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Enhanced Cellular Mobility Guided by TiO2 Nanotube Surfaces

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ABSTRACT

The in vitro endothelial response of primary bovine aortic endothelial cells (BAECs) was investigated on a flat Ti surface vs a nanostructured TiO_2 nanotube surface. The nanotopography provided nanoscale cues that facilitated cellular probing, cell sensing, and especially cell migration, where more organized actin cytoskeletal filaments formed lamellipodia and locomotive morphologies. Motile cell protrusions were able to probe down into the nanotube pores for contact stimulation, and focal adhesions were formed and disassembled readily for enhanced advancement of cellular fronts, which was not observed on a flat substrate of titanium. NO_x and endothelin-1 functional assays confirmed that the nanotubes also up-regulated an antithrombic cellular state for maintaining vascular tone. The enhanced endothelial response to TiO_2 nanotubes is significant for a potential modification of vascular stent surfaces in order to increase the rate and reliability of endothelialization and endothelial cell migration onto the stent for repairing arterial injury after activation.

Background and Significance. According to a recent report by the American Heart Association, approximately one million coronary stent procedures are performed every year in the United States.[1] Ideally, preceding the stent insertion, the inner lining of the artery should grow over the stent struts. However, once the stent is implanted into the artery, the time course of arterial healing varies from patient to patient and all patients are at a risk for formation of a blood clot, known as thrombus, inside the vessel at the stented site. Late stent thrombosis may occur months or even years after implantation and has become a complex clinical problem due to a lack of endothelialization, or coverage of endothelial cells over the inner stent wall, where the stent fails to be fully integrated in the vessel.[2,3] The delayed endothelialization of the intracoronary stent is believed to be the major factor for the risk of late stent thrombosis because the exposed stent surface acts as a nucleation site for thrombosis to arise.[4]

Once inserted, the stent is in direct contact with the endothelium and endothelial cell recruitment, migration, and coverage are critical for arterial healing and complete endothelialization.[5] To stimulate a positive reaction upon stent deployment and injury to the vessel wall, there is a need for an increase in the tissue/material interaction to stimulate faster wound healing. The main criteria for stent surfaces are primarily that they should not facilitate platelet aggregation and they should demonstrate thromboresistivity.[6] In the material selection of a vascular stent, the material should have surface properties that enable full integration of the stent as a part of the vessel and facilitate structurally sound endothelialization. The surface property of a material affects the rate of cellular migration, which is a major requirement for the success of stent implantation and ultimately the rate of endothelialization. Previous studies by Sprague et al. demonstrated that grooved surfaces double the migration rate of endothelial cells over polished and smooth controls.[7] The aim of this study however is to examine a different type of surface topography, nanotubular surfaces, based on the cellular response of primary bovine aortic endothelial cells (BAECs).

Even though it has been reported that endothelial cells function better on periodic or patterned nanostructures opposed to random nanostructures,[8] the concept of nanostructured surfaces has not extensively been looked at for potential stent surfaces. Additional studies have shown how nano cues may effect endothelial sensing, spreading, and attaching,[9] indicating that a nanostructured stent surface may be a promising approach to faster endothelialization. With this hypothesis, the use of a nanotubular surface has been explored in this work for the endothelialization with BAECs in vitro. The nanotubular surfaces have been evaluated based on cellular migration, adhesion, spreading, morphology, and functional properties compared to flat controls for potentially improving stent endothelialization.

The two main surfaces that are compared in this work are flat titanium (Ti) and the nanostructured surface of titania (TiO_2) nanotubes prepared by anodization. The polystyrene plastic cell culture plate (commercially available substrates often optimized for cell culture, from Nunc catalogue no. 150628) was also used as another base substrate. The

