A Potential "In-Stent Restenosis Model" Evaluating the Kinetics of Smooth Muscle Cell Proliferation on Metallic Surfaces In Vitro

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Summary

This article reports on the idea and first experimental experiences with a novel in-vitro test designed to characterize the proliferative response of smooth muscle cells to various metallic surfaces. The results show that fulfillment of the technical preconditions such as adherent growth of the cells on a given surface, as well as the selection of a reduced content of fetal bovine serum in the culture medium, allows one to obtain meaningful proliferation kinetics for different surfaces. As a "proof of principle" experiment, it was found that coating steel or glass with a-SiC:H results in reduced direct smooth-muscle-cell stimulation in comparison with uncoated 316L-steel. Whether this test was predictive of in-stent restenosis, and whether it therefore may turn out to be a useful screening tool for the development of new stent generations, needs to be further established.

Key Words

Restenosis, stents, biocompatibility, coronary arteries

Introduction

Coronary stents have revolutionized daily practice in interventional cardiology such that stents are currently implanted into 70 % – 80 % of all coronary arteries treated for stenosis. The excessive use of stents, as well as routine stenting of all types of lesions, has led to a new "diagnosis" in interventional cardiology, referred to as *"in-stent restenosis"*. Restenotic stented lesions present a significant challenge for modern cardiology because simple repeated balloon angioplasty leaves the patient with a probability above 50 % for another restenotic episode. Thus, there is no effective cure so far for this new, man-made disease.

Coronary brachytherapy, i.e. the focal application of ionizing radiation to coronary arteries, is currently poised to enter into widespread clinical use [1]. Due to favorable results in a variety of randomized trials, it is considered a breakthrough in the treatment of in-stent restenosis [2]. However, the application of brachytherapy is limited by demanding logistics in the clinical setting. Another approach to prospectively reduce in-stent restenosis rates has been to make the metallic surface of stents more biocompatible. The a-SiC:H semiconductor surface (Tenax stent, Biotronik, Germany) prevents electron transfer processes between the stent surface and serum proteins; this results in reduced thrombogenicity [3]. Moreover, this surface was shown to reduce corrosion in comparison with uncoated conventional stents made of 316L steel [4].

However, the most recent approach to prevent in-stent restenosis has been to utilize the stent as a platform for *local drug delivery*. The basic idea is to deliver an antiproliferative agent from the stent struts into adjacent vascular tissue to counteract restenotic smooth muscle cell proliferation. This approach requires coating of the stent with a polymer suitable to store sufficient quantities of an appropriate agent, and to release the agent from the surface over an extended period of time. The suitability of such drug-eluting stents is currently being investigated in prospectively randomized clinical trials.

So far, the only means of suitable preclinical testing of new stent generations is the implantation into porcine coronary arteries with histomorphometric assessment of the induced in-stent restenosis after 28 days. Such testing requires sufficient resources to perform large animal experiments, is technically demanding, expensive, and gives results only after a minimum delay of 5 weeks. As such, it does not allow effective screening of a variety of designs within a short period of time. We sought to establish a test procedure that would predict the capability of a new stent surface to induce less restenotic neointimal proliferation in a cell-culture model. To prove the viability of the principle behind this type of testing, we investigated the a-SiC:H semiconductor surface in comparison with uncoated 316L medical-grade steel.

Materials and Methods

The proposed monolayer proliferation assay was designed to model, in a geometrically simplified test environment, the interface between the metallic foreign-body "stent" and the tissue in the vessel wall. The key elements of the assay include a flat disc-like specimen representing the stent material, and a monolayer of smooth muscle cells growing on this surface (Figure 1). *The speed of proliferation is intended to be a surrogate for in-stent restenosis.* The design of this test directly implies two distinct preconditions. First, it is of paramount importance that the test cells proliferate on the test specimens while remaining completely adherent to the surface throughout the test period. Second, the growth factors in the Fetal Bovine Serum (FBS) additive should not stimulate the test cells to maximum proliferation to the extent that the influence of the test surface is overwhelmed.

