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INFLUENCE OF TOPOGRAPHY OF ENDOVASCULAR STENT MATERIAL ON SMOOTH MUSCLE CELL RESPONSE

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INFLUENCE OF TOPOGRAPHY OF ENDOVASCULAR STENT MATERIAL ON
SMOOTH MUSCLE CELL RESPONSE

A Thesis
Presented to
The Graduate School of
Clemson University

In Partial Fulfillment
of the Requirements for the Degree
Master of Science
Bioengineering

by
Vipul Taneja
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Accepted by:
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ABSTRACT

Although implantation of endovascular stents for the treatment of coronary and peripheral artery diseases has been one of the most rapidly used medical interventions, significant limitations in terms of a restenosis rate of 10-30% still persists. Neointimal hyperplasia characterized by a phenotypic shift of smooth muscle cells from contractile to synthetic type, has been deemed to be the predominant cause of restenosis. A number of stent surface parameters, including topography, have been attributed to play an important role in stent performance. Although endothelial cell and thrombotic response to surface roughness have been well evaluated, the effect of topography of the outer stent struts on smooth muscle cells has not been reported yet. The objective of this study, therefore, was to examine the effects of topography of a regularly used endovascular stent material (316L stainless steel) on smooth muscle cell phenotype as an indicator of neointimal hyperplasia and restenosis.

A model simulating the contact of stent material in the arterial wall was developed by utilizing 316L stainless steel of varying topography. Topography of as received 316L stainless steel was modified to get electropolished and micro-grooved surfaces using electropolishing and metallographic grinding respectively. Surface characterization was done using non contact profilometry, EDS and SEM. Cell morphology was analyzed using confocal microscopy, while a standard cell proliferation assay and DAPI cell counting were employed to study cellular growth. Also, a cell based

ELISA assay was developed to quantify smooth muscle α -actin expression as a marker of contractile phenotype of smooth muscle cells.

It was observed that cells grown on micro-grooved surfaces were significantly more elongated than the cells on both of the other surface types. Ascertained by repeated proliferation studies, cells grown on micro-grooved surface demonstrated as much as 44% lower cell count when compared to the electropolished surface. Results of ELISA assay indicated upto 63% higher α -actin expression in cells cultured on micro-grooved surface compared to the electropolished surface. Furthermore, cells on electropolished surface demonstrated a loss of 47% smooth muscle α -actin content per cell between day1 and day 4, while that on micro-grooved surface remained the same.

Since contractile smooth muscle cells are characterized by elongated spindle shaped morphology, low proliferation rate, and high α -actin expression, our results suggest that micro-grooves on 316L stainless steel promote contractile phenotype in smooth muscle cells when compared to the current industry standard - electropolished surface.

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CHAPTER ONE

INTRODUCTION AND BACKGROUND

1.1 Introduction

Cardiovascular diseases are the leading cause of mortality in the United States and Europe, accounting for approximately half of all deaths [1,2]. Furthermore, coronary heart disease accounts for more than 50% of all the deaths due to cardiovascular diseases making it the single leading cause of death in United States today [3]. Atherosclerosis which is a progressive build up of atheromatous plaque in arteries has a preference for developing in coronary arteries leading to coronary artery disease. This condition is commonly treated using balloon angioplasty which, in most cases, is done in conjunction with the deployment of a metallic stent. Stents exert a continuous radial pressure on the diseased artery resulting in compression of atherosclerotic plaques and thus assist in maintaining vessel patency. Although implantation of stents for the treatment of coronary and peripheral artery diseases has been one of the most rapidly used medical interventions, significant limitations in terms of restenosis (re-narrowing of stented artery) rate of 10-30% still persist [4]. This makes research into the improvement of stent design a very important endeavor.

A number of strategies have been proposed to optimize stent performance including drug loaded polymer coatings, pre-endothelialization of stents, gene delivery, and variations in stent geometry to name a few [5-8]. However, none of these strategies have

been completely successful in eradicating the problem of restenosis. This might be related to the current lack of a basic understanding of how a biomaterial interacts with the tissues at a cellular or a molecular level. Therefore, in order to improve stent performance, one must gain an understanding of the fundamental interactions between a stent/coating surface and the vessel wall. This understanding can then be used in an effort to optimize various parameters of stent design in order to minimize occurrence of restenosis.

Since the interface between the host tissue and foreign biomaterial plays an important role in implant biocompatibility, recent research in implant design has been focused on studying and engineering implant surface properties. The specific chemistry and topography of a biomaterial surface determine how the living host tissue interacts with the implant. Starting nanoseconds after implantation, proteins adsorb on the implant surface, and this adsorption is affected by the surface chemical nature and topographical features [9]. Surface-bound proteins may then mediate cell interaction dictating cell response. Numerous studies have documented that substratum topography affects the ability of cells to orient, migrate, proliferate, and to even induce changes in gene expression [10-11]. The application of micro- and nanotechnology, therefore, to the biomedical arena has brought a tremendous potential in terms of developing new diagnostic and therapeutic modalities.

Although microfabrication was mainly applied to semiconductor materials, techniques to translate micromachined structures from inorganic to organic polymeric materials were only introduced by Brunette in 1986 [12]. Since then, several different techniques have been developed to create micro and nano-structured surfaces [13-14] opening up tremendous opportunities in substrate development for medical implant design.