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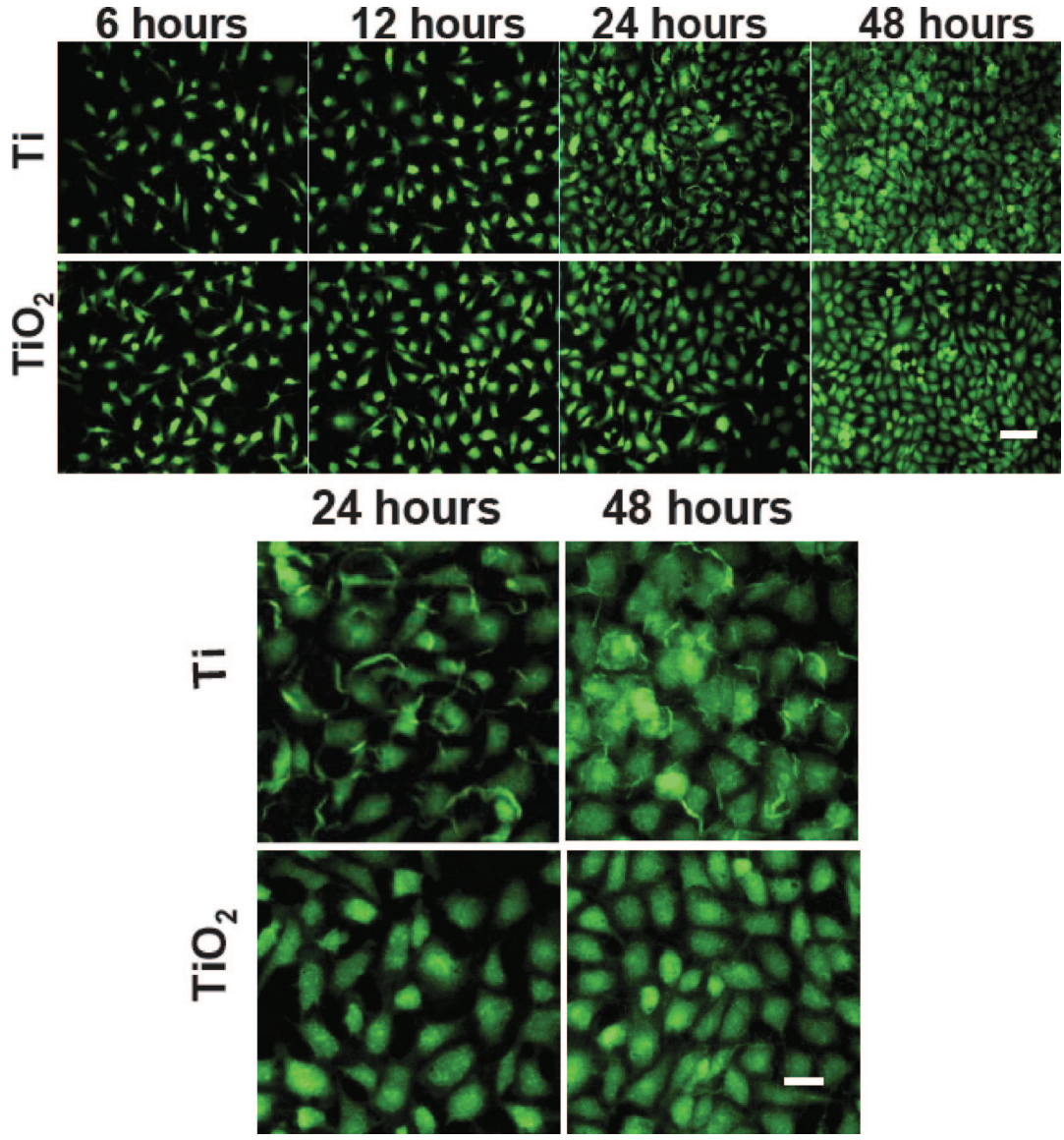

Figure 1. FDA viability of BAECs on flat Ti and TiO_2 nanotube substrates after 6, 12, 24, and 48 h of culture. The upper panel demonstrates that the cells are proliferating over the 48 h period and practically all the cells are alive on both Ti and TiO_2 surfaces (scale bar is 100 μm). The lower panel is a magnified view (scale bar 30 μm) of the 24 and 48 h images showing a flat monolayer of cells on the TiO_2 surface similar to natural endothelium and an aggregated, disrupted, and possibly detached status of cells on the Ti surface.

vertically aligned yet laterally spaced nanotubes have pronounced topological features and increased surface areas that play a significant role in cell behavior.[10–13] It has been demonstrated that the TiO_2 nanotubes induce enhanced hydroxyapatite formation[10] and allow for filopodia of growing cells to actually go into the nanotube pores for increased spreading and propagation.[11] In addition, Ti and its alloys are widely used implantable materials and have since gained popularity as a stent material, demonstrating several ideal properties needed for coronary stents including biocompatibility, stability in a surface oxide layer, and MRI compatibility.[6] This study illustrates that, for bare metal stents, such as Ti stents, it may be beneficial to consider surface modification techniques such as anodization in order to improve the quality and rate of endothelialization after implantation.

Cell Viability. The endothelium is derived of a layer of flat cells where the individual cells are anchored together to form a continuous monolayer linked together through cell-to-cell junctions.[14] Because the restoration of the endothelium after stent implantation is critical, the Ti and TiO_2 surfaces were compared according to cell viability and monolayer formation. Flouroscein diacetate (FDA) staining, Figure 1, revealed that the bare metal, Ti surface may not provide adequate conditions for monolayer formation. Although the cells proliferated over time and fully covered both surfaces, the BAECs on the flat Ti did not appear anchored, as indicated by cellular extensions seeming to be detached from the surface. The BAECs on the Ti surface looked aggregated and bunched up, possibly more disease prone. On the other hand, BAECs on the TiO_2 nanotube surface seem to be flatter and less aggregated with fewer detached cellular extensions.

