Polyethylene and Silicon Carbide Coated Steel Promote Less Complement Activation and Platelet or Leukocyte Adhesion than Medical Steel and Silicone Rubber Material

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Summary

Despite their positive effect on short and long-term complications after catheter-based coronary interventions, stents still carry the risk of promoting subacute thrombosis and restenosis, partly due to blood activation on the stent surface. Metals are used for the construction of most stents, but they seem less blood-compatible than polymers or semiconducting amorphous hydrogen-rich phosphorous doped silicon carbide (a-SiC:H), although seldom has a direct comparison between metals and surface coatings been made. In particular, interaction of inflammatory components on metals has been rarely examined. The effect of medical grade stainless steel (AISI 316L) was compared with amorphous silicon carbide coated stainless steel (a-SiC:H) and with polydimethylsulfoxide (PDMS) and low density polyethylene (LDPE) polymers on human platelets, granulocytes, and complement activation by biochemical assays after standard incubation conditions. Platelet and granulocyte binding to the materials was studied using immunofluorometric assays (anti GpIb, anti GPIIbIIIa and anti CD11b labelled antibodies, respectively). Elastase release was measured by means of an enzyme linked immuno assay. Complement activation was tested by C5a generation and complement convertase activity on the material surface. We found that platelet and granulocyte binding, as well as granulocyte release and complement activation were in the order of PDMS > medical steel > LDPE > a-SiC:H. We concluded that medical steel has blood compatibility characteristics between a positive and negative reference polymer. An a-SiC:H coating turns the blood compatibility to low levels of adhesion and activation, similar or even better than the negative reference polymer LDPE.

Key Words

Blood compatibility, complement, granulocyte, platelet adhesion

Introduction

Restenosis in stented arteries frequently occurs and is the result of intimal hyperplasia. Intravascular stenting results in damage to the endothelium, basal membrane, and media of the artery. Circulating granulocytes adhere to and migrate into the damaged arterial wall. Exposure to the implanted stent further activates granulocytes, which to some extent depend on the composition of the implanted stent. The appearance of stents in the early 1990's [1] brought a significant reduction in short- and long-term complications [2,3], and changed dramatically the scenario of catheter-based coronary interventions [4,5]. Since then, new stent models have been continuously developed; however, despite the appearance of several fascinating innovations, many have failed to provide better clinical results than their ancestor, the Palmaz-Schatz stent [6-8].

The late reappearance of coronary stenosis at the site of intervention (restenosis) is a problem that has not been completely solved by stents; it still affects 10 – 30 % of the percutaneously treated, atherosclerotic lesions [9]. In-stent subacute thrombosis and restenosis arise from the same source: the immediate local deposition of

platelets and granulocytes onto the bare metal surface can lead to abrupt lumen obliteration, but also enhances the release of cytokines and growth factors, thus promoting the gradual proliferation of smooth muscle cells into the vessel wall [10]. Additionally, activated granulocytes are responsible for release of enzymes and free oxygen radical products, which causes further damage to the artery wall. Information about the extent of granulocyte activation after intravascular stenting may provide valuable support for improving coronary implanted stents. Information can be provided by measuring platelet and granulocyte adhesion and activation, as well as complement activation induced by the material itself.

In general, polymers are thought to be more blood compatible than metals; however, few comparative studies between polymers and metals are available [11]. However, first investigations gave evidence that the biocompatibility of metallic surfaces can be improved by an a-SiC:H-coating [12]. Therefore, we studied flat sheet surfaces of medical steel, silicon carbide coated medical steel, and two polymers, which are known in our laboratory as positive and negative reference materials for blood compatibility testing.

Materials and Methods

Study Design

A comparative study was established to assess patterns of blood-material interaction by measuring activated cell deposition and release as well as complement activation in the incubation medium.

Materials

AISI 316L stainless steel and amorphous silicon carbide coated stainless steel were obtained from Biotronik (Berlin, Germany). Low density polyethylene (LDPE) was obtained from Goodfellow (UK), and polydimethylsulfoxide (PDMS, silicone rubber) came from Eriks (The Netherlands). All biomaterials had the same macro-structure, since all pieces were rectangular in shape with a size of 5.0 mm wide by 15.0 mm long by 0.1 mm thick. Prior to use, the materials were washed with a detergent (RBS) and methanol.