This chapter will take a systematic approach to gaining an understanding of vascular stenting as the primary modality for treatment of atherosclerosis, and the major complication of using vascular stents, neointimal hyperplasia leading to restenosis. This chapter also attempts to simplify the direct mechanotransductive pathways explaining how mechanical forces may be transduced to the cell nucleus and the current thinking as to how the nucleus turns these mechanical cues arising from topography into changes in cell behavior. Cellular response to synthetic topography is then discussed with an emphasis on vascular cells for synthetic vascular graft applications. Finally, the emphasis on the need to evaluate effects of surface topography of outer stent surface on smooth muscle cell (SMC) response is laid. A detailed understanding of these interactions could ultimately offer a powerful intrinsic tool to control the adverse SMC response seen during in-stent restenosis.

1.2 Endovascular stents

Predominant treatment options for atherosclerosis include bypass grafting, balloon angioplasty, and endovascular stenting. While bypass grafting is mainly limited by available autologous veins or arteries, efficacy of balloon angioplasty is questionable with a restenosis rate of 30-40% [4]. Stents, which are commonly metallic, lattice-like scaffolds are inserted into stenosed arteries following balloon angioplasty, thereby exerting a continuous radial pressure on the diseased artery wall resulting in compression of atherosclerotic plaques and thus in maintaining vessel patency.

Even though introduced in the 1960's, endovascular stents have triggered a substantial interest in the last two decades because of the high frequency of restenosis or re-closure of the vessel lumen. Stents have found extensive use in coronary applications, predominantly because of the fact that coronary arteries are the most common site for endovascular stent insertion and thus the enormous investment by stent manufacturing companies in developing coronary stents. However, with increase in peripheral occlusive diseases, increasing focus is being put on developing specialized stents, such as drug eluting stents (DES) for peripheral arteries as well.

Several criteria are important for stent design including biocompatibility, flexibility, radial strength, percentage of lesion covered, radio-opacity, minimal shortening at the

opening, cost, and surface properties such as topography, composition, free-energy/hydrophobicity, heterogeneity, and electrical potential [15-16].

Stents can be classified on the basis of a number of parameters. Based on geometry, stents can be wire mesh, slotted tube, ring, coil, or corrugated ring type. Based on method of deployment, stents can be divided into two major classes: (i) balloon expandable stents, and (ii) self expandable stents. Balloon expandable stents are manufactured in a crimped state and expanded in the vessel using a balloon. Self-expanding stents, on the other hand, are manufactured at the vessel diameter (or slightly above) and are crimped and constrained to a smaller diameter until the time of deployment, where the constraint (usually a sheath) is removed and the stent deployed.

Stents can also be distinguished into bare-metal or coated stents. Polymer coated drug eluting stents are discussed in the next section. Although drug eluting stents came up as an improvement to bare metal stents by limiting restenosis, they appear to invoke a thrombogenic response that leads to late thrombosis and eventual vessel occlusion [17]. A new generation of stents in the form of biodegradable stents offers a promising alternate to permanent metal stents, by presenting a way to deliver greater amount and controlled release of drugs, while being degraded when they have scaffolded the vessel for a substantial period of time. However, there are still pertinent issues regarding inflammation, mechanical properties, and toxicity that need to be addressed before the bright future of bio-degradable stents can be realized [18].

1.3 In-stent restenosis and drug-eluting stents (DES)

Following stent deployment, it is common to experience significant local damage to the inner most lining of arteries, the endothelium. This injury often results in a near complete denudation of the endothelial layer [19] which is manifested in the exposure of underlying thrombogenic collagen and sub-endothelial matrix that is accompanied by the loss of inhibitory effects of the endothelium on thrombus formation [15]. With the use of anticoagulant and anti-platelet therapy, thrombolization to such an extent as to occlude the vessel lumen is relatively rare. However, thrombus formation and inflammation process going on at the site of stenting which leads to high local concentration of growth factors such as cytokines and platelet-derived growth factor (PDGF) triggers a mitogenic and chemotactic response from SMCs [17]. It should be noted that the loss of endothelium also exposes the underlying medial layer of arteries to not only shear force due to blood flow but also to mechanical loading due to tightly appositioned stent struts. The over proliferation of smooth muscle cells to the various afore mentioned stimuli coupled with copious amounts of extracellular matrix (ECM) that these cells produce forms a neointimal tissue generally known as intimal hyperplasia which has been regarded as one of the major mechanisms responsible for restenosis. Figure 1 depicts the progression of in-stent restenosis.

Figure1: Proposed mechanism of restenosis (adapted²⁰)

Intimal hyperplasia which is clearly one of the major mechanisms responsible for restenosis is largely caused by a change in phenotype of vascular smooth muscle cells from a quiescent contractile phenotype to a proliferative synthetic phenotype [21-23]. Contractile SMCs are quiescent, morphologically smaller (figure 2) and more elongated than their counterpart. They demonstrate low migration, proliferation, and ECM protein synthesis.

Figure 2: Morphology of contractile and synthetic SMCs (adapted²⁵)

Additionally, contractile SMCs express relatively higher levels of contractile proteins such as smooth muscle α -actin, calponin, myosin heavy chain and smoothelin, which contribute to the cells' ability to contract [24-25]. In contrast, synthetic SMCs are marked by large morphology, higher cell proliferation, increased extracellular matrix production, and loss of the ability to contract. Thus, blocking of SMC proliferation and/or the overall phenotype shift from contractile to synthetic is seen as a useful way to reduce the incidence of restenosis and has been a major motivation for the development of drug-eluting stents.

Drug eluting stents (DES) rely on the delivery of drugs from a polymer coating on the stent with targets including cell proliferation and migration, inflammation and platelet activation [74]. Polymers that have been the recent subject of experimental testing as stent coating materials include polyethylene-co-vinyl acetate (PEVA), poly n-butyl methacrylate (PBMA), phosphorycholine (PC), polycaprolactone (PCL), poly(styrene-b-isobutylene-b-styrene), poly lactic acid-co-glycolic acid (PLGA) etc [28,30]. While DES have been shown to reduce in-stent restenosis [26-27], in no study published to date has the use of drug-eluting stents reduced mortality or myocardial infarction. This is in all likelihood because with a delay in growth and migration of smooth muscle cells, these drugs also bring about a simultaneous delay in re-endothelialization, potentially increasing the chances of acute and late thrombus formation [29].

