Reduced Deposition of Blood Formed Elements and Fibrin onto Amorphous Silicon Carbide Coated Stainless Steel

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Summary

Reduced blood activation and reduced adhesion of blood elements in contact with stent material increases the chances for uncomplicated implantation, by minimizing early occlusion due to thrombosis and late occlusion due to release of growth factors and granulocyte activation. Amorphous silicone carbide, which is known to have antithrombotic effects, can be applied as a coating onto existing stent materials. This in vitro study evaluates the properties of a-SiC:H coating on stainless steel as compared to uncoated stainless steel, by exposure of the stents to circulating human blood. The coating fully covered the basis stainless-steel material, as deduced from XPS measurements. A (although not significant) reduction of activation of the clotting system was found after use of the coated stent, which was substantiated by significantly reduced platelet and granulocyte deposition on the coated stent surface as compared to the uncoated case. We conclude that the acute response of stainless steel on blood activation can be quenched by a-SiC:H coating.

Introduction

Intracoronary stents have proved to be a valuable approach to treat occlusions during percutaneous transluminal coronary angioplasty and to reduce restenosis [1]. However, the incidence of thrombosis in early studies was high, up to 25% [2].

Thus, aggressive antiplatelet and anticoagulation therapy is used during and after stent implantation [3]. Consequently, hemorrhagic complications related to the antithrombosis therapy are frequently seen [4]. This motivates the need to develop stents with a non-thrombogenic surface, thereby obviating the need for aggressive systemic inhibition of platelet function and anticoagulation and to prevent restenosis which is often observed distal of the stent. This latter effect has been related with the release of growth factors among others from activated platelets [5].

Stents were initially made from stainless steel, which is known to be a prothrombotic material. Likewise, other stents made with different metal composition were developed. Currently, stents are also made from nickel-titanium alloy, gold or tantalum, which have the advantage of a higher radio-opacity than stainless steel. Moreover, tantalum was initially reported as being less thrombogenic than stainless steel [6]. Later studies, however, showed a high incidence of thrombotic stent occlusion in tantalum stents [7]. In baboons no difference was found between stainless steel and tantalum with regard to platelet and fibrin deposition [8]. The deposition of these blood components has consequences for early stent occlusion by thrombosis, but may also result in an increased late restenosis rate, particularly when simultaneously granulocyte deposition and activation take place onto the stent surface.

Thus, attempts have been made to apply coatings on the basic stent material, in order to exclude the direct effects of the metal. Amorphous silicon carbide (a-SiC:H) has been reported to reduce fibrin deposition, which may result in reduced platelet and leukocyte adherence as well [9].

To assess the thrombogenic characteristics of stents with or without a-SiC:H coating, an in vitro circulation model was developed. By measuring blood activation products formed during contact with the stent and by evaluating the stent surface by scanning electron microscopy or XPS it was possible to measure platelet and clotting activation and deposition of platelets, granulocytes and proteins in general under standardized conditions.

Materials and Methods

Experimental setup

A standardized closed loop system was constructed by means of a 3 mm internal diameter silicon rubber tubing precoated with albumin and a roller pump. Part of the circuit was immersed in a water bath to ensure a blood temperature of 37°C. This circulation model fulfils the requirements of the International Organization of Standardization (ISO 10993-4) for hemocompatibility testing. Blood was obtained on the experimental day from a healthy donor and immediately anticoagulated with 1 IU heparin/ml to prevent coagulation and to mimic the clinical situation.

A stent was placed in the silicon-rubber tubing, after which the supporting balloon was inflated with 10 atmospheres to fixate the stent. The closed-loop system was then filled with 4 ml blood out of one of the aliquots assuring that the circuits contained no air. Blood was circulated at a flow of 40 ml/min. for 15 minutes. Thus, the total blood volume of the circuit will pass the stent 150 times. This resembles the presence of the first 150 hours in the human coronary artery post implantation, considering a coronary flow of 80 ml/min and a total blood volume of approximately 5000 ml. After circulation, blood was collected in EDTA (0.01 mM final concentration) or indomethacin (0.05 mg/ml)-citrate for biochemical evaluation and the stent was removed carefully from the silicon tubing and flushed with saline to allow antigen detection and scanning electron microscopy. a-SiC:H coated stainless steel stents (n = 8, Tenax®; BIOTRONIK, Germany) and uncoated stainless-steel stents with the same design (n = 8) were used. Additionally circulation experiments (n = 8) were performed without a stent in the loop to obtain a negative control.