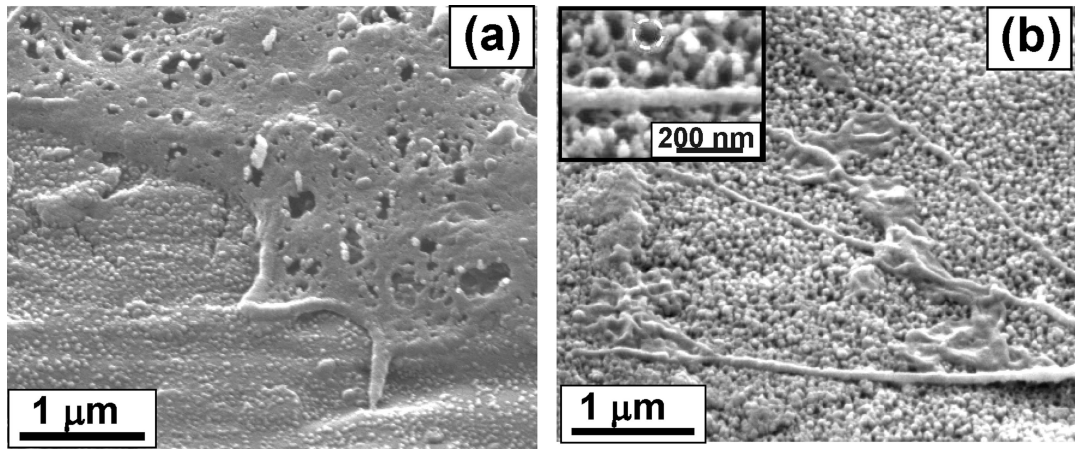

Figure 2. (a) SEM micrographs of BAECs on Ti and (b) on TiO_2 surfaces after 2 h of culture. A much more pronounced protrusion of filopodia with significantly longer configuration and a high degree of contact is seen on the TiO_2 nanotube surface. (The white dashed circle in the inset of Figure 2 shows the TiO_2 nanotube features.)

Further examination with increased statistics may be needed, but the cells on the TiO_2 surface appear to be evenly spread and formed a more complete monolayer than that on the Ti surface because they were seemingly more stable in the FDA images.

Scanning Electron Microscopy (SEM) for Substrate and Cell Morphological Examination. In the SEM micrographs, the nanotube structured surface on the anodized Ti metal substrate is evident. The typical dimensions of the hollow TiO_2 nanotubes as fabricated in this work are on the average ~100 nm outer diameter and ~70 nm inner diameter with ~15 nm in wall thickness and ~250 nm in height. The ~70 nm inner diameter is suitable for sufficient integrin functioning and activation, being in the approximate desired range between 53 and 73 nm needed for proper cell attachment reported by Arnold et al.[15]

Figure 2 show comparative SEM images of endothelial cells cultured (for 2 h) on flat Ti vs TiO_2 nanotube surfaces. The flat Ti substrate has a much lower surface area and does not render much topological cues and hence most likely could not provide for adequate attachment conditions for cellular probing, as indicated by Figure 2a, which shows filopodia that are not particularly pronounced. After two identical hours of incubation, the BAECs on the nanotubular surfaces, Figure 2b, show much more pronounced protrusion of filopodia with significantly longer configuration and a high degree of contact (also see the higher magnification insert in Figure 2b). The filopodia are also probing the surface and form more intimate contacts with the nanotubes, sometimes protruding into the nanotube holes. A similar behavior was also observed for osteoblast cells.[11] The interplay between the cell and the nanotubes allows for enhanced cellular propagation and an overall increase in cell/substrate interaction. The nanotubes may also facilitate movement because there are more holes, edges, and ledges that act as nano cues, which adds to the growing body of data that shows cellular responses to nanotopography.[10–16]

Clearly, the difference in surface structure has an effect on the response of the BAECs on flat vs nanotube surfaces. In relation to a cell migration scenario, such as that in wound healing after stent deployment, the nanotube surface has an advantage because the nanotube structure provides a better environment for typical migration processes that involves extension of leading cell edges, adhesion, and pulling forward. The nano features initiate cell probing and contact stimulation for cellular advancement. Because migration of cells onto the stent surfaces is essential for endothelialization and vascular remodeling, the three-dimensional environment may give topographical cues that allow for faster cell communication and migration. An additional aspect noteworthy to mention is that the unique geometry of nanotube arrangement with ~10 nm spacing between adjacent nanotubes[11] still allows fluid spaces even after the endothelial cells cover the nanotube top surface, thus enabling a continued supply of natural blood underneath the attached endothelial cells, as they are normally exposed to blood flow regularly in the artery. It is hypothesized that such a condition may also contribute to a healthier environment for the cells to thrive. It would be interesting to investigate the comparison of the nanotube samples with different diameters for endothelial cell culture so as to elucidate the possible effect of such gap spacing between the nanotubes; this would allow more light to be shed on the nature of topography versus chemistry in cell behavior.

SEM observations of endothelial cells cultured for longer incubation period of 6 h, Figure 3, indicate a striking difference in the formation of extra cellular matrix between the flat vs nanotube surfaces. There is a deposition of high density, rough material on the nanotubular surfaces. The nanotubes are well covered with extracellular matrix (ECM) type material, which was also observed in a recent study using the TiO_2 nanotubes for bone cell culture.[17] It may be that the nanotubes are actually regulating the cells by facilitating additional ECM deposition because the ECM distribution and quantity is much greater on the TiO_2 nanotube surface compared to the flat Ti surface. The ECM deposited upon the flat Ti surface appeared to be found in aggregated clumps and appeared sparser in comparison to the nanostructured substrates, however, an assay would need to be conducted to be able to quantify this difference.

