivity. Final results were expressed as cells/area (cm^2) of test surface.

Endothelial Cell Migration Studies

Human aortic endothelial cells (HAEC) obtained from Clonetics (San Diego, CA, USA) were cultured and their migration evaluated using a previously described in vitro migration model (Fig. 1).[12] In brief, a model arterial surface was created using a 1% v/v collagen type-I solution (Collaborative Research, Waltham, MA, USA) cross-linked with ammonium to form a firm gel. After rinsing, HAECs were seeded onto the gels and cultured to attain confluence. Sterile test materials were individually implanted directly on the surface of the endothelialized gel such that the top surface of each piece was flush with the sur-

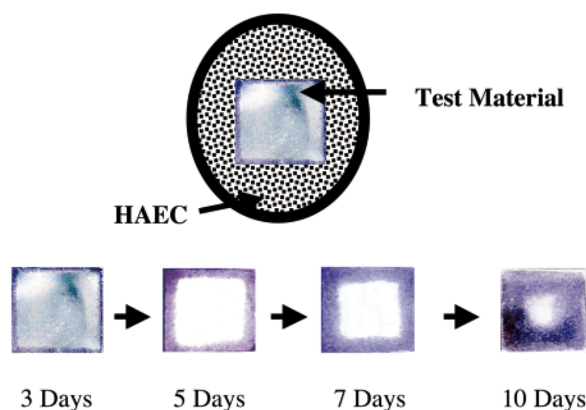

Figure 1 ◆ Diagram of HAEC migration from culture surface onto test material.

rounding gel surface. The gels were incubated at 37°C for 10 days to allow endothelial cell migration onto the test surfaces. The distance of cell migration onto each piece of material was measured using a microscope-based micrometer as the perpendicular distance from the midpoint of each edge to the leading edge of migrating cells toward the center of the square.

Material Biocompatibility Score

To integrate the results gained from each of the 4 individual parameters measured for each material, a scoring system was devised to allow a more effective comparison among the different biomaterial surfaces examined. The long-range goal of development of this scoring system was to provide a basis for prospectively evaluating novel biomaterial surfaces for potential intravascular biocompatibility. The results within each of the 4 parameters (fibrinogen, monocyte, and platelet binding; endothelial cell migration) were divided into quartiles from lowest to highest values. The lowest quartile of the fibrinogen, platelet, and monocyte binding assays was assigned a 4, indicating that these surfaces exhibited the least binding of these blood elements. For each material, the scores from these 3 binding assays were summed and then averaged to yield a mean thromboinflammatory score. With respect to the endothelial migration results, a score of 4 was assigned to the highest quartile of values. The final score was attained by summing the results of the average thromboinflammatory score and the endothelial migration score. Materials with the highest score exhibited the lowest fibrinogen, platelet, and monocyte binding and the highest rate of endothelial migration onto their surfaces.

RESULTS

Fibrinogen Adsorption

A wide range of fibrinogen adherence was observed among the various surfaces (Fig. 2). Stainless steel demonstrated the lowest level of fibrinogen adherence with the exception of polyHEMA-coated stainless steel. Elgiloy, Corethane, Teflon, and nitinol all exhibited similar amounts of fibrinogen binding relative to stainless steel. Though the levels of fibrinogen binding to nitinol were not significantly higher than stainless steel, the two components of this shape-memory alloy (nickel and titanium) both exhibited significantly higher levels of binding.

Similarly, the component metals of stainless steel, L605, and Elgiloy individually exhibited higher fibrinogen adherence than their alloy. Gold exhibited more than twice the level of fibrinogen binding relative to stainless steel ($p<0.01$). DLC, silicon carbide, and turbostratic carbon bound very high levels of fibrinogen, ranging from 3 to nearly 18-fold increases relative to stainless steel. Among the polymers examined, only polyester exhibited significantly higher levels of fibrinogen uptake relative to stainless steel, while the polyHEMA surface bound significantly less ($p<0.01$) than stainless steel.