1.4 Topography and mechanotransduction

It is well understood that that cells react to the topographic structure of their environment. The term “cell guidance” was first introduced by Weiss and Garber in 1952 [31] to describe how cells follow a topographical cue. In reaction to grooved topography in particular, cell guidance is believed to occur when cells are inhibited from crossing a step, and rather follow the step edge generally becoming elongated and aligned [10]. How the cells perceive such steps or other topographical features can be explained with the principles of mechanotransduction, the mechanism by which cells convert mechanical

signals into biochemical responses. For topography, these forces are considered to be generated by changes in cell spreading.

Such changes in cell morphology/spreading due to topographical cues can be attributed to two antagonist processes: (1) active force generation through conversion of chemical energy, and (2) passive resistance of the cell and reactive forces [31]. Some examples of the first category include contractile forces generated at cell-substrate contact [32], and active protrusive forces arising from polymerization of cytoskeleton elements [33], which for example drives lamelliopodium extension in fibroblasts or axon growth in neurons [31]. In the second category, lie all the mechanical processes that resist the described active forces, for example the viscoelastic strength of the overall cell structure. Another example in the second category would be the reactive forces that a cell would experience while attached to a topographical cue (Newton's third law of motion which states that "for every action, there is an equal and opposite reaction."), which would be consequently exerted on the cell membrane and transmitted to the rest of the cell via its cytoskeleton [34]. These active processes as well as reactive forces have been shown to affect organelle displacement including deformation of nucleus [35]. Changes in shape of nucleus may, in turn, alter the rate of transport of molecules through nuclear pores, and/or chromosome positioning etc. [36] which could ultimately affect many aspects of cell behavior. By what routes do these mechanical effects alter cellular behavior is what the principles of mechanotransduction attempt to explain.

1.4.1 Cell shape governs cell fate

Cell shape changes with changes in mechanical forces that a cell is subjected to [11,41]. This change is not kept to morphology alone, but has been shown to have effects on cellular proliferation, phenotype changes, and the overall cell fate. This is highly relevant for studying cellular response to topography, because reaction of cells to topography is usually manifested in a change in cell shape, although it is, by no means limited to just a change in cell shape.

Cell shape or distortion represents a visual manifestation of their underlying physical force distributions since cells spontaneously change their shape when the mechanical integrity of their substratum is altered. This is best explained with Ingber's three-dimensional tensegrity model [38]. Thus considering the fact that applied mechanical stimulus to cells causes change in intracellular force distribution leading to changes in cell shape, and tying it with the observation that a variety of nuclear functions including DNA and RNA synthesis can be controlled by perturbing cell shape [13,39], it is likely that changes in mechanical force distribution could have substantial consequences on cell fate in terms of apoptosis, differentiation, or growth etc. Ingber and collaborators observed a mechanochemical response in endothelial cells by controlling cell spreading using different densities of fibronectin as cell substratum [40]. Extensive cell spreading on high density fibronectin ($>500 \text{ ng/cm}^2$) resulted in proliferative and

non-differentiated cells, while round cells on low density fibronectin ($<100\text{ng/cm}^2$) exhibited detachment and apoptosis.

Chen and collaborators [11] showed that cell spreading controls growth versus apoptosis in capillary endothelial cells by using micro-patterned substrates that contained ECM-coated adhesive islands of varying size. In their following experiments, these authors patterned adhesive/non-adhesive regions to control cell spreading while maintaining constant cell/ECM contact area, for increasing cell-ECM contact area might increase integrin binding and accessibility to matrix-bound growth factors amongst other things. They found that cell spreading or cell shape per se was the critical determinant that switches cells between life and death and between proliferation and quiescence. These findings indicate that controlling substrate topography could be exploited to dictate cell shape which ultimately could determine the cell fate. An additional level of control to direct cell behavior is presented by the proteins that adsorb on a biomaterial surface, as it is believed that the proteins mediating cell attachment convey the underlying chemistry and topography through their own specific geometry and conformation [14].

1.4.2 How does topography exert mechanotransductive effects on cells

As discussed earlier, topography results in changes in cell spreading. The accompanied change in cell morphology leads to cytoskeleton tension rearrangements [42,43]. Furthermore, the resulting changes in cytoskeleton organization that mediate cell

spreading are driven by mechanical tension that is generated within actomyosin filaments inside the cytoskeleton [41,44]. This balance of intracellular mechanical forces that accompany cell spreading and drives cytoskeletal restructuring controls downstream signaling cascades by both direct transmission of forces to the nuclei as well by indirect routes. To this end, mechanotransduction can be divided into two major categories: direct and indirect [45]. Direct mechanotransduction takes into account the conformational changes in cell cytoskeleton to signal nucleus about changes in the extracellular environment like topography, and strain etc. Indirect mechanotransduction utilizes signaling events tied in with focal adhesions, G-proteins and tyrosine kinases etc. to relay information to the nucleus through various signaling cascades.

The exact mechanisms by which cells sense, signal, and respond to topography remain unclear, but direct mechanotransduction is currently being investigated in relation to topography. Indirect mechanotransductive events in cellular response to topography, however, have not been evaluated yet due to their intertwined nature and thus the associated complexity.