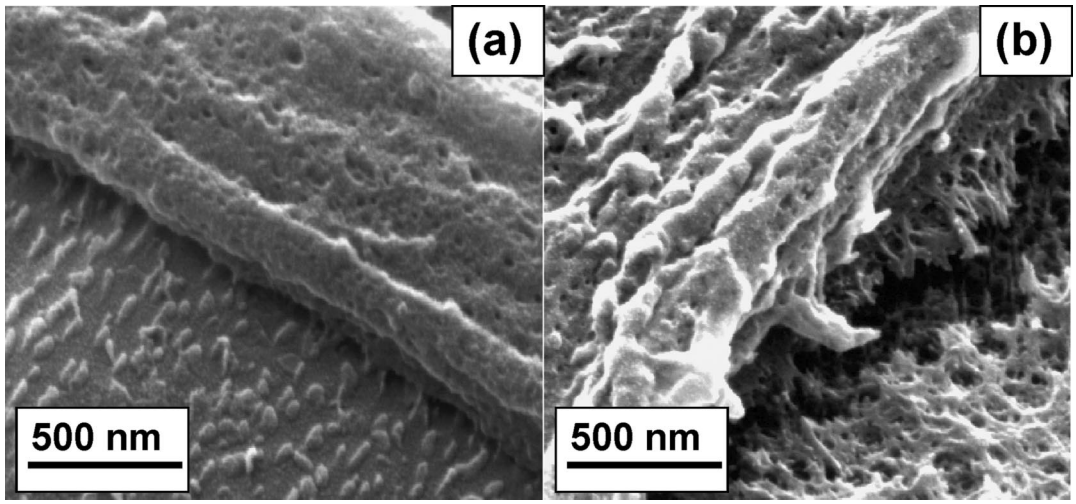

Figure 3. SEM micrographs after 6 h of incubation. (a) BAECs are deficient in ECM material on the flat Ti controls compared to (b) where a coarse ECM material is deposited on and filled in the nanotubes on the TiO_2 surface.

Furthermore, it is speculated that the increase in ECM on the nanotube surface may play a critical role in endothelial homeostasis because it helps form a more natural basal membrane for the cells to be imbedded, a type of "vascular bed" so to speak. The enhanced ECM formation may reduce possible thrombus formation as well because the stent surface is now well coated. The ECM itself serves as a type of substrate for cell adhesion and migration. The composition, density, and distribution of ECM exert critical regulatory influences on the cells through interaction of ECM.[18] The abundance of ECM on the nanotubes may also trigger faster cell signaling and endothelialization. The nanotubes may thus function as a tool for manipulating ECM distribution and ultimately cellular responses.

In addition, endothelial cells are known to produce several secretary products which are vital for maintaining vessel physiology including many ECM products such as fibronectin, laminin, collagen (I, II, III, IV, VIII, XVIII), and proteoglycans.[19] The nanotubes have the advantage over the flat surface because, as the endothelial cells will eventually completely cover the surface, there will be a reserve of ECM nutrients for preserving the health of the cells and vessel physiology, whereas only a limited supply of ECM would be available on the Ti substrate. The naturally present pores within nanotubes can be utilized as a nanodepot to store biomolecules or drugs for controlled slow release (e.g., for drug eluding stents), the rate of which should be controllable with the nanotube diameter and aspect ratio.

Actin/Vinculin Immunofluorescence and Morphological Analysis. The results for actin showed that the BAEC morphology and cytoskeleton organization depended on the different substrate surface topologies. The images in Figures 4 and 5 suggest that the cells on the nanotubes were much more motile because of the more prominent lamellipodia and fewer stress fibers that formed. This implies that the nanotubes were able to provide cues that organized the actin more efficiently for cell locomotion because all the cells have formed advancing fronts. The nanotubes may act as signals for initiating cell migration because the protrusion of prominent lamellipodia is the very first step in the process of cellular movement. The nanotubes possibly even transmit signals between cells more rapidly, speculated by the abundant cell/substrate contacts and ECM cues, as the images show cells crawling toward each other. The nanotubes up regulated cell to cell communication that is not apparent on the Ti and polystyrene controls because cellular interconnects and elongation toward each other were lacking. The actin images show that the nanotube surface has the advantage over the flat surfaces because it allows for more dynamic and coordinated changes in cytoskeleton organization, locomotion, and cell-to-cell communication.

The morphological analysis based on the actin observations shown in Figure 6 further imply that the nanotubes are influencing cell area and elongation. The cell spreading area was significantly lower for BAECs on the TiO_2 surfaces compared to the flat controls. As well the minor/major axis ratio was significantly lower on the nanotube surfaces, implying a more elongated, unidirectional, and polarized shape for facilitating migrational traction forces. Further analysis with a greater number of cells is needed to confirm this implication, however, it has already been demonstrated that the cell shape, rather than the cell spreading area, determine the efficiency of cell migration. Endothelial cells on 15 um patterned surfaces had a noticeably lower area and lower shape index (the value 1 for perfectly round and 0 for a straight line) but much higher migrational velocities than cells on larger micropatterned and nonpatterned surfaces.[18] This suggests that the cells on the nanotubes are probably migrating at faster speeds than on the flat surfaces and endothelialization rates would also be enhanced by the nanotube surface.