A pattern of platelet adherence (Fig. 3) similar to that for fibrinogen adherence was observed, with some notable exceptions. Tantalum bound less than half the number of platelets per surface area and the least among the substrates examined. Nitinol binding levels were also significantly less ($p<0.05$) than the level bound to stainless steel. Elgiloy and L605, which had exhibited levels of fibrinogen binding similar to stainless steel, bound significantly more ($p<0.01$) platelets. The other materials that had higher levels of fibrinogen binding also exhibited higher platelet adher-

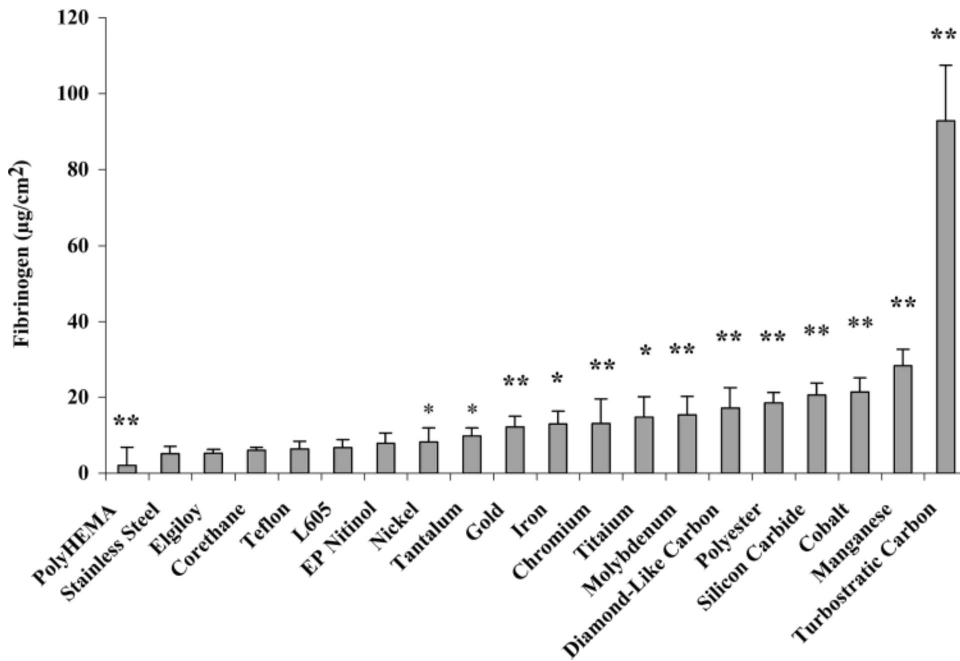

Figure 2 ◆ Binding of human fibrinogen I 125 in plasma to test materials. Results are expressed as mean ± 1 standard deviation. *P<0.05 and **p<0.01 (n=7) by Student *t* test with reference to stainless steel.

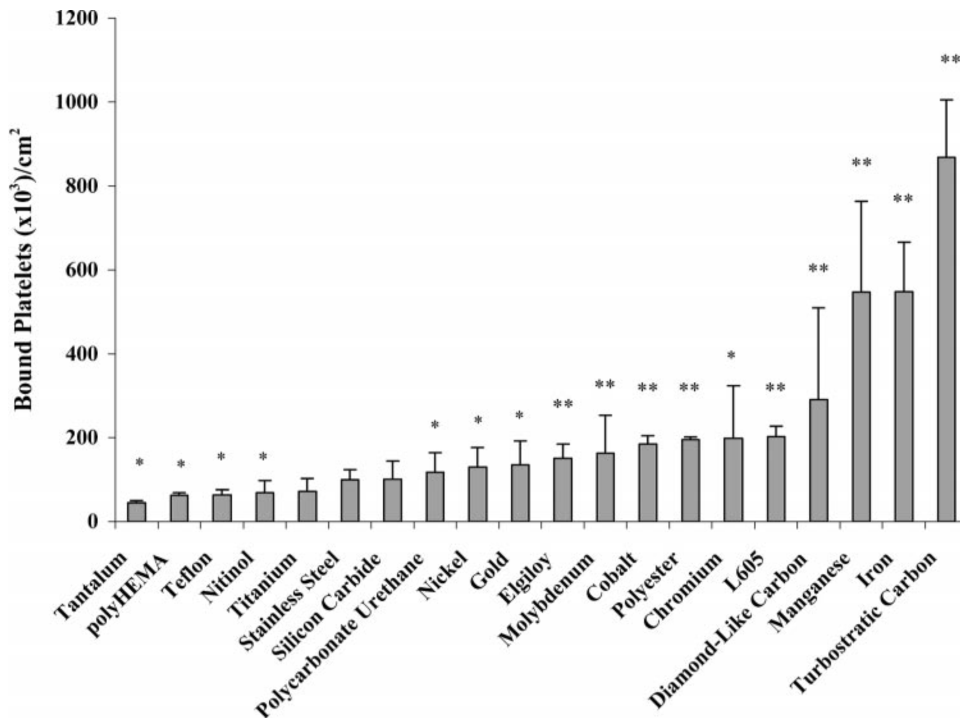

Figure 3 ◆ Binding of ^{111}In-labeled human platelets in plasma to test materials. Results are expressed as mean ± 1 standard deviation. *P<0.05 and **p<0.01 (n=7) by Student *t* test with reference to stainless steel.

