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Novel micropatterns mechanically control fibrotic reactions at the surface of silicone implants

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ABSTRACT

Over the past decade, various implantable devices have been developed to treat diseases that were previously difficult to manage such diabetes, chronic pain, and neurodegenerative disorders. However, translation of these novel technologies into clinical practice is often difficult because fibrotic encapsulation and/or rejection impairs device function after body implantation. Ideally, cells of the host tissue should perceive the surface of the implant being similar to the normal extracellular matrix. Here, we developed an innovative approach to provide implant surfaces with adhesive protein micropatterns. The patterns were designed to promote adhesion of fibroblasts and macrophages by simultaneously suppressing fibrogenic activation of both cell types. In a rat model, subcutaneously implanted silicone pads provided with the novel micropatterns caused 6-fold lower formation of inflammatory giant cells compared with clinical grade, uncoated, or collagen-coated silicone implants. We further show that micropatterning of implants resulted in 2–3-fold reduced numbers of pro-fibrotic myofibroblast by inhibiting their mechanical activation. Our novel approach allows controlled cell attachment to implant surfaces, representing a critical advance for enhanced biointegration of implantable medical devices.

1. Introduction

The number of implanted medical devices such as pacemakers, glucose sensors, and joint replacements prosthesis is steadily increasing to address the needs of an aging population. These devices dramatically improved the life of millions of patients worldwide. However, in select applications such as diabetes and support of the central nervous system, sensors for continuous monitoring and treatment of chronic conditions, clinical translation is complicated due to foreign body reactions [1–8]. Depending on the surface material, recipient site, and contact duration, implants induce fibrotic reactions that manifest clinically as scar-like capsules around the device [3]. Fibrotic encapsulation not only reduces the function and half-life of the device but can severely affect the functionality of the host tissue in critical situations such as infections, often requiring additional surgery for explantation, adding risks for the patients and increasing the costs for the health care system [9].

To improve implant device acceptance and function by reducing foreign body and fibrotic reactions, we need to understand the mechanisms of tissue–biomaterial interactions. Several parameters have been investigated to control foreign body reactions such as functionalization with growth factors [10]. However, including such biological clues in the implant material has not yet been sufficient

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to guide healing for a variety of reasons. First, the release of signaling molecules must follow a precisely controlled time and concentration course in order to avoid a paradoxical down regulation of specific receptors. Second, the biophysical properties of the material is a powerful factor controlling the behavior of cells such as terminal differentiation or activation, even overriding specific actions of growth factors. Since controlling the orchestrated expression of soluble molecules *in vivo* is a daunting if not impossible task to date, we set out to manipulate the biophysical properties of cell interactions with the extracellular matrix (ECM) to guide cell behavior on the biomaterial surface. Our strategy principally aims in controlling the activation of host-tissue resident mesenchymal cells into myofibroblasts in the peri-prosthetic wound.

Myofibroblasts are characterized by the neo-expression of α-smooth muscle actin (α-SMA) and the excessive production of collagen. It is the incorporation of α-SMA into microfilament bundles (stress fibers) that confers high cell contractile activity [11]. Myofibroblast forces are transmitted to and perceived from the ECM at sites of large, "supermature" focal adhesions (FA) that are termed 'fibronexus' *in vivo* [12–15]. FA mechanosensing is the basis for spontaneous myofibroblast activation upon adhesion to sufficiently rigid surfaces [11]. Since abnormal interaction of fibroblasts with implant surfaces is a possible major cause for the development of implant encapsulation, a variety of specific surface coatings have been developed to improve biointegration [3,16], such as adding Arg-Gly-Asp (RGD) sequences to mimic the integrin signaling domain of fibronectin. However, we hypothesize that the specific size and distribution of the adhesion sites - rather than their sheer presence in the substrate itself – is the key aspect to guide cell attachment and behavior.