Blood Collection

Venous blood samples were collected from five healthy volunteers who signed an informed consent agreement before donating blood. These volunteers received no medication within the two-week period preceding blood collection. A total of 60 ml of blood was collected from each volunteer. All blood was collected in tubes containing heparin (1 IU/ml final concentration). The collected blood samples were processed as follows. A small aliquot was used immediately for cell counts (RBC, WBC, HCT, HGB, and PLT; Cell-Dyn 610, Sequoia Turner, USA). This procedure was performed on all samples to confirm normal hematological variables of each sample before it was used in the incubations. The remaining blood sample was divided into two aliquots. The aliquot of whole blood was incubated with the biomaterials. The other aliquot was centrifuged at 1500 g for 20 minutes at 4 °C to prepare platelet-poor plasma, and additionally for 2 min at 10,000 g to obtain platelet-free plasma (PFP).

Incubation of Human Blood or Plasma with Biomaterials

The pieces of biomaterial were incubated with fresh blood for the determination of antibody binding (GpIb, GpIIbIIIa and CD11b) and for the determination of elastase release during a period of one hour at 37 °C to allow equilibration of protein and cell deposition. Following incubation, the pieces of biomaterials were carefully collected, washed, and stored in a freezer at -80 °C until they were used for the binding experiments. Following incubation, the blood supernatants were collected in individual tubes containing EDTA, and the samples were centrifuged (at 10,000 g for 2 min at ambient temperature) to prepare plasma. The plasma was stored in a freezer at -80 °C to be used at a later time for elastase measurements. Other pieces of biomaterial were incubated with plasma for the determination of background antibody binding against GpIb, GpIIbIIIa and CD11b receptors, and C5a generation. Following incubation, the plasma supernatants were also collected into individual tubes containing EDTA. The samples were then centrifuged (at 10,000 g for 2 min at ambient temperature) to prepare the plasma. This plasma was stored in a freezer at -80 °C to be used at a later time for C5a measurements.

Evaluation of Complement Activation via C5a Measurements

C5a is a split product of complement protein C5. Activation of complement proteins can be initiated soon after blood collection, as well as by the container

in which the incubations with biomaterials are performed. C5 activation is a step further downstream the complement cascade and, therefore, is expected to suffer less from the non-biomaterial mediated activation than for instance C3 activation. Concentrations of generated C5a were determined by radio-immunoassay on plasma that had been incubated with the biomaterials. The assay was carried out in accordance with the instructions on the manufacturer's kit (Amersham, UK).

Determination of Complement Convertase Activity of Biomaterials

This experiment used frozen stokes of porcine plasma, which was collected from healthy pigs. Porcine plasma was used because of its higher convertase activity than human plasma. The pieces of biomaterial were incubated for 15 minutes at 22 ± 1 °C with porcine plasma for the determination of CCA. Following incubation, the pieces of biomaterials were washed and incubated with a chromogenic substrate to determine the activity of convertase complexes. This enzymatic test is based on the formation of complement convertase complexes on the surface of biomaterials after incubation with (porcine) plasma. Because this test is performed on the biomaterial, it accurately reflects the complement activating properties of the material. Concentrations of surface-bound CCA were determined by enzymatic conversion of specific substrate S2527 due to C5-convertase activity.

Elastase Release

Leukocyte activation is an important method for evaluating activation of blood that has been in contact with medical devices. Activated granulocytes release elastase, both by degranulation and by neo-formation, and therefore elastase can be used as a marker for leukocyte activation.

Elastase release during incubation of the materials was determined by immunoassay and in accordance with the manufacturer's instructions (Milenia Biotec, Germany)

Deposition of Cells from Blood

Evaluation of Platelet Adhesion via GpIb and GpIIbIIIa Determination: CD42 is a protein complex expressed on the surface of platelets and consists of three glycoproteins, one of which is GpIb. GpIb, which is constitually exposed on the platelet membrane and is platelet specific, is not known to be involved in the binding of platelets to biomaterial. Therefore, this receptor is detectable with antibody binding, even after platelet adherence to bio-materials, and will reflect the total number of platelets bound to the biomaterial. Available GpIb present on the surface of platelets was determined by binding a fluorescent-labelled anti-GpIb antibody (M753, Dakopatts, Denmark). Antibody labeling was performed with Sm-DDTA. After binding the antibody, all biomaterials were washed and immersed in enhancement solution; then the release of the samarium-labelled antibody was counted by fluorometry (Delfia, Wallac Oy, Finland). Due to the high sensitivity of this time-resolved fluorescence-counting methodology, it was possible to use small surface areas [13]. Each measurement consisted of 1000 excitations and photon emissions per second from samarium (cps = counts per second)