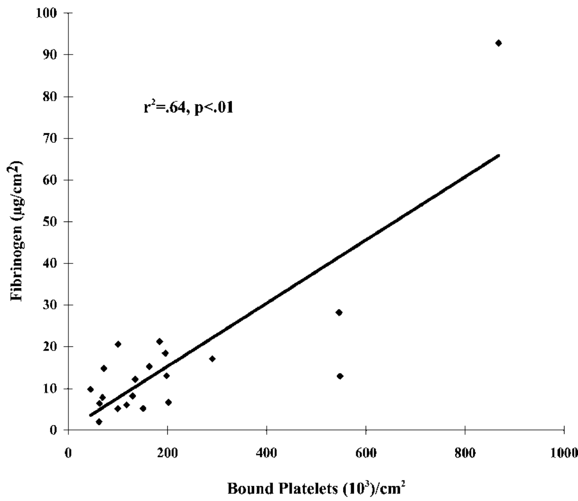

Figure 4 ◆ Relationship of the number of platelets to the amount of fibrinogen bound to different test materials as determined by linear regression analysis.

ence. PolyHEMA and Teflon exhibited low platelet adherence, while Corethane and polyester both had significantly increased binding. Based on this apparent overall similarity in fibrinogen and platelet binding patterns, the two trends were compared using linear regression. A significant correlation (Fig. 4) between levels of fibrinogen and platelet binding was observed across all test materials ($r^2=0.64$, $p<0.01$).

The pattern of monocyte binding to the different test substrates (Fig. 5) was also strikingly similar to that observed for fibrinogen binding. Stainless steel, along with cobalt, bound the least monocytes among the metals. Interestingly, cobalt and the two alloys containing large amounts of cobalt, Elgiloy and L605, all exhibited comparatively low levels of monocyte binding relative to other substrates, even though L605 levels were significantly higher ($p<0.05$) than steel, cobalt, and Elgiloy. Nitinol, which bound similar or lower levels of fibrinogen and platelets, respectively, exhibited 50% higher levels of monocyte binding than stainless steel ($p<0.05$), but still ranked low among the metals examined. Consistent with fibrinogen binding, turbostratic carbon, DLC, silicon carbide, and polyester exhibited high levels of monocyte adherence, while polyHEMA, Teflon, and Corethane all exhibited relatively low binding levels with respect to stainless steel. Regression analysis again revealed a significant relationship be-