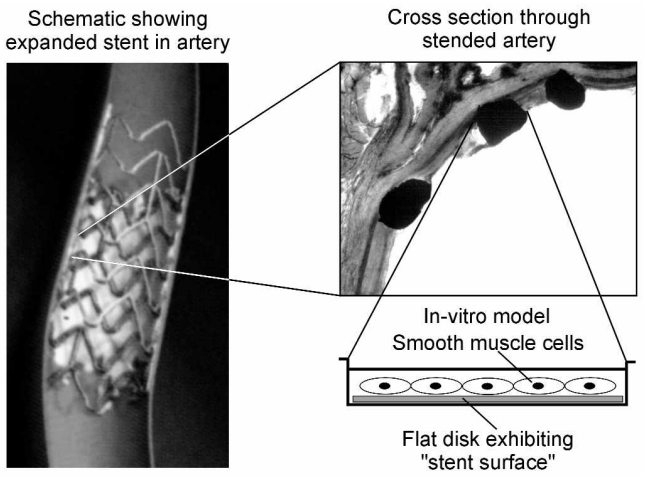


Figure 1. Illustration showing in macroscopic view (a) and in a histologic section (b) how the contact interface between the stent strut and the artery wall is modeled by a cell-culture assay using a flat disc on the bottom of the culture-well dish with smooth muscle cells growing on that surface (c).

Surfaces and Materials

All investigations were made using test specimens consisting of flat circular discs with a diameter of 3.5 mm suitable for insertion into the wells of conventional 6-well plates designed for cell-culture studies. The following materials were studied in particular:

- *316L-steel:* These discs were made of medical-grade stainless 316L-steel. The surface of the discs was electro-polished using a protocol routinely applied to refine the surface of medical stents at the end of the production process. These specimens were considered representative of many modern uncoated stents from various manufacturers.
- *316L-steel with a-SiC:H coating:* The core body of these specimens was identical to the 316L-steel discs described above. However, the surfaces were coated with amorphous silicon carbide (a-SiC:H) [5]. These specimens were designed to represent the silicon-carbide-coated Tenax stent.
- *a-SiC:H coating on glass:* The same coating was applied to glass discs to obtain specimens with an a-SiC:H coating that remain translucent for light microscopy.

Cells

Primary Bovine Aortic Smooth Muscle Cell (BASMC) cultures were obtained by outgrowth from medial explants from bovine thoracic aortas obtained within 4 hours of slaughter. Initial outgrowths as well as established cells were maintained in culture using Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10 % fetal bovine serum (FBS) in a humidified incubator equilibrated with a 5 % CO_2 atmosphere. Media were replenished every 3 – 4 days and cells were passaged every 7 – 10 days. All experiments were performed using cells of passage 10 or less. These cells were observed to exhibit typical morphological characteristics of vascular smooth muscle

tissue in vitro, and demonstrated specific immunoperoxidase staining by a monoclonal antibody selective for smooth muscle α-actin.

Definition of an Appropriate Level of Humoral Proliferative Stimulation

The media for the cell-cultures contain additives, including a certain amount of FBS, usually at a concentration of 10 %, which is suitable for providing a very strong proliferative stimulus. We performed an individual series of proliferation experiments under standardized culture conditions and increased the content of FBS stepwise from 0 % to 10 % for study of its impact on proliferation speed.

Verification of BASMC Adherence to the Test Surfaces

The proposed assay aims at precise quantification of cell proliferation by direct counting of the target cells after removal from the test surface. It is therefore essential that cells would reliably adhere to the surface over the entire observation period (up to 20 days) so that there would be no losses due to detachment of cells. To verify this precondition, we centrifuged aliquots of the cell-culture supernatant at any observational time-point and evaluated the fluids for detached cells. More interestingly, we prepared glass discs with the a-SiC:H surface. Due to the very thin a-SiC:H layer, the specimens were still translucent enough that direct microscopic control of cell morphology was feasible.