Evaluation of Activated Platelet Adhesion via GpIIbIIIa Determination: GpIIIa is one of the membrane receptors of platelets. During platelet activation, this receptor combines with GpIIb. This complex (GpIIbIIIa) plays a role in platelet aggregation and acts as a receptor for fibrinogen. This receptor is detectable with antibody binding and reflects the number of activated platelets bound to the biomaterial sample. Available GpIIbIIIa was determined by binding a fluorescent-labeled, anti-GpIIbIIIa antibody (PAC-1, Becton Dickinson, USA) present on the surface of platelets.

Evaluation of Granulocyte Adhesion via CD11b Determination: CD11b is a membrane receptor expressed on granulocytes, on a subset of T-cells, and on monocytes. Among others, this receptor binds to complement C3b. CD11b is used as a marker for the presence of these white blood cells, mainly granulocytes, which can contribute to delayed wound healing responses after binding to implanted materials. Available CD11b was determined by binding of a fluorescent-labeled, samarium-labeled antibody for a leukocyte CD11b receptor (M741, Dakopatts, Denmark) to assess leukocyte binding. However, as with the GpIb and GpIIbIIIa antibodies, a small amount of "non-specific" antibody binding does occur. Therefore, these experiments were also performed in cell-free plasma, and the differences between the measurements were used to determine antibody specific binding. The obtained fluorescence units were converted into pg of antibody bound per cm^2 of biomaterial.

Statistical Analysis and Presentation of the Results
Variables are reported as mean and standard deviation. Paired t-tests were performed for the CCA test and for the immunofluorocytometric parameters to assess any differences in platelet and leukocyte binding to the four biomaterials. Friedman's two-way analysis was performed on the C5a and elastase data. All statistical analyses were performed using Systat 8.0 (SPSS, USA).

Results

Plasma Assays
C5a release in plasma showed a large variability after exposure to different plasma samples, which resulted in a somewhat lower C5a production after exposure of a-SiC:H and polyethylene as compared to PDMS ($P < 0.05$) (Table 1). By means of the CCA, a significant reduction of complement activation onto silicon carbide coated steel and LDPE was observed as compared to medical steel and PDMS ($P < 0.01$). Also, stainless steel appeared to generate less surface-bound complement convertase activity than PDMS ($P < 0.05$) (Figure 1). Due to differences in donor blood, elastase release showed large standard deviations. On average, medical steel and a-SiC:H induced the lowest elastase release, but only after a-SiC:H incubation a significant reduction of elastase, as compared to LDPE, was observed ($P < 0.05$) (Table 1).

	C5a (ng/ml)		Elastase (μg/ml)	
	mean	sd	mean	sd
Medical steel	199	67	157	96
a-SiC:H-coated steel	146	24	134	33
PDMS	218	44	272	140
LDPE	149	53	205	55

Table 1. C5a and elastase generation after in vitro incubation of biomaterials.

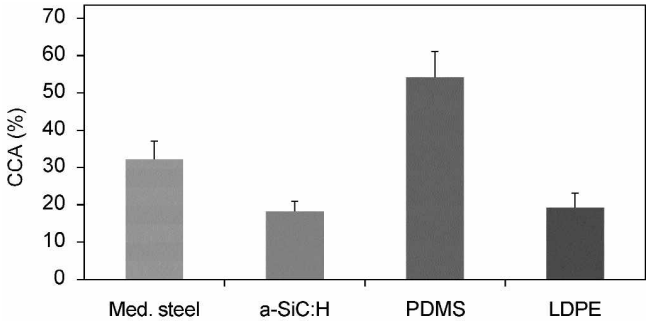

Figure 1. Complement convertase activity (CCA) shown on the surface of medical steel, amorphous silicon carbide coated medical steel (a-SiC:H), polydimethylsulfoxide (PDMS), or low density polyethylene (LDPE). CCA on PDMS was significantly higher than on the other materials; CCA was significantly higher on medical steel than on a-SiC:H and LDPE.