Using fibroblast culture models, we have previously shown that the size of FAs is directly proportional to the level of intracellular tension and the activation state of myofibroblasts. Formation of too small FAs prevents fibroblasts to attach and survive, formation of too large FAs translates into excessive tension development and ultimately myofibroblast activation [17]. Here, we identified specific anti-fibrotic protein micro-patterns and transferred them onto silicone polymer surfaces in order to modulate cell–implant interactions. To stably transfer cell-adhesive proteins with micrometer resolution onto compliant silicone substrates, we developed an innovative stencil technology. Proteins are deposited in the desired pattern through the openings of a stencil, produced by photolithography. The principle of our novel implant surface coating procedure is applicable to most implant materials and shapes and we demonstrate that it is effective to suppress myofibroblast and inflammatory cell activation at the surface of silicone implants in cell culture and animal experiments.

2. Materials and methods

2.1. Cell culture and reagents

Primary rat fibroblasts were explanted from subcutaneous tissue and used between passages P2 and P5 as described before [18] and cultured in standard Dulbecco's modified Eagle's medium (DMEM; Life Technologies), supplemented with 10% fetal bovine serum (Sigma–Aldrich), and penicillin/streptomycin (Life Technologies). Myofibroblast activation was induced using 2 ng/ml TGF-β1 (R&D Systems, Abington, UK) added once for 5 days to the culture medium. Murine lineage RAW 264.7 macrophages were cultured in standard medium and culture dishes. Macrophages were activated by adding LPS for 7 days to cells grown on coated/uncoated silicone substrates.

2.2. Hard stencil production

To create a 'hard microstencil' silicon mask, exhibiting arrays of openings with characteristics of FAs at the bottom of a micro-reservoir, we used a silicon-on-insulator (SOI) 380-2-50 wafer, dry etching, and soft lithography (Fig. 1a). Briefly, SOI wafer with the following dimensions have been used: upper silicon layer of 10 μm thickness, silicon oxide of 1 μm thickness and a lower silicon layer of 380 μm thickness. After cleaning the SOI with piranha solution, a 2 μm layer of oxide was grown by wet oxidation. This layer served as a reinforcement mask for the photoresist during the etching processes. To create the microstructure bottom side of the stencil we used a mask with small dimension holes and a 3 μm layer of AZ9260 photoresist. The mask pattern was transferred to the photoresist in a classical photolithography step. To achieve a high aspect ratio for the holes, which was recognized to be important for protein transfer, we applied an advanced dry etching Bosch process. After etching, the photoresist was removed and the wafer was turned to continue with the upper side's processing to generate the stencil reservoir. A second photolithography step was then applied, using a 10 μm layer of the photoresist AZ9260 and a second mask to produce the wide opening of the reservoir. Dry etching was again used to first remove the silicon oxide and second to etch through the thick silicon layer down to the interior oxide layer, serving to stop the etching process. Finally, photoresist and oxide layer were removed using a wet etching process to generate microstructure openings.

2.3. Pliable stencil production

To transfer proteins to curved surfaces that excluded close contact with the hard stencil, we developed an alternative method to create 'soft microstencils' (Fig. 1b). Briefly, plasma oxygen cleaned silicon wafers were used as support to deposit a 10 μm thick layer of parylene C using a parylene deposition system (Comelec C-30-S, La Chaux-de-Fonds, Switzerland). A second layer of 500 nm amorphous silicon (a-Si) was deposed using a sputter (Pfeiffer SPIDER 600, Zürich, Switzerland), serving as support for photoresist spin-coating. A photolithography step using a mask with small dimension holes was then applied to 1 μm photoresist S1805. After development of the photoresist, we applied a dry etching of a-Si layer using a silicon etcher (Alcatel AMS 200 SE, Annecy, France) and dry etching Parylene C using (STS Multiplex ICP). As terminal step, a-Si was stripped.