To further support this hypothesis, a time-dependent study of cell migration was conducted (data shown in Supporting Information, Figure S1), the result of which clearly suggests a faster cell mobility on 100 nm TiO_2 nanotube surfaces compared to flat Ti and smaller sized nanotubes of 50–70 nm diameter. This is in contrast to Park et al.'s data[12] reporting that the cell adhesion and spreading

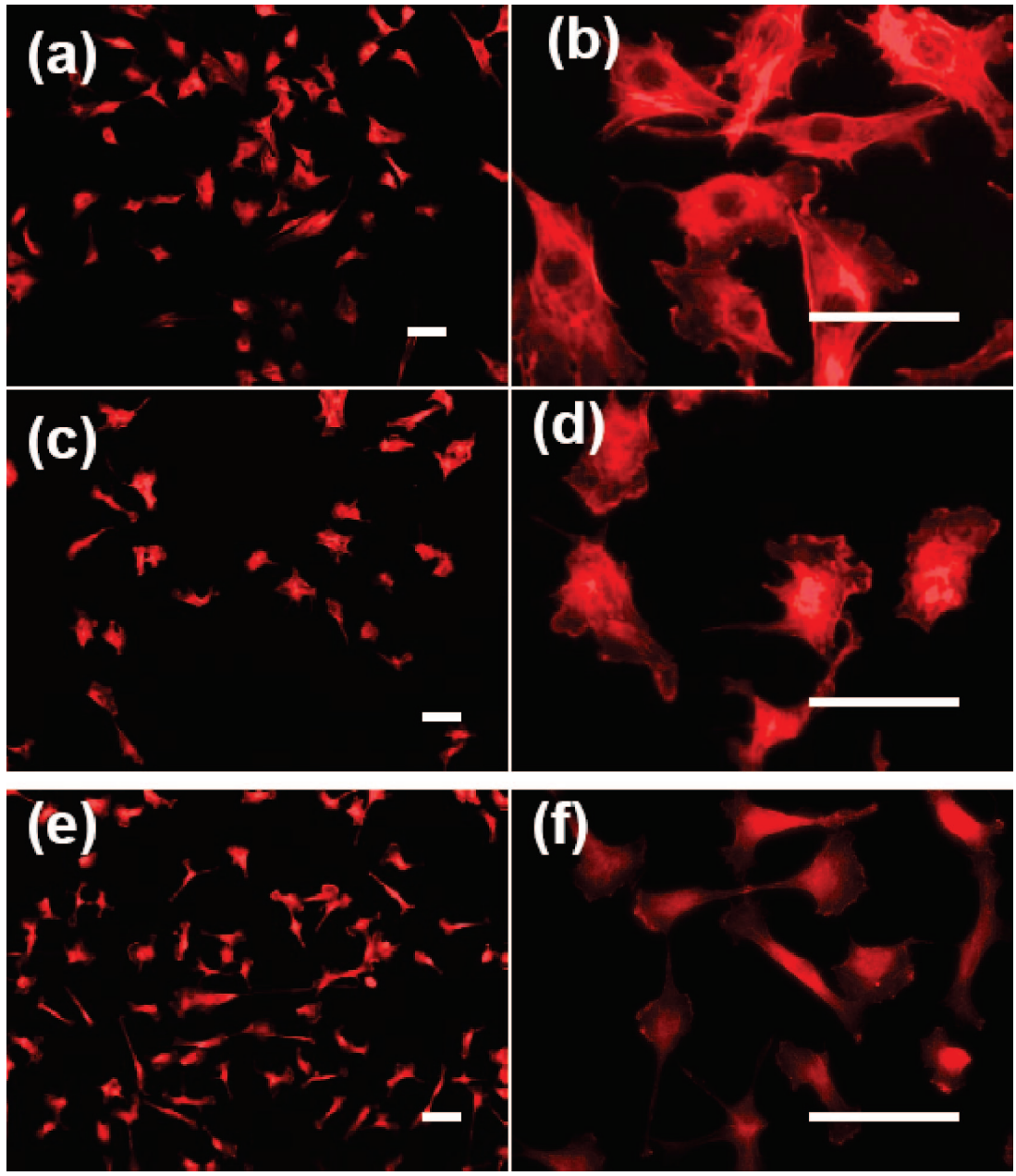

Figure 4. Immunofluorescent images of cytoskeletal actin for BAECs on polystyrene (a,b), flat Ti (c,d), and TiO_2 nanotube (e,f) surfaces after 24 h of culture. The lower magnification images show that the nanotube surface increases cell to cell interactions. The higher magnification images reveal that the nanotube surfaces trigger more organized, prominent lamellipodia for increased cell locomotion. Scale bars are 50 μm.

of mesenchymal stem cells was highest on smaller diameter ($\sim$15 nm) TiO_2 nanotubes and declined significantly with increasing pore size, showing dramatically reduced cellular activity and a high extent of cell death near $\sim$100 nm diameter nanotubes. This difference in the cell growth behavior as compared to our data might be caused by the substantially different nature of the TiO_2 nanotubes (as-anodized, amorphous phase TiO_2 nanotubes used in Park et al., vs heat-treated and crystallized, anatase phase TiO_2 nanotubes in our case) and also the type of cells involved (mesenchymal stem cells vs differentiated primary endothelial cells in our case). Further studies on the effect of TiO_2 nanotube dimensions, surface chemistry, and crystal structure on cell growth behavior of different cell types would be valuable for understanding of the nature of cell adhesion and propagation on nanotube substrates.

In vascular repair, according to ref 20, the nanotubes show qualities that are much more suitable than the flat surfaces because the cells are able to extrude prominent lamellipodia, spread, elongate, and migrate, as the actin results in this work suggest.