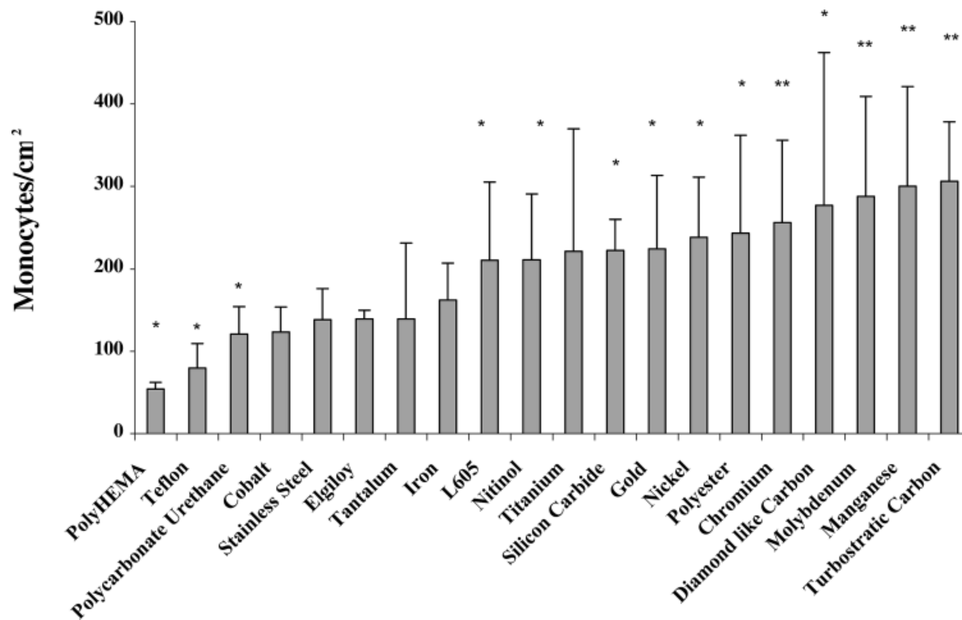

Figure 5 ◆ Binding of ^{111}In-labeled human monocytes in plasma to test materials. Results are expressed as mean ± 1 standard deviation. *$P<0.05$ and **$p<0.01$ (n=7) by Student *t* test with reference to stainless steel.

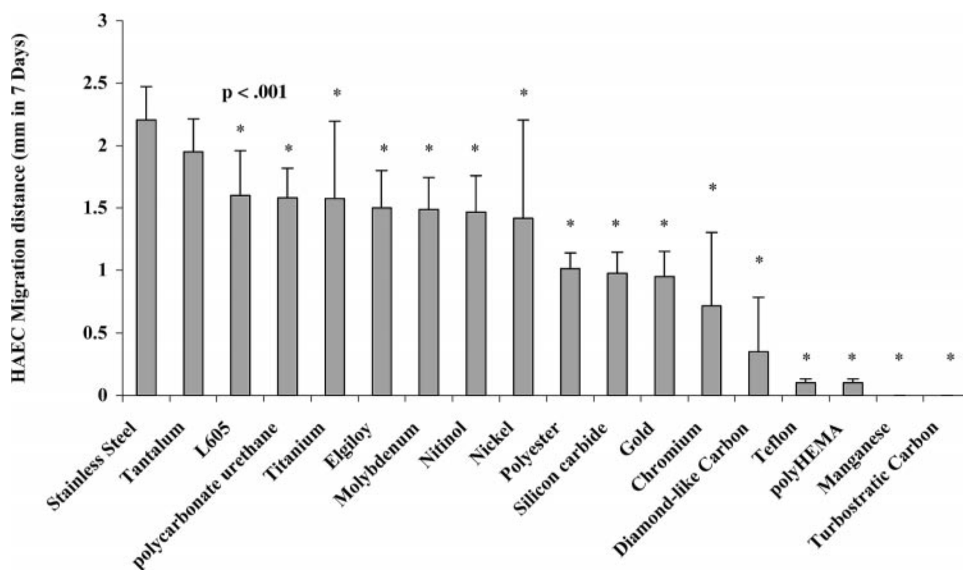

Figure 6 ◆ Relative rates of 10-day endothelial cell migration onto different test material surfaces. Results are expressed as mean ± 1 standard deviation. *$P<0.001$ by Student *t* test with reference to stainless steel.

tween the pattern of monocyte and fibrinogen binding ($r^2=0.40$, $p<0.005$).

In sharp contrast to the pattern of fibrinogen, platelet, and monocyte adherence, migration rates exhibited an almost inverse pattern (Fig. 6). Thus, the endothelial migration rate was the highest ($p<0.001$) onto stainless steel relative to all other materials, except tantalum, while only a rare endothelial cell was observed to migrate onto either manganese or turbostratic carbon. Migration rates onto gold, chromium, and manganese were significantly lower ($p<0.01$) relative to all the other metals tested. Polyester, DLC, and silicon carbide, which all exhibited relatively high levels of fibrinogen and blood cell adherence, had relatively lower rates of endothelial migration onto their surfaces. In contrast to these patterns, Teflon and polyHEMA, both of which exhibited very low protein and blood cell adherence levels, also demonstrated negligible migration of endothelial cells onto their surfaces. Even with these exceptions, regression analyses indicated that the pattern of fibrinogen binding levels across the tested substrates was inversely related to the migration rates measured ($r^2=0.34$, $p<0.05$).

While the relative performance of each material within the individual parameters gives an interesting basis for comparison, the thromboinflammatory score provided a comprehensive means for comparing a material's overall performance relative to other tested materials. The average thromboinflammatory scores (Table) ranged from a low of 1 for turbostratic carbon to 7.33 for 316 L stainless steel.

