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Reduced immune cell responses on nano and submicron rough titanium

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ABSTRACT

Current bare metal stents can be improved by nanotechnology to support the simultaneous acceleration of endothelialization and consequent reduction of immune cell responses after implantation. In our prior study, electron beam deposition was utilized to create different scales of roughness on titanium stents including flat (F-Ti), a mixture of nanometer and submicron (S-Ti), and nanometer (N-Ti). Enhanced endothelial responses (adhesion, migration, and nitric acid/endothelin-1 secretion) on nanometer to submicron rough titanium were observed compared to flat titanium. The present study aimed to further investigate the influence of nano and submicron titanium surface features on immune cells. Initial monocyte adhesion was found to be reduced on nano and submicron surface features compared to a flat surface. In a model including both endothelial cells and monocytes, it was proven that the submicron surface gave rise to an endothelial cell monolayer which generated the highest amount of NO_x and subsequently led to decreased adhesiveness of endothelial cells to monocytes. The analysis of monocyte morphology gave hints to less differentiated monocytes on a submicron surface. Furthermore, the adhesion of and pro-inflammatory cytokine release from macrophages were all reduced on nano and submicron titanium surface features compared to a flat surface. This study, thus, suggests that nano and submicron titanium surfaces should be further studied for improved vascular stent performance.

1. Introduction

The endothelial layer in arteries is important for maintaining numerous physiological functions, one of which is resistance to thrombosis formation [1]. During stenting, the endothelium is usually impaired and, in some cases, when a stent wall is not completely covered by endothelial cells after months, it acts as a reaction site for thrombus formation accompanied by an excessive inflammatory reaction [2]. This delayed endothelialization has been considered to be a major factor for late stent thrombosis and stent failure.

Thus, the design of the next generation of vascular stents demands simultaneous acceleration of endothelialization immediately after implantation and consequent reduction of immune cell responses. Besides drug-eluting stents, bare metal stent surfaces empowered by nanotechnology (or the use of constituent materials with at least one dimension less than 100 nm) have been of great interest because nanotopographies mimic the scale of the natural extracellular matrix environment of the vasculature and may support a natural endothelialization process more readily than traditional flat surfaces. So far, researchers have created nanopatterned, nanotubular, and random nanostructured surfaces on titanium-based stents and have successfully promoted endothelial cell functions without the use of pharmaceutical agents or growth factors (which undoubtedly can have undesirable side effects) [3–7].

In our prior study, electron beam deposition was utilized to create different scales of roughness on titanium stents, including flat features (F-Ti), a mixture of nanometer and submicron features (S-Ti), and nanometer features (N-Ti) [8]. We previously reported that endothelial cell adhesion, proliferation, and collagen/elastin synthesis were improved on submicron (followed by nanometer) stent surfaces compared to traditional flat surfaces [8]. We further investigated the underlying mechanism by studying initial protein adsorption, endothelial cell focal adhesion, migration, and nitric acid/endothelin-1 secretion (bioactive molecules secreted by endothelial cells indicating their thrombic or anti-thrombic state). All findings favored submicron (followed by nanometer) titanium surface features over flat titanium surfaces in terms of attracting and activating endothelial cells toward an anti-thrombic state [9]. Interestingly, platelet adhesion results showed that nano and submicron rough titanium surfaces also induced less

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attachment than flat surfaces, possibly indicating better blood compatibility [9].

However, an unanswered question about such submicron and nanometer titanium stent surface features is how they interact with immune cells recruited to the injury site immediately after implantation, which may give rise to an excessive inflammatory response, rendering the improved endothelialization identified in earlier studies mute.

Therefore, in the present in vitro study, we first assessed how circulating monocytes (precursors of macrophages) interacted with the three titanium surfaces of interest mentioned above: flat features (F-Ti), a mixture of nanometer and submicron features (S-Ti), and nanometer features (N-Ti) (Fig. 1).

We investigated not only their response to bare titanium surfaces, but also their interaction with an intermediate endothelial monolayer pre-formed on the titanium surface. Monocyte differentiation was analyzed via cell coverage and morphology after 5 days. Next, macrophage responses to the titanium surfaces of interest were investigated focusing on cell adhesion and the release of several pro-inflammation cytokines for up to 36 h of culture. Results showed significant promise for the reduction of an immune response by simply introducing nano and submicron roughness to stents (and not employing drug release which has adverse consequences in the body).