Principles of the Test

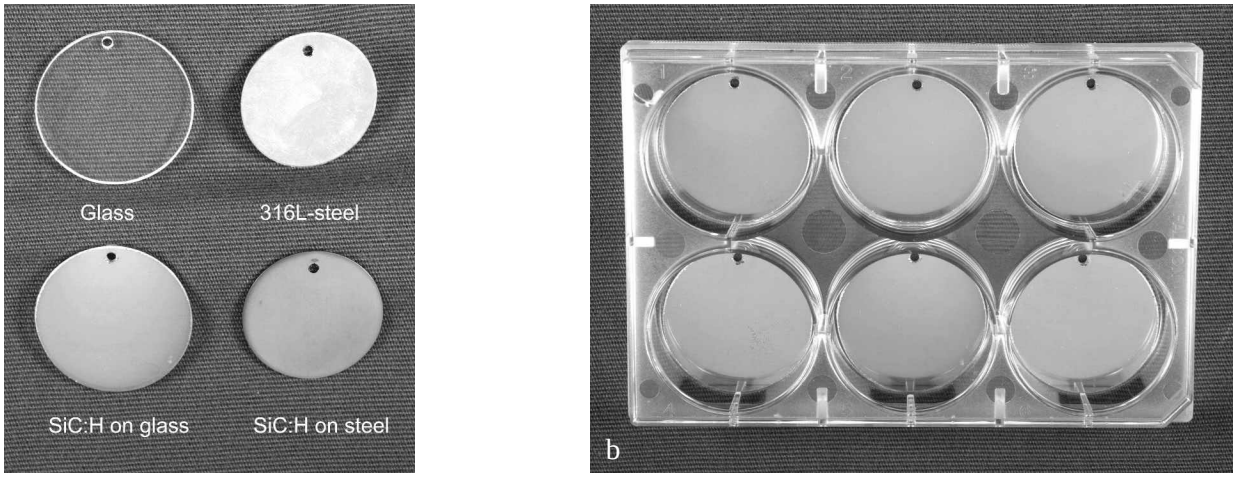
To facilitate close contact between the outer cellular membrane of the test cells and the surface of interest, BASMC were directly seeded onto the test surfaces, which consisted of flat circular discs with a diameter of 3.5 mm (Figure 2a). The outer dimensions of these disks were particularly chosen to enable snug fitting into the cell-culture wells of conventional 6-well plates (Falcon). As such, culture dishes with suitable geometry and media volumes appropriate for cell culture assays could be used (Figure 2b).

At day 0, cells were seeded onto the sample specimens at a density of 10,000/cm^2. Twelve equal specimens of each material were available. Thus, 6 individual time points could be evaluated using measurements from two independent cultures for each specimen type. The two cultures for each kind of specimen were terminated at day 0 (after 4 hours once cells were given sufficient time to attach), and at days 4, 12, 16, and 20.

Evaluation of the Assay

At termination of the culture growth, supernatants were harvested and stored at -70 °C; cells were removed from the specimens by trypsination, resuspended in a known volume, and counted visually under the microscope using a Neubauer chamber (Fisher Scientific, USA). The resulting numbers were expressed as cellular density after normalization of the raw counts to the surface area of growth (9.62 cm^2).

Two further normalization steps of the measured cellu-



Samples with “stent surface” Samples in 6-well plates

Figure 2. Photograph showing the individual specimens representing the surfaces of interest: glass, 316L-steel, a-SiC:H on glass, a-SiC:H on steel (a), and the 6-well plate for cell-culture with inserted a-SiC:H on glass specimens (b).

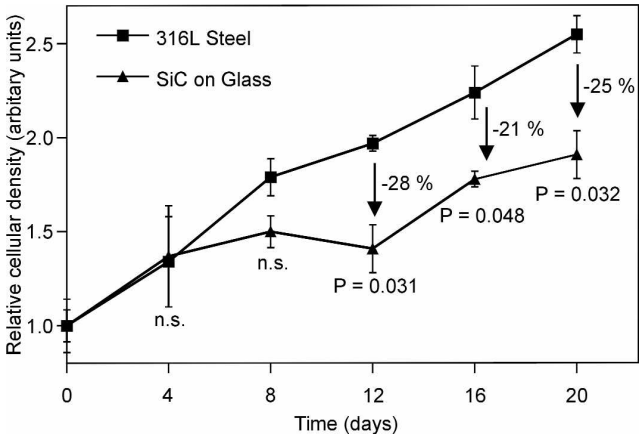


Figure 3. Graph showing the relative cellular density of cells plotted against time for a series-1 experiment comparing 316L-steel with a-SiC:H on glass. Cellular densities on a-SiC:H are significantly smaller than densities on 316L-steel at the 12-day mark ($P < 0.05$, $n = 2$). The relative reduction caused by the a-SiC:H on glass cultures in comparison with 316L-steel is also indicated.