Biochemical evaluation

In whole blood, cell counting of erythrocytes, leukocytes and platelets was performed electronically (Cell-Dyn 610, Sequoia-Turner Corp., Mountain View, Ca). In indomethacin-citrate plasma elastase-alpha-PI (leukocyte activation), b-thrombo-globulin (mechanically induced platelet activation) and prothrombin fragment F1.2 concentrations (activation of the coagulation system) were determined by enzyme linked immunosorbent assays (Merck, Darmstadt, Germany; Cayman Chemical Company, Ann Arbor, Michigan, USA and Behringwerke AG, Marburg, Germany respectively), and thromboxane-B2 (platelet activation) was determined by radio immuno assay (Kodak Clinical Diagnostics LTD, Amersham, UK). Part of the stent was subjected to an Europium-labeled antibody directed to the platelet adhesion glycoprotein IIIa receptor (GpIIIa), while another part was subjected to an Europium-labeled antibody directed to the leukocyte adhesion CD11b receptor (M753 and M741 respectively, Dakopatts, Glostrup, Denmark) for 1 hour. After removal of unbound antibody by washing, the stent was emerged in enhancement solution to release the Europium label for counting in a fluorometer (Delfia, Wallac Oy), Türkü, Finland). This methodology of time resolved fluorescence counting of Europium enables the use of small surface areas by its high sensitivity. Each measurement consists of 1000 excitations and photon emissions from Europium per second (counts per second; cps). An antibody not directed against human proteins with similar amount of Europium label was used as negative control of non-specific binding. Results were corrected for the stent weight. For scanning electron microscopy the samples were washed, fixated and dehydrated in ethanol series. After critical point drying, the samples were sputter-coated with gold and examined with FEG-SEM at 2 kV (Jeol 6301 F, Tokyo, Japan).

XPS measurements

X-ray photoelectron spectroscopy (XPS) was performed on the stainless steel and a-SiC:H samples prior to and after blood circulation and after another 14 days exposure to platelet poor plasma in the presence of Fungizon. A S-probe spectrometer (Surface Science Instruments, Mountain View, CA, USA) equipped with an aluminum anode (10 kV, 22 mA) and a quartz monochromator was used10. The direction of the photoelectron detection angle was 60° with normal to the sample. Broad scan spectra were made with a 250 x 1000 mm spot and a pass energy of 150 eV. The binding energy scale was calibrated to the C1s peak at 284.8 eV. The experimental peaks were integrated after linear background subtraction and the peaks were decomposed assuming a Gaussian/Lorentzian ration 85/15 by using the SSI PC software package. Elemental surface compositions were calculated from the integrated peak areas employing instrumental sensitivity factors as supplied by the manufacturer and expressed in atomic %.

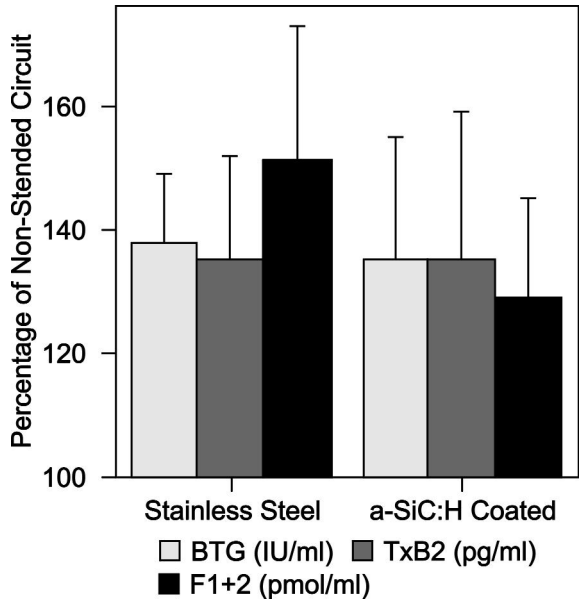

Figure 1. After correction for baseline blood activation in the circuits without a stent, ß-thromboglobulin (BTG), thromboxane B2 (TxB2) and prothrombin fragment F1+2 were increased in both stainless steel and coated stainless steel. No statistical differences were observed between the two types of stents, although F1+2 tended to be higher in the stainless steel group.