Maniotis and collaborators [36] reported that in reaction to tension, the intermediate filaments re-orient, the nucleus distorts and the nucleoli rearrange along the applied axis. This shows the mechanical integration of nucleus within the physical entity of the cell and that applied mechanical forces can lead to nuclear deformation. Intermediate filaments and microtubules are considered to play the main roles in direct

mechanotransduction by linking the plasma membrane to cell nucleus and acting as a conduit for transmittal of mechanical effects from the substrate to the cell [36,46]. Dalby's research group [10] studied response of fibroblasts to micro-grooved topographies and found that the cells aligned along the grooves and their nuclei were elongated. They also reported a large number of gene expression variations after 24 hours and 5 days compared to flat controls. These changes were predominantly in the areas of cytoskeleton, cell signaling, extracellular matrix regulation and proliferation.

Tying in the above observations on nucleus, cytoskeleton, and topographic interactions, Dalby and collaborators put forward a hypothesis of self induced mechanotransduction [10]. The authors suggest that surface topography alters nuclear morphology via direct mechanotransduction and in turn the position of the chromosomes and subsequent down-stream events including changes in the probability of gene transcription. To test their hypothesis, they conducted a study on fibroblasts on nano-columns, and found that cells on the columns had a spherical nuclei compared to an ovoid shape nuclei for cells on flat controls [10]. Centromeres in cells on nano-columns were found to be significantly closer compared to the control. Furthermore, the cells were shown to be more relaxed on the nano-columns compared to well-spread cells on flat controls suggesting that less tension was applied to the nuclei of cells on the former. Assuming, then that cellular effects to topography come mostly if not entirely from the ability of cells to spread, it is possible that a change in chromosome position following

mechanotransduction can lead to altered gene transcription which may manifest itself in changes in cell growth, proliferation, apoptosis, phenotypic changes and so on.

Another potential mechanism for direct mechanotransduction in response to topography could be that the structural changes in the nuclear envelope may influence accessibility of transcription factors [36]. However, these are only theories, and need further investigation to comprehend the role of direct mechanotransduction in cellular response to topography. As well as direct mechanotransduction, indirect mechanotransduction should also be taking place. It has been shown that integrin binding to RGD and similar peptides can signal the G-protein and kinase based signaling events, and produce many down-stream effects [47,48]. Thus, indirect mechanotransductive events entail thorough investigation too.

Ultimately, how a cell responds to topography depends on size the scale of topography - bottom-up if the grooves are in the nano range where a cell will orientate one adhesion at a time, and top-down in case of micron level grooves where the cells are forced to align and the adhesions will align as the cytoskeleton has to [Personal communication with Dr. Matthew J Dalby, University of Glasgow, UK]. Either ways, it is likely that adhesion signaling and cytoskeletal arrangements will be the key through direct and indirect routes.

1.5 Effects of synthetic micro and nano-structured surfaces on cell behavior

The interaction between cells and biomaterials is influenced by a number of physical and chemical factors, of which one of the major factor is surface topography. The influence of microtopography on cells has been known from a long time with one of the first studies using microfabricated substrates to study cellular behavior performed by Brunette and collaborators [49]. They demonstrated the use of a silicon mask-etching technique to prepare grooved surfaces to control the direction of outgrowths of human gingival explants. Subsequently, there have been many studies showing topographical control of cells and even tissues. Surface micro and nano-topography have been shown to modulate the cell morphology, adhesion, proliferation, migration, and differentiation for a variety of cells including fibroblasts, osteoblasts, macrophages, hepatocyte, BHK cells, epithelial cells, neuronal cells, endothelial cells, and smooth muscle cells. Varying cellular responses to defined topographies for a variety of cells have been reviewed elsewhere [13,50]. Response of vascular smooth muscle cells to topography has been highlighted for the purpose of this review.

1.5.1 Range of phenomena

Different types of substratum topography have been shown to influence cell behavior depending on both scale and feature type, for example micro- and nanometre scale ridges and grooves and if there are randomly or evenly distributed features or artifacts such as

pits or spikes [51,52]. Micro-topography has been shown to influence cell response as these dimensions are comparable with cellular dimensions of 10-30 μm , while nanotopography has been shown to predominantly influence cellular orientation [14,52].

1.5.2 Chemistry or topography

It becomes very important at this point to introduce a question that has been asked repeatedly in relation to response of cells to topography. The question is whether the cells are reacting to the topography directly or to the patterned substratum chemistry formed during fabrication of topography (e.g. by any etching process or otherwise).

This problem has been largely answered by the work of Britland et al. [54]. These authors used laminin as a chemical cue oriented at right angles to grooves used as topographic cues. When the grooves were 500nm deep or less, the cells reacted chiefly to the chemical cue. On deeper grooves, the topographic cue over-rode the chemical one and at 5 μm depth, the topographic effect oriented about 80% of the cells and the chemical one 7%. Thus, the ability to synthesize well-controlled topographies holds the promise to direct cell function, either without or synergistically with the use of chemical cues.

Cellular response to topography has been studied for a wide variety of cell types and topographical features including grooves, ridges, pores, wells, steps and aligned or non-aligned fibers etc. It is well established that different cell types will exhibit different

behavior on different substrate topographies. Hence, the overall trends of effect of topography on cellular response are challenging to determine and are rather cell type and application specific. For the purpose of this review, therefore, the response of vascular smooth muscle cells to micro and nano-topographies is being discussed.