2.4. Silicone implants and protein deposition

Silicone pads consisting of clinical grade silicone (Silbione LSR4305, Silitech SA, Gümligen, Germany) with dimensions 10 × 10 × 1 mm were either left uncoated (control, corresponding to clinically used silicones) or covalently coated with collagen type I (100 μg/ml, Devro Medical LTD, Glascow, UK), or human plasma fibronectin (FN, Sigma). To provide surfaces with a non-protein adhesive molecule, we used poly N-acetyl glucosamine (sNAG, Marine Polymer Technologies, Inc., Burlington, MA, USA). For complete coating, collagen, FN, and sNAG were covalently bound to silicone substrates, in consequent treatments with plasma oxygen, 3-aminopropyltriethoxysilane (APTES) and glutaraldehyde [19,20] (Fig. 1c). In brief, silicone surfaces were pre-treated with plasma oxygen (100 W, 300 mTorr, 45 s) (Tepla, Kirchheim, Germany). This layer was then covalently provided with amine groups by incubation with APTES (Sigma) (1% in water) and subsequent heating to 60 °C for 60 min. Substrates were then copiously washed with PBS, immersed with 0.1% glutaraldehyde for 20 min, washed again 3-times with PBS and covalently crosslinked with collagen for 60 min at 37 °C [20]. For patterning, we exposed the silicone surface to the same protein/sNAG functionalization procedure through a silicon microstencil, exhibiting arrays of openings on the bottom of a micro-reservoir with dimensions 2 × 2, 4 × 2, 6 × 2, 8 × 2, 10 × 2, and 20 × 2 μm, and regular spacing of 5 μm. The stencil was removed to leaving the protein patterned silicone (Fig. 1c). In one series of experiments, we used polycarbonate filters (Merck Millipore, Tullagreen, Ireland) exhibiting pores with diameters of 2, 5 and 10 μm to produce irregular patterns of adhesive islands. All protein-coated surfaces were treated with PLL-g-PEG to prevent cell attachment any non-protein-coated regions (passivation) as described before [17].

2.5. Antibodies, microscopy, and image analysis

For immunofluorescence, we used primary antibodies directed against α-SMA (αSM-1, a gift from Dr. G. Gabbiani, University of Geneva, CH), FN (Sigma), collagen type I (Acris, San Diego, CA), F4/80 (BioLegend, San Diego, CA), CD68 (Abcam, Cambridge, MA), and vinculin (hVin-1, Sigma). Primary antibodies were probed with AlexaFluor-conjugated goat anti-mouse, goat anti-rat, and goat anti-rabbit secondary antibodies (Molecular Probes, Invitrogen, Basel, CH). DNA was probed with DAPI (Sigma) and F-actin with Phalloidin-Alexa 488 (Molecular Probes) [21]. Phase contrast and epifluorescence microscopy was performed using oil immersion objectives (Plan-Neofluar 40x/1.2 Ph3, Plan-Neofluar 63x/1.4 Ph3, Zeiss) mounted on an inverted microscope (Axiovert 135, Carl Zeiss AG, Feldbach, CH) and digital CCD camera (Hamamatsu C4742-95-12ERG, Bucher Biotec AG, Basel, CH). Images were acquired with Openlab 3.1.2 software (Improvision, Basel, CH) and assembled with Adobe Photoshop CS5. To quantify the level of myofibroblast activation, the percentages of α-SMA stress fiber-positive cells of all cells were quantified in a semi-automated algorithm in Image J (U. S. National Institutes of Health (NIH), Bethesda, Maryland, USA, http://imagej.nih.gov/ij/, 1997–2013). Macrophage fusion into multi-nucleated giant cells was quantified by automatically detecting the number of nuclei within the boundaries of F4/80-positive single cells. To exclude that aggregates are quantified as single cells, macrophages were detached after 7 day growth on silicone surfaces, mechanically separated and reseeded onto standard plastic dishes to be quantified after another 6 h. Mean values ± standard deviation