DISCUSSION

In this study, a wide range of biomaterials have been compared for fibrinogen, platelet, and monocyte adherence, as well as for the relative ability of endothelial cells to migrate onto and across these surfaces. Fibrinogen adherence was directly correlated with platelet and monocyte adherence but was inversely correlated to the rate of endothelial cell migration across these test materials.

Because of its integral involvement in thrombosis, fibrinogen accumulation at sites of interventional vascular injury has been the focus of many investigations. Chinn et al.[13] found that platelet adherence to polyurethane-related polymers under both static and flow conditions was dependent upon surface-bound fibrinogen and was inhibited in the presence of fibrinogen-free plasma. Fibrino-

TABLE
Relative Scores of Biocompatibility for Tested Biomaterials

Material	Mean Thromboinflammatory Score	EC Migration Rate	Biocompatibility Score
Stainless steel	3.33	4	7.33
Corethane	3.00	3	6.00
Elgiloy	3.00	3	6.00
Tantalum	3.00	3	6.00
Nitinol	3.00	3	6.00
Titanium	2.67	3	5.67
L605	2.33	3	5.33
Pellethane	3.00	2	5.00
Nickel	3.00	2	5.00
Molybdenum	1.67	3	4.67
Silathane	3.33	1	4.33
PolyHEMA	4.00	0	4.00
PTFE	4.00	0	4.00
Silicon carbide	2.00	2	4.00
Gold	2.00	2	4.00
Polyester	2.00	2	4.00
Chromium	1.67	2	3.67
PDMS	3.67	0	3.67
Cobalt	2.00	1	3.00
Iron	2.00	1	3.00
Diamond-like carbon	1.33	1	2.33
Manganese	1.00	0	1.00
Turbostratic carbon	1.00	0	1.00

PDMS: polydimethylsiloxane, PTFE: polytetrafluoroethylene.

gen-related platelet binding has also been used as a means of evaluating the potential thrombogenicity of angiographic catheters.[14] Baier and Dutton[15] also reported a general increase in platelet binding on a wide range of materials with increasing amounts of surface-bound fibrinogen. However, fibrinogen bound to different surfaces does not necessarily follow this simple rule. Examining fibrinogen-platelet interactions on polystyrene surfaces, Tsai et al.[16] observed differences in platelet binding that were more dependent on the molecular conformation that expressed the terminal gamma chain site than on fibrinogen concentration.

Perez-Luna et al.[17] used secondary ion mass spectrometry characterization of polymer surfaces coupled with multivariate analysis to develop a correlation between surface properties and the amount of fibrinogen absorbed and retained. Surface chemical functional groups positively influencing fibrinogen adsorption were hydrocarbon and polyether, while fluorinated, methacrylic, silicone, and phenylic ring groups were found to be negatively correlated. Consistent with this prediction, we observed that polyHEMA and Teflon exhibited low fibrinogen, platelet, and monocyte adherence, while polyester demonstrated significantly higher fibrinogen binding. However, polyHEMA and Teflon also displayed almost no endothelial migration onto their surfaces. Teflon and polyHEMA represent the extremes of hydrophobicity and hydrophilicity, respectively, indicating that neither extreme promotes protein or cellular attachment.

As fibrinogen concentrations increase at stent placement sites, so also does the potential for increased monocyte recruitment. Examining the inflammatory response to a polyester implant in normal and afibrinogenemic mice, Tang and Eaton[18] demonstrated that fibrinogen adherence to the material was the requisite factor mediating monocyte/macrophage recruitment to the polyester surface. In

contrast to the platelet binding site, the known monocyte binding P1 and P2 sites are not exposed in the native plasma fibrinogen molecule.[7] Our results demonstrate that as bound fibrinogen increases across the different surfaces, a related increase in monocyte adherence is observed. Previous studies have demonstrated that these intramolecular binding sites with high affinity for the Mac-1 receptor on the monocyte/macrophage are exposed as the fibrinogen molecule alters its conformation upon surface binding.[7] Our data indicating an increase in monocyte binding associated with increased fibrinogen binding to the different test surfaces suggest that this conformational change remains consistent even on surfaces with large amounts of bound fibrinogen.