1.5.3 Response of smooth muscle cells to topography

While commercial synthetic vascular grafts have had success in replacing large arteries ($>6\text{mm}$), thrombosis and intimal hyperplasia have plagued small diameter vascular grafts ($< 6\text{mm}$) causing a substantial drop in patency rate, from 95% for large diameter vascular grafts to 30% in small diameter vascular grafts, after only 5 years [55]. Tissue engineering comes across as a promising way to synthesize functional vascular grafts, and has led to exploration for numerous design modifications [56] to not only achieve adequate mechanical properties, but to recapitulate the in vivo function of native blood vessels. The medial layer provides the main structural support of the vessel and consists of multiple layers of smooth muscle cells (SMCs) and extracellular matrix (ECM) [57]. SMCs in native blood vessels, referred to as contractile SMCs, have a spindle-shaped morphology and align in circumferential direction to cause vessel constriction or dilation [57]. When cultured in vitro, these cells lose their contractility and assume a synthetic phenotype [24-25]. Maintaining the contractile phenotype of SMCs has long been an unsolved problem in developing tissue engineered vascular grafts gaining significant attention in the last 2 decades. To this end, a number of studies have

reported the potential of topography in forming a defined architecture to guide SMC growth and development as needed. Additionally, Thakar and collaborators [58] demonstrated the potential use of micro-patterning for controlling SMC phenotype, alignment, and proliferation to produce functional tissue-engineered PLGA vascular grafts. Selected studies to control SMC growth and differentiation with topographical cues are summarized in Table1.

Table 1 Selected references showing effect of topographical features on smooth muscle cells

Cell type [Ref]	Feature type	Fabrication method	Material	Feature dimensions	Cellular response
Rat aortic [59]	Stochastic roughness	Treatment with NaOH, casting with Sylgard followed by PLGA casting	PLGA	Nano-structured; exact dimensions not listed	Enhanced cell proliferation & adhesion
Rat aortic [60]	Stochastic roughness	Compacted nanoparticles	Cp Ti, CoCrMo	Nano-structured	Increased adhesion, spread morphology
Human coronary artery [61]	Aligned fibers	Electrospinning	P(LLA-CL)	500nm diameter, random spacing	Cells aligned & migrated along fibers; were spindle shaped; showed less proliferation compared to control surfaces
Bovine pulmonary artery [62]	Nanogratings	Nano imprint lithography, molding	PMMA, PDMS	350nm linewidth, 700nm pitch, 350nm depth	Significant elongation & alignment in cytoskeleton & nuclei compared to non-patterned control
Human aortic [63]	Grooves	Soft-lithography	PDMS	10µm wide, 2.8µm deep	Elongated morphology, lower proliferation rate compared to planar control
Human coronary artery [64]	Grooves	Photolithography	PDMS	2-10µm wide, 50-200nm deep	Elongated morphology, migration along grooves
Human umbilical [65]	Grooves	Photolithography, soft-lithography	PDMS	20-80µm wide, 5µm deep	Elongated morphology, actin filaments aligned along grooves
Human aortic [66]	Channels with discontinuous walls	Deep reactive ion etching	PLGA	300µm wide, microwall 60µm high, 160µm long, separated by 40µm gaps	Elongated morphology, contractile phenotype at confluence
Bovine aortic	Grooves	Microfluidic	Collagen,	20,30,50 µm wide	Decreased proliferation, cell

[58]		patterning, photolithography	PLGA		shape index, expression of α -actin on 20 & 30 μ m collagen; similar results for PLGA with 30 μ m groove width
Rat aortic [67]	Three-dimensional micro-channels	Deep reactive ion etching	PCLLGA	10-160 μ m wide, 10/25 μ m thick walls, 65 μ m deep	Cells in wider micro-channels (80-160 μ m) showed elongated morphology, contractile phenotype compared to control surfaces.

PLGA: *poly(lactic-co-glycolic acid)*; P(LLA-CL): *poly(L -lactid-co- ϵ -caprolactone)*; PMMA: *poly(methyl methacrylate)*;

PDMS: *poly(dimethylsiloxane)*; PCLLGA: *poly(ϵ -caprolactone-r-L-lactide-r-glycolide) diacrylate*; ECM: extra cellular matrix

1.6 Implications for endovascular stent applications

Stents are metallic, lattice-like scaffolds that are inserted into stenosed arteries thereby exerting a continuous radial pressure on artery wall resulting in compression of atherosclerotic plaques and thus maintaining vessel patency. The major problem associated with use of stents is re-narrowing of the treated artery called in-stent restenosis which occurs in 10-30% of all stent procedures [4]. This has led to the development of drug eluting stents as well as other strategies aimed at limiting neointimal hyperplasia and thrombosis- the two major determinants of clinical restenosis. Since anti-platelet therapy is used to limit thrombogenesis, most of the research in this regard has been aimed at limiting neointimal hyperplasia which is caused by SMC hyperplasia, and migration following endothelium denudation caused during stent deployment [17]. Thus, majority of the strategies to improve the performance of stents have been aimed to either promote stent strut endothelialization, or to limit smooth muscle cell migration, proliferation and an overall phenotypic change from contractile to synthetic type.

In relation to endovascular stents, the degree of smoothness or flatness has been the choice of investigation for a long time. It has been shown that polishing of coronary stents results in decreased thrombogenicity as well as neointimal hyperplasia in different animal models [68,69]. Recent investigation of influence of stent topography on endothelial cells, however, revealed conflicting results with rough stent surfaces showing accelerated endothelialization rate and similar to lesser restenosis when compared to

smooth stent surfaces in both in-vivo and in-vitro studies [70-73]. Palmaz et al. [70] observed that surface grooves at microscopic scale influence the conformation and migration of endothelial cells and concluded that the endothelial cell migration rates significantly increased on surfaces with grooves ranging from 3 to 22 μ m with a maximum at surface with 22 μ m grooves compared to the flat control surfaces. In another study [71] stents with micron level parallel grooves were placed in carotid arteries of pigs. It was reported that at 1 week after implantation, textured surfaces exhibited an endothelialization rate almost double that observed on stents with smooth surfaces. Dibra and collaborators [72] conducted the first clinical study to evaluate the relationship between stent surface topography and outcome in patients undergoing implantation of electrochemically polished and sand blasted stochastically rough stent surfaces. They observed that both types of stents were equivalent with respect to late lumen loss but the results indicated a reduced restenosis for the sand blasted stent surface as compared to smooth electrochemically polished stents. Very recently, Choubey and research group [73] studied the response of endothelial cells to stainless microstructure and reported an increased endothelial cell density on stainless steel specimens with 16 μ m grain size when compared to larger grain sizes specimens.