To further investigate the migration of the endothelial cells in this study, the focal adhesion protein vinculin was compared on the different surfaces. Vinculin is necessary for binding cell surface integrin receptors to the ECM adhesion molecules, which in part controls the process of cell spreading and movement. Vinculin is a major part of focal adhesion complexes. These focal adhesion complexes

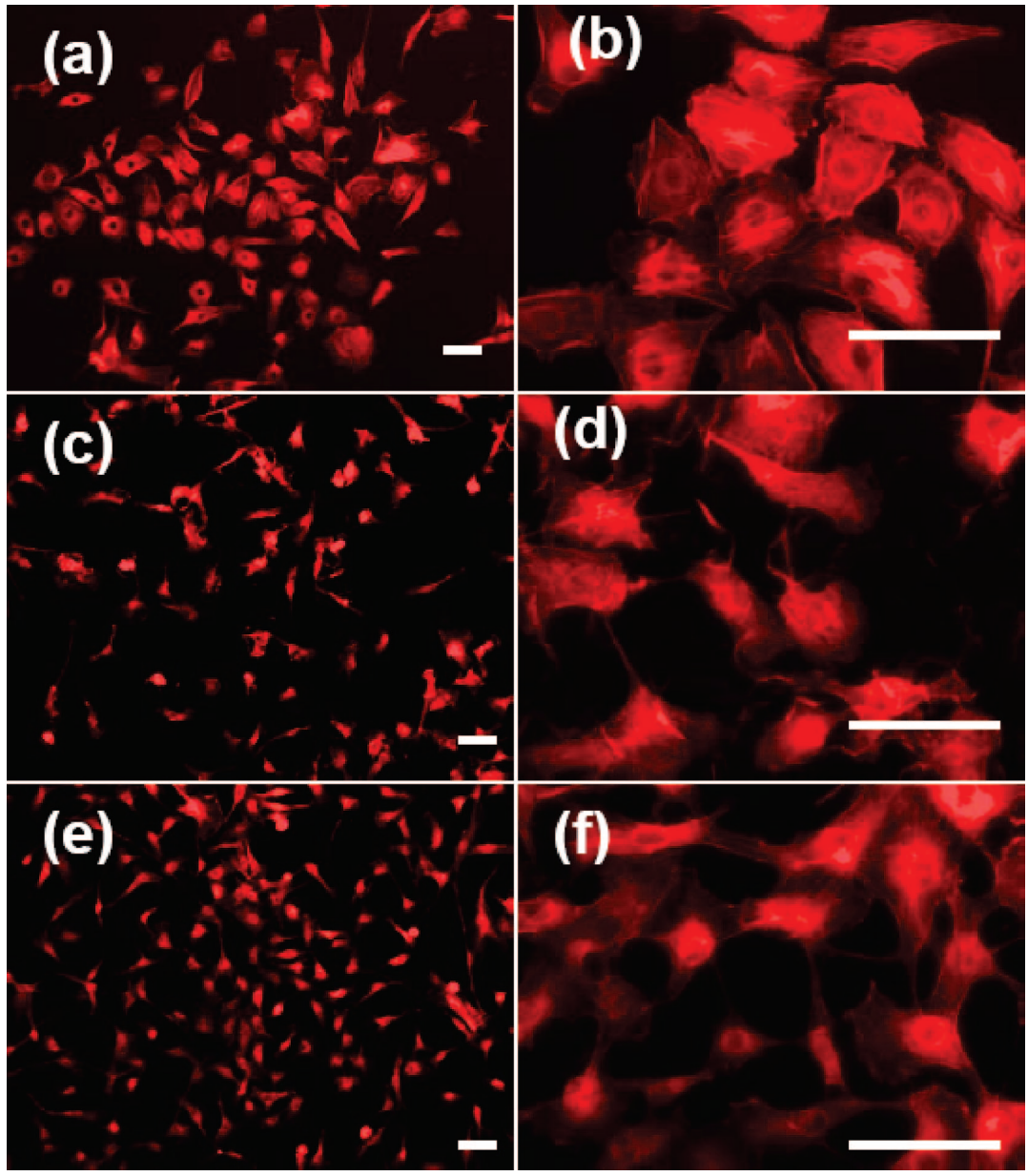

Figure 5. Immunofluorescent images of cytoskeletal actin for BAECs on polystyrene (a,b), flat Ti (c,d), and TiO_2 nanotube (e,f) surfaces after 48 h of culture. The BAECs on the nanotube surface are more elongated and mobile. The nanotube surface generates greater cell communication, interaction, and movement where BAECs on flat controls are spread wider with more stationary morphology. Unstable and broken cell extensions are seen on the Ti surfaces. Scale bars are 50 μm.

are the binding sites at the end of the cytoskeleton. When the cells are on the move so are these complexes, which allows the cell adhesion system to be very dynamic. Migrating cells have to form and disassemble focal adhesions readily. The vinculin results in Figure 7 show that the focal contacts were not large dash adhesions, like the ones on flat Ti, but rather smaller dot adhesions on the nanotube surfaces. Previously, Dalby et al. showed that nano islands caused fibroblast focal adhesions to be smaller and less pronounced than flat control surfaces, similar to other work with ordered nanotopography and our findings.[9,21] It was claimed that this was an indicator of more motile cells that could still be spreading. The BAECs on the TiO_2 nanostructures are probably actively forming and disassembling their focal adhesions for migrational purposes. The distribution of focal adhesions on the nanotube surfaces are primarily in the leading edges of the lamellipodia compared to the uneven distribution of adhesions on the Ti substrate. This agrees with the migrational study[18] and further suggests that the nanotubes facilitate a polarized distribution of contact with a lamellipodial protrusion in the front and the detachment of the tail in the back for accelerated cellular locomotion.