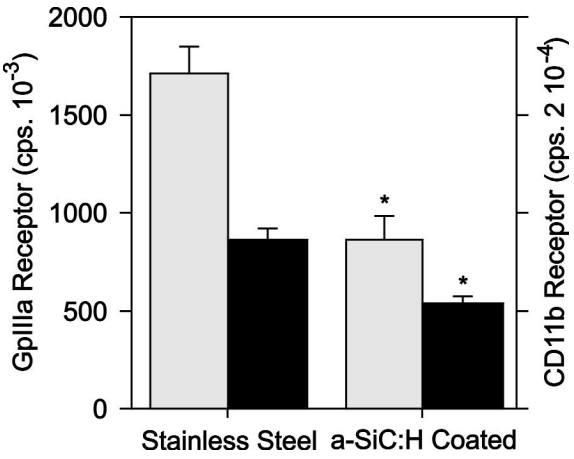

Figure 2. Platelet receptor GpIIIa receptor antigen and granulocyte CD11b receptor antigen were significantly higher on stainless steel stents than on a-SiC:H coated stents: (p<0.05).*

Results

Cell count

The cell count of platelets and leukocytes showed no significant reduction after blood circulation.

Activation of the clotting system and platelets

The clotting cascade was activated to some extent, shown by the release of fragment 1+2 from prothrombin (Figure 1). More F1+2 was formed in blood during contact with stainless steel as compared to a-SiC:H, although not statistically different.

The release products from platelets, ß-Thromboglobulin and Thromboxane, were found identically after stainless steel and a-SiC:H stent exposure (Figure 1) and both these components were significantly produced in the presence of a stent.

Deposition onto the surface

Platelet and leukocyte deposition onto the stent surfaces were higher on stainless steel than on a-SiC:H. These differences were found to be statistically significant ($p < 0.05$) (Figure 2). Interestingly, a statistically significant correlation was found between the platelet release product ß-thromboglobulin and platelet receptor GpIIIa antigen binding on the stent surface (Figure 3).

The presented sensitive technique, based on the detection of platelet and leukocyte-specific receptors, was supported by electron microscopy and by XPS analysis. A typical example of electron microscopy visua-

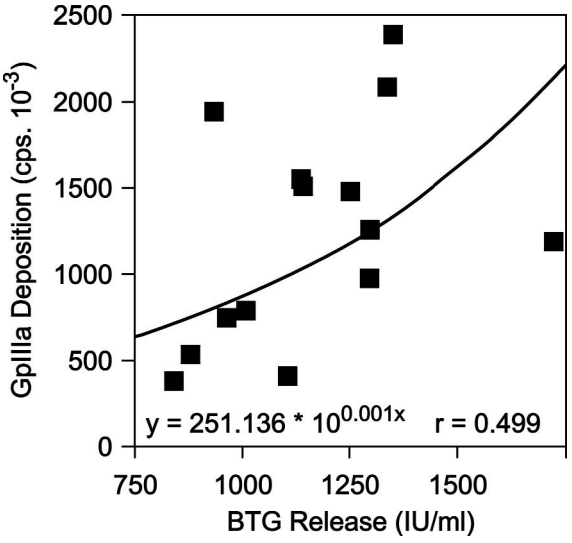

Figure 3. The deposition of platelet receptor GpIIIa plotted against the release of ß-thromboglobulin (BTG), revealed a statistically significant correlation p < 0.05.