As discussed in the previous section, response of SMCs to texture has been evaluated for polymeric surfaces potentially for vascular graft applications. However, to the author's best knowledge, response of stent strut roughness on smooth muscle cells has not yet been reported. It has been well established that stent deployment causes injury to

the blood vessel including endothelium denudation. This subjects the underlying smooth muscle cells to additional mechanical forces, either from a stent strut or due to shear from blood flow, which they usually do not experience in healthy arteries. It, therefore, becomes pertinent to analyze how these cells respond to the new mechanical stimuli and if it has a role in the development of neointimal hyperplasia. It should be noted that with the mechanical stimuli, the cells also get exposed to blood components and circulating growth factors, cytokines etc. which would affect the progression of restenosis as well.

SMCs have been shown to experience a phenotypic change from a quiescent contractile phenotype to a synthetic phenotype following stenting [21]. Increased proliferation of synthetic SMCs, and the copious amount of ECM they produce soon starts obstructing the blood flow to progress restenosis. What effect does shear/mechanical loading due to tightly appositioned struts has on smooth muscle cell proliferation, migration, and the overall phenotypic change is a question that needs to be addressed in order to improve the stent design. Assuming that cells do respond to altered mechanical forces, another question that finds relevance here is that “What are the mechanisms involved in signal transduction- direct mechanotransduction or indirect signaling?” Once deciphered, the information could be used to control this signal transduction and thus cell fate.

1.7 Conclusion

In conclusion, the chapter introduced vascular stents, the problem of restenosis, and tried to explain how cells respond to topography, in particular by direct mechanotransduction, what effect does micro and nano-topography has on cells, particularly endothelial and smooth muscle cells, and finally the need to investigate and control smooth muscle cell response to stent topography. Clearly, controlling substratum topography represents a powerful intrinsic method to control the abilities of cells to orient, migrate, and proliferate, as documented by the studies summarized above. Thus, after careful investigation of response of SMCs to defined stent topographies by both direct and indirect mechanotransductive pathways, their morphology, cytoskeleton and the gene expression could potentially be directed to limit neointimal hyperplasia and ultimately clinical restenosis.

CHAPTER TWO

RESEARCH RATIONALE

2.1 Overview

Intimal hyperplasia is triggered by endothelium denudation caused during stent deployment and advanced with the subsequent smooth muscle cell hyperplasia and hypertrophy [15,17,19,21]. The limitations and problems associated with the clinically used stents, therefore, provides the impetus for exploration of pro-healing therapeutic strategies that prevent smooth muscle migration, proliferation, or the overall phenotypic shift without delaying stent strut and inter-strut re-endothelialization, with the overall objective of maintaining vessel patency.

Topography, as discussed in Chapter 1, has been shown to affect SMC phenotype, proliferation, and alignment. This study was designed to study the effects of micro-grooves on the surface of one of the most commonly used intravascular stent material 316L stainless steel on qualitative (contractile vs. synthetic phenotype) as well as quantitative (cell proliferation) smooth muscle cell response. As seen in some of the research with textured polymers, if cells become spindle-shaped in the grooves, it is possible that direct mechanotransduction could alter gene transcription and result in a phenotype shift. A decrease in proliferation due to restrictions of spreading could also be caused. Or, the cells could just be elongated, without any phenotypic change as seen by Thakar and collaborators [24] However, the response of SMCs to textured stent could

very well be different from that observed with polymers, and could manifest itself in a different response because of the concurrent inflammation process going on at the site of stenting.

Prospective use of topographical cues for stent application, particularly in relation to the outer surface, remains to be evaluated. This project aimed at exploring the potential of topographical cues embedded on stent surface as an implicit way to direct smooth muscle cell response to prevent restenosis. As discussed previously, SMCs are believed to experience a phenotypic shift from a contractile to a synthetic phenotype following stent deployment. Spindle shaped contractile cells which have been shown to proliferate slowly, and produce less ECM represent a more favorable phenotype compared to their rapidly proliferating counterpart – synthetic SMCs. This shift in phenotype was studied for SMCs grown on different topographies of 316L stainless steel, with an overall objective of researching optimal stent strut topography to preserve contractile phenotype of smooth muscle cells.

2.2 Specific Research Hypothesis:

We postulated that micro-grooves on 316L stainless steel would affect smooth muscle cell response manifested in cell morphology, cell proliferation, and cell phenotype.

Contractile smooth muscle cells are characterized by low cell proliferation, elongated spindle shaped morphology, and high expression of contractile markers such as α -actin. These three characteristics of smooth muscle cells were evaluated and compared against cells grown on the current industry standard - electropolished 316L stainless steel.