NO_x and Endothelin-1 Production. Healthy endothelial cells are fundamental for correct maintenance of the vasculature to ensure under normal conditions the appropriate regulation of platelet activation and to avoid blood coagulation.[22] In the endothelium, nitric oxide (NO_x) is continuously synthesized in order to maintain vascular homeostasis. It is an important vasodilator, as it is also called the vascular endothelium relaxation factor, and it inhibits platelet ag-

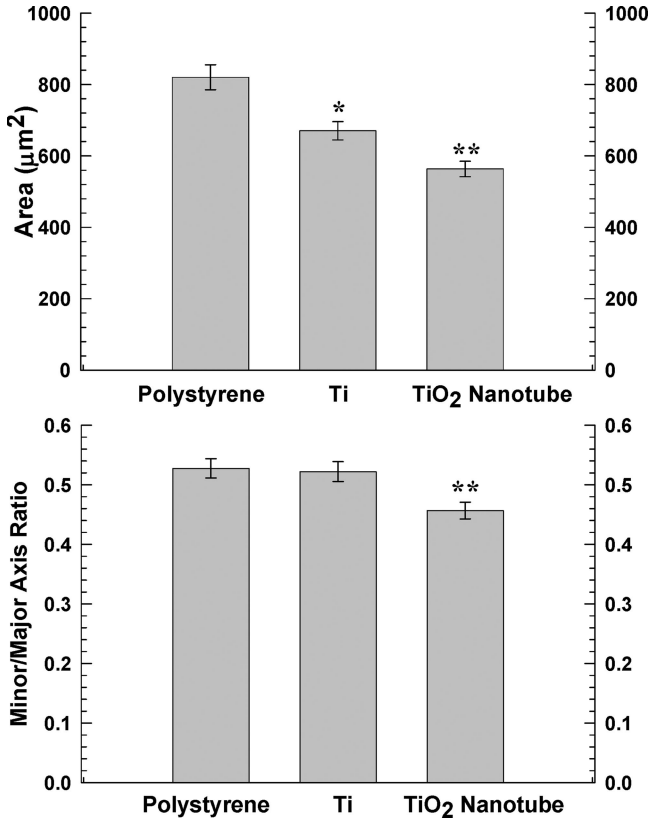

Figure 6. Analysis of BAEC morphology on polystyrene tissue culture plates, flat Ti, and TiO_2 nanotubes. The spreading area and minor/major axis ratio are calculated from at least 100 cells from three different fields ± SEM. * denotes $p < 0.05$ when compared with cells on polystyrene. ** denotes $p < 0.05$ when compared with cells on polystyrene and cells on Ti.

gregation.[5] On the other hand, the release of endothelin-1 counteracts NO_x and is a vasoconstrictor that promotes platelet aggregation.[23] A stent surface must be able to condition the cells for proper functioning so that the cells are producing normal biologically active substances and the surface must provide for a homeostatic environment. For this reason, the levels of NO_x and endothelin-1 produced by BAECs in reaction to the TiO_2 nanotubular and flat Ti surfaces were also investigated. The physical stimuli of the nanotopography of the TiO_2 nanotubes caused a greater increase of the in vitro NO_x/endothelin-1 ratio, see Table 1 and Figure 8, compared to flat controls. The TiO_2 surface induced a lower platelet aggregating endothelial state, increasing the vasodilating bioactivity and bioavailability indicative of the augmented NO_x levels per cell in the media. This improves vascular tone and controls blood flow, a central role of endothelial cells in vivo. Although NO_x levels alone produced by the cells increased on both the flat Ti and TiO_2 nanotube surfaces about the same, the peptide levels of endothelin-1 are significantly increased in BAECs on the flat Ti substrate, counteracting the healthy arterial environment. The aim of these experiments was to verify if NO_x and endothelin production by endothelial cells may be affected by the presence of the nanotube structure. The results obtained are encouraging and suggest that the use of the nanotube structure could up-regulate antithrombic conditions.