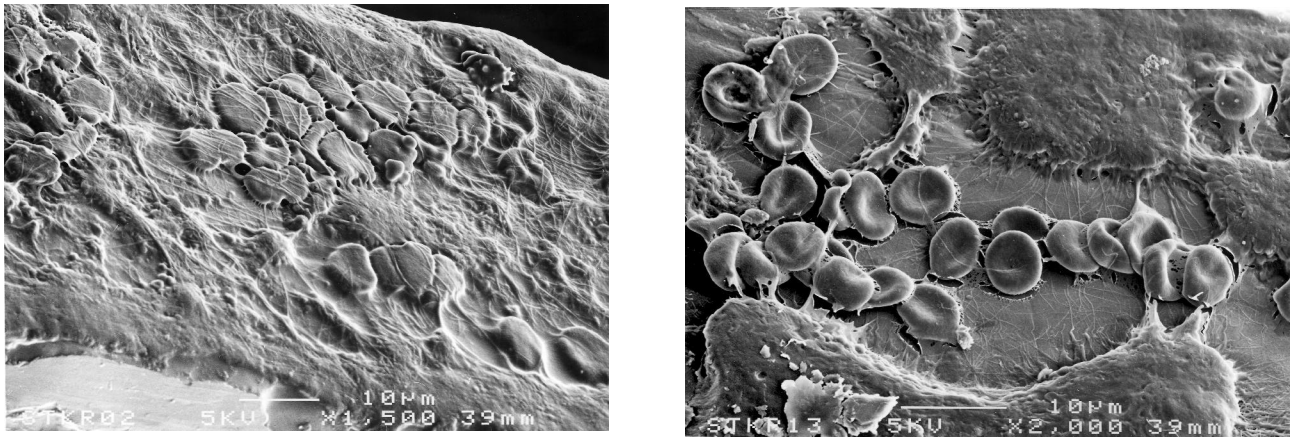

Figure 4. (a) Scanning electron micrograph (1500x) of the stainless steel surface showing areas with a dense layer of blood proteins and formed elements, covered by fibrin strands; (b) Scanning electron micrograph (2000x) of the a-SiC:H coated surface showing areas with thrombi and erythrocytes, but not densely packed and not extensively covered with fibrin.

lization of a stainless steel surface shows a very dense layer of formed elements on the surface, covered with fibrin ("smear"). In contrast, the a-SiC:H surface shows multiple clean areas or a loose cell deposit without the fibrin network (Figure 4). A more detailed picture shows extensive fibrin polymerization onto stainless steel (Figure 5).

Surface analysis

The atomic constitution of the outer surface revealed, as expected, the presence of chromium in case of the stainless steel material, whereas chromium was not detectable on a-SiC:H coated steel (Figure 6). This indicates a completely covered steel surface.

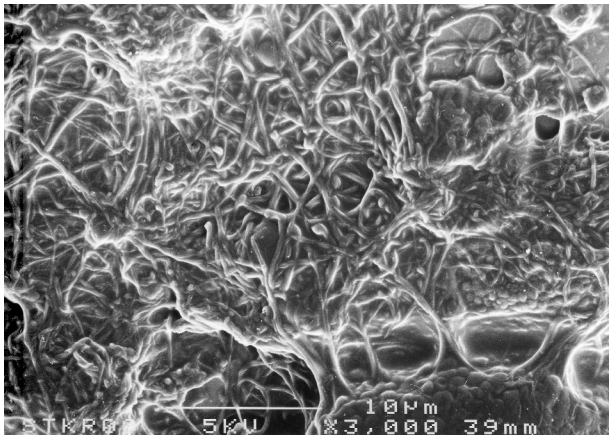

Figure 5. Detailed scanning electron microscopy picture (3000x) of the stainless steel surface with degranulated platelets and fibrin.

After the circulation experiments, the chromium percentage was reduced by 70%, while the nitrogen concentration increased markedly. On a-SiC:H coated steel nitrogen concentrations also increased but to a smaller extent as compared to the uncovered case. In our setup nitrogen can be considered to represent protein deposition.

XPS analysis performed 14 days later after continued exposure of the stents to blood plasma showed a further increase of nitrogen to approximately 10% (Figure 7). Simultaneously, the percentage of surface markers chromium and silicon decreased.

Discussion

In order to modify the surface characteristics of a stent, a thin layer of a-SiC:H appeared sufficient to completely hide the underlying metal scaffold. This modification resulted in a significant improved hemocompatibility, mainly shown in this in vitro study by reduced deposition of blood elements.

Despite the presence of 1 IU/ml of unfractionated heparin in blood during the experiments the clotting cascade was activated to some extent. The thrombin formed after release of F1+2 will likely have been inactivated rapidly by heparin-antithrombin III, but has exerted an effect on the polymerization of fibrinogen to a fibrin network as shown by electron microscopy. We used this concentration of heparin to match the clinical situation and to have a non-occluding but still critical model [11].

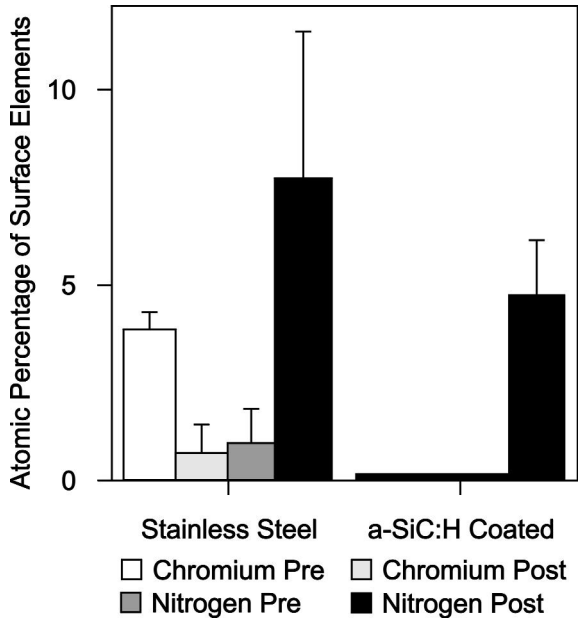

Figure 6. Chromium, used as a marker for stainless steel was found before circulation to almost 4%, whereas a-SiC:H completely covered chromium. After circulation the exposed chromium concentration was reduced to 0.7% and replaced by an increased nitrogen concentration, representing protein deposition. On a-SiC:H also nitrogen was deposited, though to a lesser extent.