2. Materials and methods

2.1. Fabrication of titanium surfaces with nanometer to sub-micron roughness

This method has been previously described in [8,9]. All materials were also fully characterized for roughness and chemistry (showing no difference) in previous reports [8,9]. Briefly, pure titanium (99.8% purity: T-2069, Cerac Inc.) was deposited on glass coverslips (12-550-15, Fisher-Scientific) with 50 nm of thickness using an e-beam evaporator (in a vacuum state: 10^{-7} Torr) to generate flat titanium (F-Ti) surfaces. The default e-beam deposition rate used in this experiment was 2 Å/s with an e-beam current density of 60–70 mA/cm^2. A high deposition rate (20 Å/s with an e-beam current density of 130 mA/cm^2) was used to generate nano surface topographies (N-Ti) with a deposition of 50 nm thickness on F-Ti. In addition, to generating sub-micron surface features (S-Ti), the same e-beam current density was used with a deposition of 1 μm thickness on F-Ti. The e-beam energy was set to 7 keV during all experiments. These substrates were characterized by scanning electron microscope (LEO 1530VP, Zeiss) and atomic force microscopy (Dimension 3100, Veeco). All samples were sterilized by autoclaving before the cell studies described below.

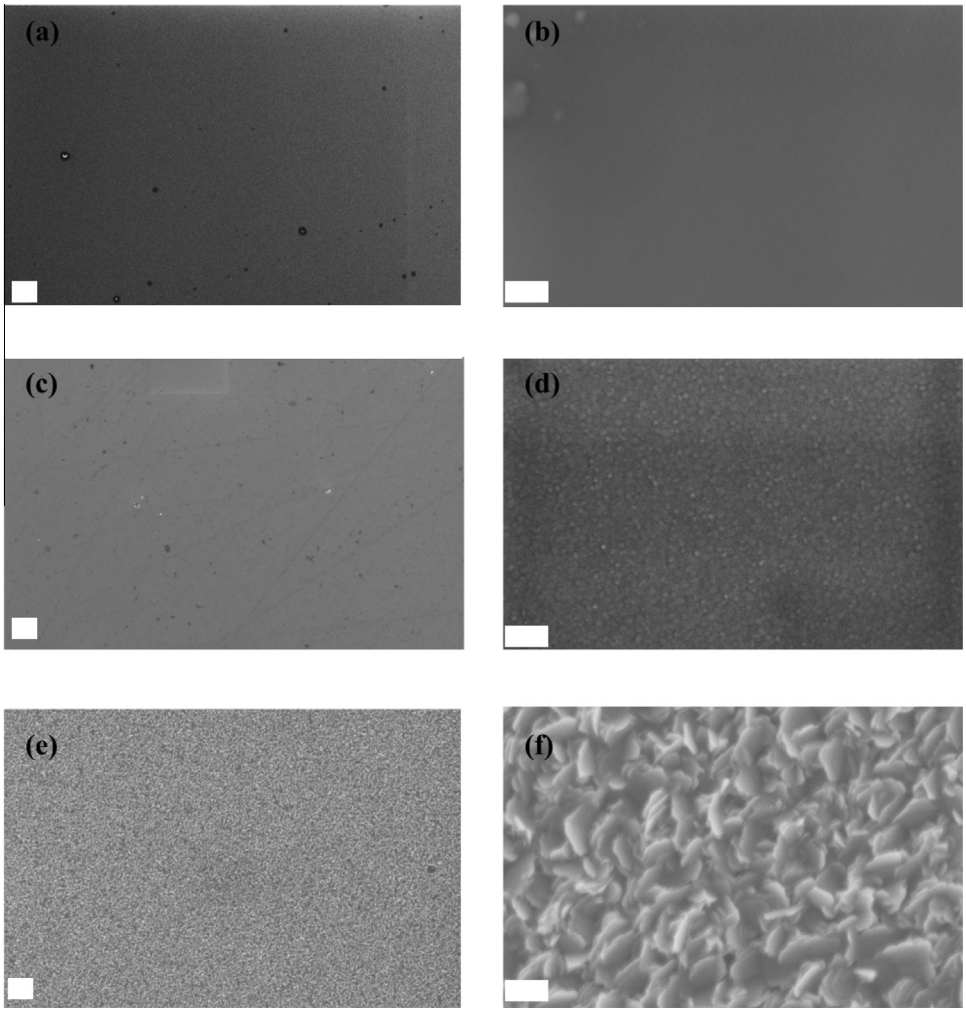

Fig. 1. SEM images of titanium surface roughness. (a) Flat surface (F-Ti), bar = 2 μm; (b) flat surface (F-Ti), bar = 200 nm; (c) nanorough surfaces (N-Ti), bar = 2 μm; (d) nanorough surfaces (N-Ti), bar = 200 nm; (e) sub-micron rough surfaces, bar = 1 μm and (f) sub-micron rough surfaces (S-Ti), bar = 200 nm.