CHAPTER THREE

MATERIALS AND METHODS

3.1 Surface Preparation

Annealed 0.005” thick 316L stainless steel specimens (Brown metal company, Rancho Cucamonga, CA) were divided into three groups – electropolished, as received, and micro-grooved. While a smooth surface finish was obtained by electropolishing (Team Metal Finishing, Toccoa, GA), the topography of “as received” samples was not modified. Micro-grooved surfaces were produced by pasting the as received metal pieces on to a plastic microscopy slide and placing it on a contact surface grinder (Exakt D-2000, Exakt Technologies Inc., Oklahoma, OK). Metallographic grinding for two minutes using 60 grit silicon carbide paper (Buehler, Lake Bluff, Illinois) produced near parallel grooves on the surface. Using wire EDM, 1.5 cm diameter discs with a 0.3x0.3 cm square lip were cut from the stainless steel specimens. The square lip was folded to be perpendicular to the disc surface to facilitate sample handling. The metal discs were first cleaned with 100% acetone for 20 minutes in an ultrasonic cleaner. They were then placed in a 1% (by vol.) detergent solution (Liquinox brand, Alconox Inc.) ultrasonicated for another 20 minutes. The discs were then put through three 15 minute ultrasonic rinse cycles in ultra pure de-ionized water, where the water was completely exchanged after each rinse. Air-dried samples were then sterilized using standard procedures of ethylene oxide sterilization.

3.2 Surface characterization

Non-contact profilometry (NT-2000, Veeco, WYKO Corp., Tuscon, AZ) was conducted on each cleaned sample of stainless steel on eight different areas at a magnification of 25X and a cut-off area of 736 μ m x 480 μ m. These measurements allowed for calculation of various roughness parameters for all of the samples used for this study including roughness average (R_a), and root mean square roughness (R_q).

Scanning Electron Microscopy (SEM, Model S-3400, Hitachi, Japan) was performed to visually evaluate the metals surface characteristics. This characterization tool was utilized to both observe the topography of the metal and to guarantee that material preparation by-products no longer remained on the metal surface.

Electron dispersive x-rays (EDS, Model S-3400N, Hitachi, Japan) was used to ascertain a quantitative break down of elements on the sample surface. This procedure was employed to ensure that the metal being used was in fact 316L stainless steel, with Palmaz Schatz Stent (Boston Scientific, Natick, MA) used as reference for 316L stainless steel. Additionally, EDS was conducted to assure that surface treatment did not alter the material composition.

3.3 Cell culture

Using an approved protocol by the Institutional Animal Care and Use Committee, aortas were harvested from 6-10 weeks old female Sprague-Dawley rats. Adventitia and fat layers were peeled off, while endothelium was gently scraped off to leave only medial layer of aorta. Rat aortic smooth muscle cells (RASMCs) were collected using collagenase type II (Worthington Biomedical, Lakewood, NJ), Elastase (Worthington Biomedical, Lakewood, NJ), and soybean trypsin inhibitor (Worthington Biomedical, Lakewood, NJ). The cells were maintained in Dulbecco's modification of Eagle medium (DMEM, 10-013-CV; Mediatech, Herndon, VA) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (F-4135; Sigma-Aldrich, St. Louis, MO) and 1% antibiotic-antimycotic (A5955, Sigma-Aldrich, St. Louis, MO) under standard cell culture conditions (37°C, 5% CO₂ with 95% air, humidified environment). 15000 RASMCs were seeded per disc at passage 4-7, with medium replaced every second day.

3.4 Confocal microscopy for F-actin and cell nuclei

At days 1-4, the cells were fixed in a 2% paraformaldehyde solution for 20 minutes, incubated with glycine for 5 minutes, followed by permeabilization with 0.1% TritonX-100. Cytoskeletal F-actin and cell nuclei were stained using rhodamine-phalloidin (Invitrogen, R415) and 4',6-diamidino-2-phenylindole (DAPI) (Molecular Probes, D-1306, Eugene, OR) for one hour and 10 minutes respectively. Samples were

thoroughly washed with PBS and refrigerated after covering with Vectashield Mounting medium (Vector Laboratories, Burlingame, CA) until imaged using confocal microscopy (Nikon LV-UDM, Nikon Instruments Inc., Melville, NY).

3.5 Quantification of cell elongation

Measurements of the cells including length, width, minor axis, major axis, and elongation were performed using NIS Elements 3.1 (Nikon Instruments Inc., Melville, NY). The elongation of cells was calculated as the ratio between MaxFerret and MinFerret (maximum or minimum perpendicular distance between parallel tangents touching opposite sides of the profile of the chosen cells), with 1.0 being a complete circle. A minimum of 137 cells per group were measured for analysis from random points from three samples of each of the three experimental groups. The experiment was repeated once.

3.6 Cell proliferation

3.6.1 CyQuant Cell Proliferation assay

At day 1, 2, 3, and 4, Cell Titer 96 AQueous ONE Solution Cell Proliferation Assay (MTS) (Promega Corporation, G-3580, Madison, WI) was used to evaluate cell proliferation. This was done by adding 60 μ l of the AQueous One assay solution to each

metal disc immersed in 300 μ l of serum free DMEM. After incubating for 2hrs, the supernatant was collected and 100 μ l was dispensed into three wells of a 96 well plate for each of the individual metal discs. The absorbance was then measured at 490 nm (Beckman Instruments, Inc., Model# DU[®] 640B, Fullerton, CA). Two samples per group were used, and the experiment was repeated three times.

3.6.2 DAPI Cell count

At day 1, 2, 3, and 4, cells were fixed with cold methanol for 15 minutes, permeabilized with 0.1% TritonX-100, and stained with 4',6-diamidino-2-phenylindole (DAPI) (Molecular Probes, D-1306, Eugene, OR) for 10 minutes. Samples were thoroughly washed with PBS and images were captured for eight random areas on each of the three discs of all three surface types (Nikon AZ-100, Nikon Instruments Inc., Melville, NY). Cell number was counted for each disc and represented as cell count per field of view.