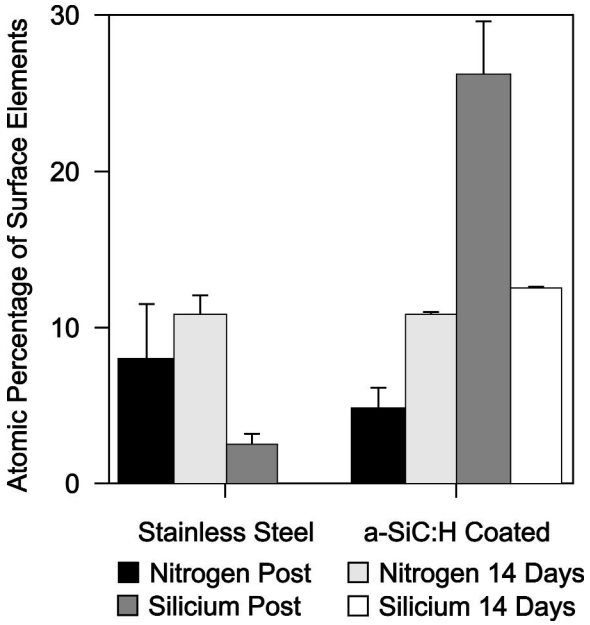

Figure 7. When the stents were left after the blood circulation experiment in platelet poor plasma, the nitrogen concentration further increased in both types of stents to a similar level. Simultaneously, the concentrations of chromium and silicium, as surface markers for the original material, reduced by half.

Next to a difference in fibrin deposition, the a-SiC:H coating also inhibits adherence of formed elements. These elements, particularly platelets and granulocytes, may adhere almost passively to the material, but the presence of fibrin indicates that at least one of the major platelet activators, thrombin, was formed at the surface of the uncovered material to a non-negligible extend. Thus, platelets were likely activated after adherence, which not only promotes further thrombus formation but also the release of platelet-derived growth factors which enhances intimal thickening of the artery distal of the stent. The observation that platelet adherence (GpIIIa deposition) significantly correlated with platelet release (ß-TG) supports this suggestion. Simultaneously to this effect of platelets, the presence of granulocytes on the stent surface hampers restoration of damaged endothelial cells, endothelial nitric oxide and prostacyclin production and endothelial overgrowth of the stent surface. Clearly, a reduction of granulocyte adherence as shown by CD11b-antibody binding is favorable to a normal healing process after implantation. A further study on the expression of platelet [12] and granulocyte receptors in circulating blood could be of interest in relation to the different surface characteristics observed in this study.

ß-TG and thromboxane production by stents, both coated and uncoated, is an indication for two mechanisms of activation: mechanical and biological. The mechanical component is obviously similar in both stented circuits, since the geometry of the stents were identical and produced by the same manufacturer. ß-TG is the main variable expressing the mechanical damage of platelets, although its release is also increased after platelet aggregation. Evidence exists about the important role of the stent geometry [13]. Thromboxane release seems not reduced by the a-SiC:H coating. It could indicate a similar response of platelets after contact with the material surface, but also a similar secondary response to platelet agonists released during blood contact. ADP, produced among others by damaged red blood cells, likely is an important agonist in our in vitro model.

XPS analysis demonstrated a more rapid deposition of proteins on stainless steel, while after two weeks both

types of surfaces, stainless steel and a-SiC:H were similarly covered with protein. Our experimental design does not allow to study long term effects on whole blood, since blood must be freshly collected in order to guarantee intact platelets and granulocytes. The nature and thrombogenicity of the late protein deposit cannot be shown as yet, and warrants further investigation, particularly as fibrinogen and possibly other adhesive proteins can adhere in a pro- or anti-thrombogenic manner. We conclude that the acute response of stainless steel on blood activation can be quenched by a-SiC:H coating.

Acknowlegdement

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