Immunofluorocytometry
Leukocyte binding as assessed by CD11b antibody binding, and was significantly reduced on silicon carbide coated steel and LDPE, as compared to stainless steel and PDMS ($P < 0.01$) (Figure 2). Platelet binding as assessed by GpIb antibody binding was significantly higher on PDMS than on the other three materials ($P < 0.01$); of the three materials, a-SiC:H showed the lowest binding (Figure 3). GpIIbIIIa binding, indicating the presence of activated platelets, showed the highest binding onto PDMS, and the lowest binding on a-SiC:H and LDPE; however, this difference was not statistically significant (Figure 4). The activity of complement convertase on the material surfaces appeared to correlate with the amount of granulocytes bound to the surfaces ($P < 0.001$), while saturation of granulocyte binding was observed at higher convertase activity (Figure 5).

Discussion

Both complement activation and immunofluorocytometry on the material surface of the two polymers, the metal and the semiconducting a-SiC:H clearly demon-

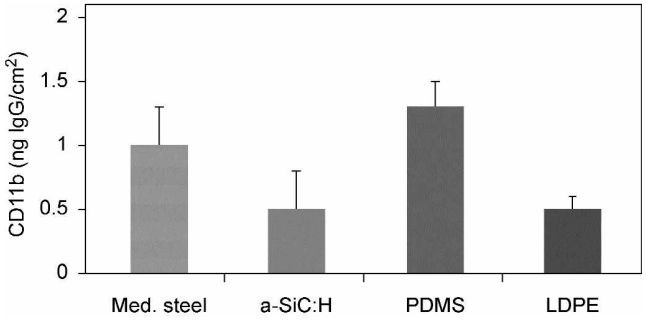

Figure 2. CD11b antibody binding, representing mainly granulocyte binding, showed significant differences between the four materials, as indicated in Figure 1.

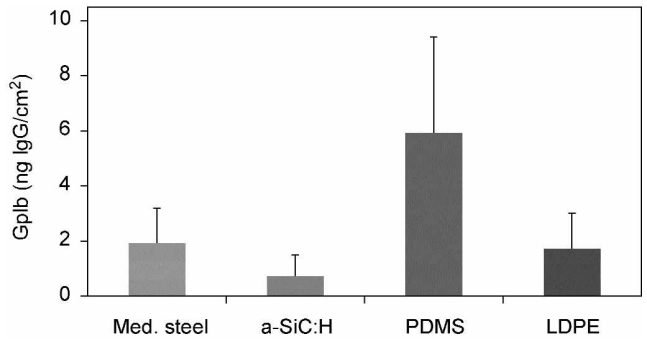

Figure 3. GpIb antibody binding to the materials (see Figure 1) was significantly higher on PDMS and medical steel than on a-SiC:H. In addition, PDMS was higher than LDPE.

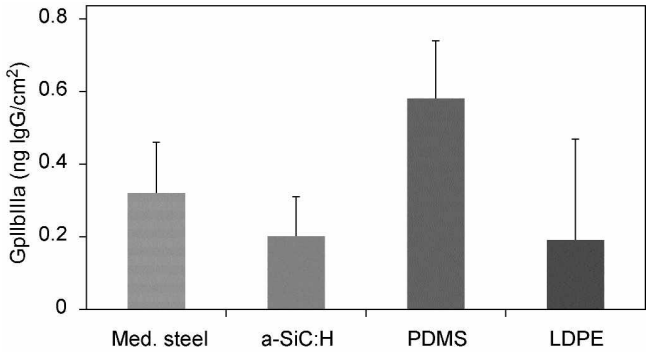

Figure 4. GpIIbIIIa antibody binding to the materials (figure 1), representing the activated platelet fibrinogen receptor, was significantly higher on PDMS than on medical steel, and significantly lower on a-SiC:H and LDPE.