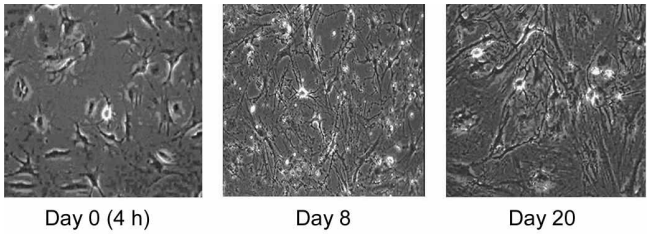


Figure 5. Light microscopy of bovine aortic smooth muscle cells growing on α-SiC:H on glass at days 0, 8, and 20. Cells were completely spread out and exhibited pseudopodia indicative of adherent growth at any given time point.

lar densities were performed. First, all densities for any given time point were related to the measured density of the same specimen at day 0. Thus, a factor was obtained that directly indicated the multiplication rate of the cells in the culture. It was found that this multiplication rate was variable depending on the number of passages the cells had gone through before. Therefore, it was necessary to relate the multiplication rate at any time point to a "standard" measured with the same cell line in the same experiment. We chose to utilize 316L-steel specimens as standard and expressed the relative cellular densities of any sample at any time point as a percentage of the 316L-steel density. Thus the final results of the test were independent of the initial cellular density as well as the age of the utilized cell line. Details of the evaluation are given in Figure 3.

Statistical Methods

All individual cellular densities at any given time point are given as mean values ± standard deviations calculated from double measurements using two independent cultures each. Mean values of cellular densities from different samples at the same time point within

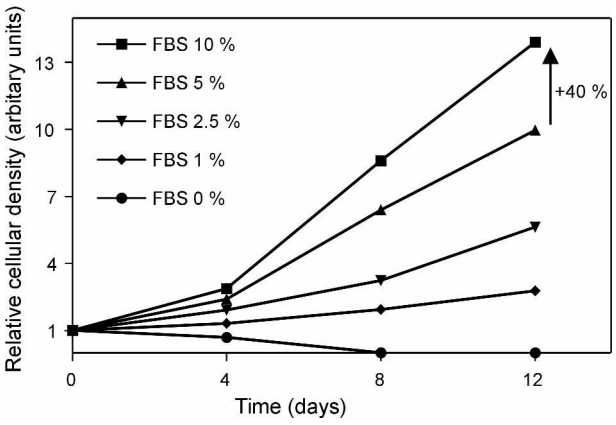


Figure 4. Results of an experiment designed to measure the impact of various concentrations of fetal bovine serum on smooth muscle cell proliferation. Note that the 5 % concentration allows the assessment of stimulation by 40 % in addition to the assessment of reduction. Therefore, a 5 % content of fetal bovine serum was chosen in all further experiments.

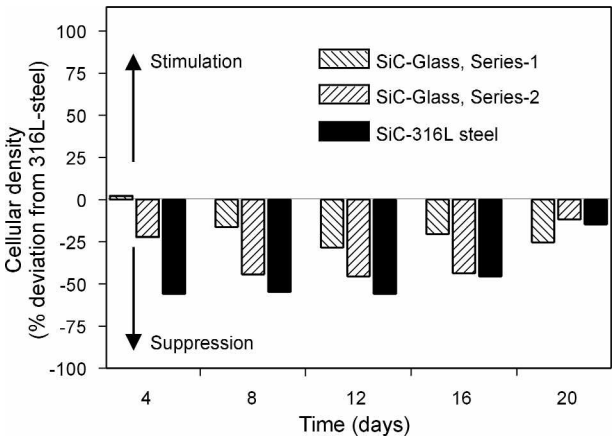


Figure 6. Bar graph diagram showing reduction of smooth muscle cell proliferation by the a-SiC:H surfaces on glass and 316L-steel for any given time point. Please note that all bars indicate a relative reduction in proliferation compared with uncoated 316L-steel (expressed in %).

the same experimental series were compared using Student's t-test.