There are few studies comparing relative monocyte adherence to different prosthetic materials. Several polymer materials have been compared for their relative ability to modify monocyte activation and cytokine secretion once they are bound.[19,20] Examining monocyte adherence to a DLC-coated glass surface in vitro, Linder et al.[21] reported a low level of monocyte adherence, but this level was compared only with adherence to the uncoated glass surface. Using a porcine animal model to evaluate the potential benefits of a DLC-coated stent, an increased macrophage presence was noted by De Scheerder et al.[22] in response to double-layer DLC-coated stainless steel stents compared to bare stents or those receiving a single coat of DLC.

Endothelial migration rates onto the materials examined were demonstrated to be in direct contrast to the parallel increases in platelet and monocyte adherence with increased bound fibrinogen. Ahmed et al.,[23] examining cultured Schwann cells, also reported a decrease in cell migration across surfaces related to relative increases in the amount of surface fibrinogen. Platelet and monocyte binding sites both reside in the relatively hydrophobic gamma chains of fibrinogen, while the endothelial sites are near the terminus of the more hydrophilic alpha c chains. Thus, if the gamma chains are bound to the surface, this may leave the alpha chains untethered and available for interaction with endothelial integrins. Likewise, if the alpha chains are tethered to the surface, the gamma sites may be more available for platelet and monocyte binding. Thus, factors such as surface charge, hydrophobicity, or chemistry could greatly influence this orientation. Comparing bound fibrinogen conformation on titanium oxide and hydroxyapatite, Yongli et al.[24] concluded that electrostatic surface charge was the primary surface characteristic modulating fibrinogen conformation. Studies utilizing surfaces with graded hydrophilicities have also demonstrated that orientation of bound fibrinogen can be dictated by this quality.[25]

In this study, gold, chromium, DLC, manganese, and carbon all exhibited characteristics and overall biocompatibility scores that would predict poor performance as an intravascular biomaterial. Williams et al.[26] also found that gold, platinum, copper, and silver adsorbed significantly higher amounts of fibrinogen than other metals, while stainless steel ranked amongst the lowest. Erikson and Nygren[27] observed that graphite and gold exposed to human capillary blood bound fibrinogen predominantly, along with an extensive accumulation of activated platelets and leukocytes. Graphite was more thrombogenic, while gold was more inflammatory based on quantitative assays of CD 62 (platelets) and CD 11b (PMN) expression. Clinically, Kastrati et al.[28] reported an increase at 30 days in in-stent restenosis and a decrease in event-free survival in patients receiving gold-coated compared to uncoated stainless steel stents. Park et al.[29] also reported a higher restenosis rate at 6 months in a large randomized study in patients receiving a gold-coated versus the uncoated NIR stent.

Based upon our study, nitinol and stainless steel might be predicted to perform similarly with regard to restenosis. In a randomized clinical trial comparing the efficacy of a nitinol to a stainless steel stent, Han et al.[30] reported no significant differences in either angiographic or clinical restenosis. The PARAGON randomized trial also found similar angiographic restenosis rates among patients receiving steel or nitinol stents, but it reported higher rates of clinical restenosis in patients who had received the nitinol stent.[31] In the only randomized, prospective, double-blind study comparing the potential benefits of

DLC-coated stents with stainless steel coronary stents in 347 patients, Airoldi et al.[32] were unable to demonstrate any significant difference in the incidence of restenosis.

As described above, an overall biocompatibility score was developed and applied to the results observed for each material to enable a more direct comparison. These relative scores not only indicate that this index may be valuable in assessing the intravascular biocompatibility of the materials tested, but it may also provide a possible basis for predicting novel surfaces as they become available. Based on current available experimental animal and clinical data, we would suggest that a material should attain a score >5.0 to be considered a good candidate for likely success in further animal or human trials.

The test surfaces used in this study were not always representative of similar surfaces in actual clinical or experimental use. Since vascular biocompatibility may vary according to surface processing, the results of this study can provide only a general idea about biocompatibility of materials and material groups used in the fabrication of vascular stents. Ideally, all test surfaces should be prepared using exactly the same procedures applied by the manufacturer in the fabrication of their device.

Conclusions

These results demonstrate that the different materials used in intravascular implantable devices, such as stents, exhibit a wide range of fibrinogen and cellular binding levels among all materials but a consistent predictable level for each individual material. This predictability provides a basis upon which new materials could be initially evaluated for thrombogenicity, inflammatory potential, and endothelialization in the laboratory prior to in vivo testing.

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