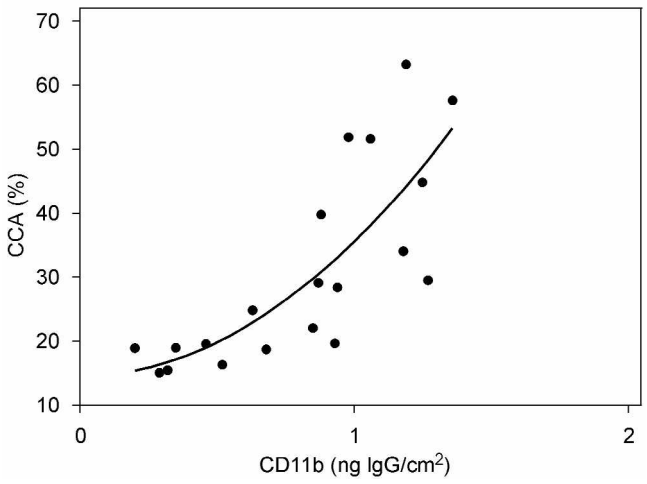

Figure 5. Correlation between complement convertase activity (CCA) on the biomaterial surfaces and the adhesion of granulocytes. (R = 0.78, P < 0.001).

strated that stainless steel induces a high degree of activation, which has properties similar to a positive polymer material like polydimethylsiloxane (PDMS). In contrast, a-SiC:H coating of medical steel abrogated this activating potential completely, which results in a surface with similar or even better biocompatibility characteristics than LDPE. These results are important for the application of materials for the construction of stents.

It has been hypothesized that the deployment of a stent in the coronary system can have repercussions on circulating blood cells and coagulation [14]. However, their metal structures can also have a direct effect on leukocyte adhesion through an inflammatory-mediated mechanism. As demonstrated in the present study, activation of complement and leukocyte deposition may also make an important contribution to the local effects of stents, which could result in significant impairment of re-endothelialization. Then, through the release of cytokines and growth factors, smooth muscle cells are induced to migrate and replicate, and intimal hyperplasia and restenosis can develop [15]. In the present study, we demonstrated that surface characteristics are an important issue in modulating blood-stent interaction.

Some studies have suggested that composition, surface covering, and/or electrical charge of stents [16-18] could be of importance in determining their hemocompatibility. Our study clearly demonstrates that differences of material surface properties can be detected by means of sensitive and discriminative assays. In addition, it shows that complement activation on the material surface (CCA) highly correlates with granulocyte binding. This result makes sense, since CD11b interacts with C3b from the complement system.

Clinical Implications of Blood-Stent Interaction

When deciding which stent to use for daily interventional procedures, hemocompatibility is often overshadowed by the ease of implantation and the cost-effectiveness of the device, while the impending short- and long-term consequences of blood-stent interaction receive less notice. Achieving convincing clinical proof for any new stent is difficult and carries the drawback of the unavoidable long wait for conclusive data. To cope with the continuing technological evolution of coronary interventional devices, manufacturers often release stents that lack satisfactory, scientific proof of their performances.

Furthermore, the population of cardiopathic patients

targeted for percutaneous interventions is evolving continuously and rapidly. Lately, stenting procedures have been used safely and effectively in the treatment of patients suffering from acute coronary syndromes (unstable angina, acute myocardial infarction). Many pivotal trials have demonstrated that blocking the activated GPIIbIIIa and leukocyte (Mac-1) receptors in these patients has a positive influence on their short- and long-term outcome after stenting. In addition, a more favorable prognosis can be reached by reducing the risk of subacute thrombosis, optimizing the blood flow in the tributary microcirculation, and possibly preventing the development of restenosis [19]. Furthermore, patients who are affected by diabetes mellitus are advanced in years, or in whom particularly complex coronary lesions have been detected, seem to benefit by stent usage more than by PTCA alone [20-22].

In this continuously evolving scenario, the propensity of a stent to tether platelets and leukocytes as shown for the a-SiC:H stent coating in this study, and eventually to promote subacute thrombosis and restenosis, becomes then a very important bias in predicting their outcomes.

Study Limitations

Our experimental model appears discriminative, efficient, and cost-effective in the assessment of the hemocompatibility of materials before their clinical use. A limitation of our model could be represented by the absence of circulating blood and an endothelial layer. By the release of cytokines and the expression of adhesion molecules, endothelium plays a major role in mediating the interplay between the injured vessel wall and blood cells after coronary stenting [23]. This omission can somehow alter the likelihood of our experimental representation. Nevertheless, the surface properties of the materials remain relevant in various implant conditions, perhaps more than the release products.

Conclusion

Complement activation, immunofluorometry, and elastase release demonstrate that medical steel activates an inflammatory response and platelet adhesion significantly more than the low responder LDPE. An a-SiC:H coating turned medical steel into a non-activator. This evidence supports the excellent, immediate, and long-term clinical results achieved with a-SiC:H coated stents.

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