Results

Variation of the FBS-content in the Cell-culture Medium

As expected, the proliferation rates exhibited a positive correlation with the FBS content in the culture medium (Figure 4). With 10 % FBS, cellular proliferation was the most rapid, reaching a thirteen-fold density over twelve days. In contrast, cells without FBS in the medium progressively died, such that these cultures did not contain any living cells after day 8. Cultures containing 5 % FBS showed an intermediate proliferation rate with a nine-fold multiplication. As such, the assay would be able to indicate not only a reduction of smooth muscle cell proliferation but also a stimulation of up to 40 %. We consequently chose a 5 % FBS content for all further experiments.

Adherence of the Test-cells to the a-SiC:H Surface

Microscopic evaluation of centrifuged aliquots of the cell-culture supernatants documented that they were cell-free at all time points. This finding was additionally confirmed by microscopy of the cells showing secure attachment to the a-SiC:H surface on the glass specimens. Four hours after seeding, the cells were spread out on the surface and had already developed pseudopodia indicative of adherent growth (Figure 5). The same morphologic appearance was observed at later time points, together with an increased density of the proliferating cells.

Comparison of the a-SiC:H Surface with 316L-steel

We found a marked reduction of the proliferative response of smooth muscle cells growing on various a-SiC:H surfaces in comparison with 316L steel. Both Figure 3 and the bar graph in Figure 6 indicate reductions in proliferation ranging from 25 % to 52 %. The effect was most significantly pronounced on day 12, with a tendency to flatten out over longer test periods, most likely due to contact inhibition of cells reaching confluence.

Discussion

This is the first report on a new in-vitro test for study of the impact of metallic surfaces on the proliferative response of the cell lines involved in in-stent restenosis. It was initially unclear if the preconditions for such a test, the establishment of adherent cellular growth, and the selection of an appropriate concentration of fetal bovine serum could be successfully implemented. However, it turned out that bovine aortic smooth muscle cells adhered securely to the investigated surfaces. Moreover, the stimulatory effect of fetal bovine serum (at a concentration of 5 %) in the culture medium was not overwhelming, so that the proliferative activity of the test cells was found to be different for the various investigated surfaces, and that this difference was consistently noted in each of three distinct experimental series.

Under the particular conditions of this in-vitro test, the a-SiC:H surface induces significantly less direct stimulation of the proliferative activity of smooth muscle cells in comparison with uncoated 316L-steel. However, the mechanism by which this finding may be explained was not elucidated in this study. In the light of the known effect of the a-SiC:H surface on fibrinogen, i.e. the complete prevention of any electron transfer between the metal and the fibrinogen molecule [3], it may be speculated that a reduced electron exchange with the cellular membrane of smooth muscle cells may contribute to the observed reduction in proliferation. It is known that the cellular membrane may process electrical signals to modulate the c-AMP concentration in the intracellular space [6]. However, little is known regarding the distinct role of c-AMP in cell-cycle regulation of restenotic smooth muscle cells.

Strictly speaking, the results of this study are only applicable to one particular mechanism contributing to in-stent restenosis, i.e. direct smooth muscle cell activation by the stent metal. Other contributing effects like humoral stimulation by cytokines released from activated macrophages, the formation of an extracellular matrix, or the progress of re-endothelialization of the stent struts remain unaddressed so far.

However, the proposed test design is particularly attractive because it has the potential to overcome some of these limitations. In addition to the study of direct smooth muscle cell stimulation, it would also allow the investigation of other cell-lines involved in restenosis, e.g., macrophages and endothelial cells, since both are known to proliferate while adhering to a variety of surfaces. Another characteristic that has not yet been evaluated is that the supernatants are available for further biochemical analysis. As such, the test may

offer the option of opening another field of biochemical research to elucidate the mechanisms underlying in-stent restenosis.

Conclusion

The presented test assay successfully documents that the a-SiC:H surface reproducibly induces less direct stimulation of smooth muscle cells than uncoated 316L-steel. Whether this finding is of prospective importance for the development of new stents, and whether it allows screening for particular surfaces, needs to be further established by a series of porcine experiments using the same 316L-steel stent with and without a-SiC:H coating.

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