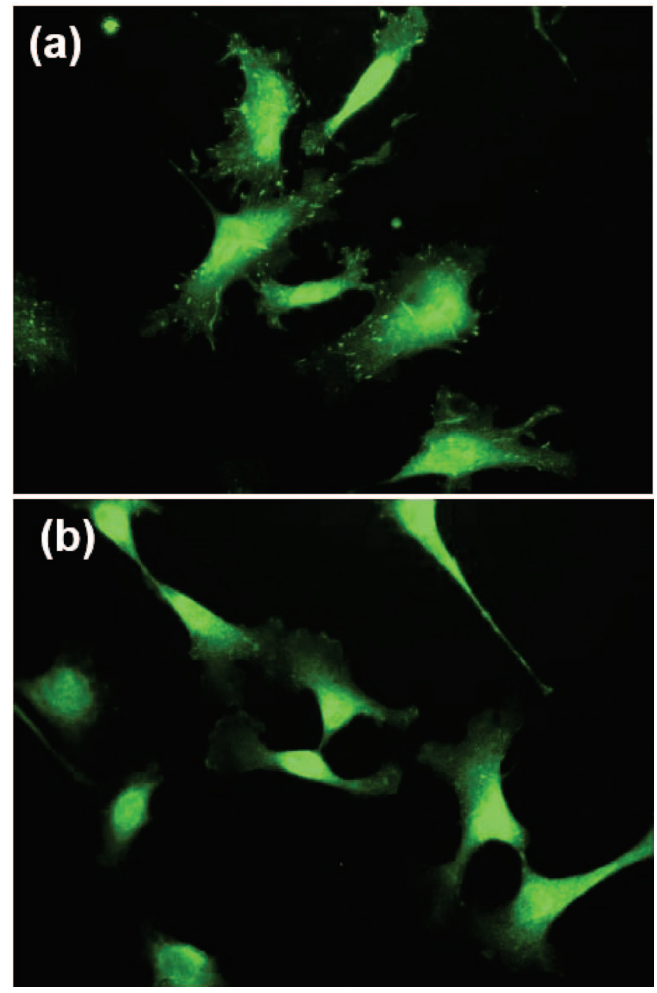

Figure 7. Immunofluorescent staining of vinculin at focal adhesions. (a) Cells on flat Ti and (b) cells on TiO_2 nanotubes.

Table 1. NO_x and Endothelin-1 Levels Released in the Culture Media for Polystyrene, Flat Ti, and TiO_2 Nanotube Substrates (Average of 4 Samples)

	polystyrene	flat Ti	TiO_2 nanotube
NO_x (μmol/cell)	2.88×10^{-5}	1.06×10^{-4}	9.12×10^{-5}
endothelin (pg/cell)	3.51×10^{-3}	8.82×10^{-3}	6.02×10^{-3}
NO_x/endothelin-1 ratio	8.21×10^{-3}	1.20×10^{-2}	1.51×10^{-2}

These results reveal that a nanostructured surface like TiO_2 nanotubes would provide for better cellular functioning than a bare metal Ti stent.

The beneficial effect of the TiO_2 nanotube structure for enhanced endothelialization, much increased extracellular matrix formation, and a substantially raised level of nitric oxide/endothelin ratio may possibly be utilized as a safer stent surface with a reduced probability of late thrombosis. Another possible approach of using the nanotube surface may be that a pre-endothelialization in vitro with a patient's own endothelial cells on the stent surface covered with TiO_2 nanotubes or similar nanostructures can be introduced to ensure complete endothelial coverage prior to stent implantation. The latter approach would be more applicable for the cases of nonemergency type stent implantation.

Conclusions. Primary bovine aorta endothelial cells were able to interact more efficiently with the TiO_2 nanotube

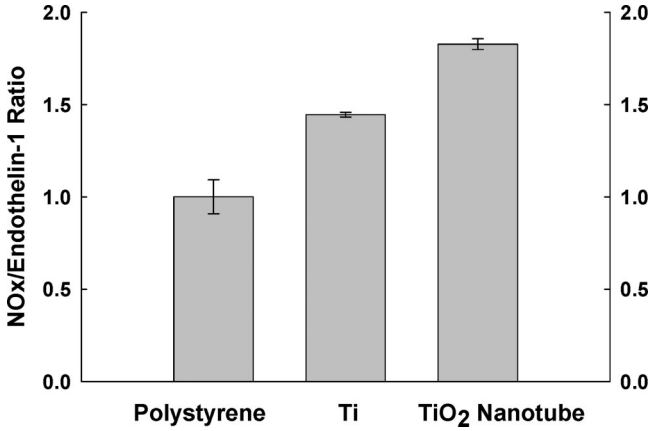

Figure 8. NO_x/endothelin-1 ratio with respect to the average of the polystyrene ± SEM.

surface with enhanced cellular migration and functioning as compared to a flat Ti surface. The combined nanotopography and nano cues caused an enhanced endothelial response in vitro. BAECs were able to probe the surface and interlock cellular extensions into the nanotubes for advanced sensing, elongation, and migration. The nanotubes also stimulated an increase in ECM deposition and induced a more natural "vascular bed". Actin filaments formed unidirectional cytoskeletons and more organized lamellipodia on the nanotube surface. Small dot instead of large dash vinculin staining proved that motile cells were observed on the nanotube surface. Last, the nanostructures increased the functionality of the cells by substantially increasing the NO_x/endothelin-1 ratio in the media. These beneficial effects seem to suggest that the nanotube structure with enhanced endothelialization, much increased extracellular matrix formation, and substantially raised level of nitric oxide/endothelin ratio may be useful as a vascular stent material with a reduced probability of late stent thrombosis.

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Supporting Information Available: All materials and methods: TiO_2 fabrication; primary bovine aorta endothelial cell culture; cell counting; FDA viability; scanning electron microscopy for substrate and cell morphological examination; immunofluorescence of actin and vinculin; data analysis for cell morphology; time-dependent cell mobility assay; NO_x and endothelin-1 functional assays; time-dependent study of cell migration on various substrates. This material is available free of charge via the Internet at http://pubs.acs.org.

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