3.7 Smooth muscle α -actin expression

α -actin expression in smooth muscle cells grown on stainless steel discs of varying topography was analyzed using a modified cell-based ELISA assay [75]. Briefly, at indicated times, the media was removed, and the cells were fixed in cold methanol. They were then incubated with a blocking solution that consisted of 40mg/ml bovine

serum albumin in PBS, 6% FBS, and 0.05% Triton-X for 45 minutes at room temperature. Primary mouse anti- α -actin antibody diluted 1:200 in the blocking solution was then added to the metal discs for 2 hours at room temperature followed by a 2hr treatment with biotin conjugated bovine anti-mouse IgG also diluted 1:200 in the blocking solution. After extensive washing, the cells were incubated with streptavidin-conjugated alkaline phosphatase for 45 minutes at 37°C. Chromogenic substrate (3 mM p-nitrophenyl phosphate, 0.05 M Na₂CO₃, and 0.05 mM MgCl₂) was then applied to produce color, and the absorbance read at 405 nm with a microplate reader (Beckman Instruments, Inc., Model# DU[®] 640B, Fullerton, CA). The cells were then DAPI stained and imaged to facilitate cell counting. Lastly, absorbance due to non-specific binding was subtracted from that of the experimental groups, with the net absorbance normalized against cell count on each disc giving a number directly related to the α -actin content per cell.

3.8 Immunofluorescent staining

Smooth muscle cells on stainless steel discs were fixed with cold methanol and blocked for 45 minutes at room temperature with a blocking solution that consisted of 40mg/ml bovine serum albumin in PBS, 6% FBS, and 0.05% Triton-X. Cells were then treated with primary mouse anti- α -actin antibody and fluorescent tagged donkey anti-mouse secondary antibody, both diluted 1:200 in the blocking solution, for 2 hours and 3 hours respectively at room temperature. The cells were then stained with DAPI

(Molecular Probes, D-1306, Eugene, OR) to assist in distinguishing individual cells during fluorescent microscopy (Nikon LV-UDM, Nikon Instruments Inc., Melville, NY).

3.9 Statistical Analysis

Data were evaluated using an ANOVA statistical analysis paired with LSD/Tukey analysis. This was done using SAS statistical analysis software (SAS Institute Inc., Cary, NC) with $p < 0.05$ indicating a significant difference.

CHAPTER FOUR

RESULTS

4.1 Surface characterization

Figure 3 shows representative topographical images of the surface types of interest to the present study. It is evident that metallographic grinding produced near-parallel grooves, while the surface features on electropolished and as received surfaces were randomly present throughout the surface. In addition to the topographical images, quantitative roughness data were acquired from the profilometry analysis, and are summarized in Table 2. The average R_a (mean $\pm$ SD) for electropolished, as received and micro-grooved surfaces was found to be 89.2 ± 8.1 nm, $179.\pm 9.9$ nm, and 2009.8 ± 361.4 nm respectively. A groove depth range of $10.56\text{ }\mu\text{m}$ and $18.0\text{ }\mu\text{m}$ with an average of $12.7\text{ }\mu\text{m}$ was measured in the micro-grooved surface samples. The relatively higher variability in all roughness parameters for micro-grooved surfaces is attributed to the heterogeneity of surface profile imparted by metallographic grinding.

Table2: Roughness values of three surface types

	R_a (in nm)	R_q (in nm)
Electropolished	89.2 ± 8.1	110.8 ± 9.7
As Received	179 ± 9.9	226.7 ± 11.7
Micro-grooved	2009.8 ± 361.4	2474.1 ± 4

(a)

(b)

(c)

Figure 3: Representative topographical images of (a) Electropolished surface, (b) As Received surface, and (c) Micro-grooved surface.

Figure 4 shows SEM images of electropolished, as received, and micro-grooved surfaces in topography mode. For the micro-grooved surfaces, it was rather difficult to distinguish between individual “scratches” and many of them collectively formed larger micro-grooves of width 25-30 μ m. Since this surface had grooves of width and depth in the micron range, this surface was called the micro-grooved surface.

EDS results did not show any significant differences in elemental composition between electropolished surface, as received surface, and Palmaz stent made from 316L stainless steel. Although, the results showed approximately 2.5 wt% carbon in all specimens tested, this is attributable to contaminants present because of imperfect vacuum present in the testing system. EDS spectra for all three surface types are shown in figure 5.

(a)

(b)

(c)

Figure 4: Representative SEM images of (a) Electropolished surface, (b) As Received surface, and (c) Micro-grooved surface (images taken at accelerating voltage of 20kV).

Figure 5: EDS spectra for electropolished (a), as received (b), and micro-grooved (c) surface

4.2 Cell morphology

Confocal microscopy of smooth muscle cells grown on stainless steel discs showed a clear difference in SMC morphology on different surface topographies. While cells on electropolished and as received surface were round and less elongated, cells on the micro-grooved surface were found to be aligned in the direction of grooves and assumed an elongated spindle-shaped morphology starting as early as 24 hours of incubation. This trend was observed up to day 4, at which point the experiment was stopped due to near confluence on the discs. Figure 6 shows representative images of for all four time points.

Quantitative data for cell elongation were obtained by image analysis, and is shown in figure 7. Cells on all time points were found to be statistically more elongated on micro-grooved surfaces (p value <0.0001) when compared to both electropolished and as received surfaces. However, the difference in cell elongation between electropolished and as received surface types was not statistically significant on any of the four time points.

E: Electropolished; A: As received; M: Micro-grooved; 1-4: Day1-Day4; Red: F-Actin; Blue: Nuclei

Figure 6: Representative confocal micrographs of F-actin (Red) and cell nuclei (Blue)
stained SMCs Magnification: 400X; Bar represents 50μm

Figure 7: Effects of surface topography on cell elongation. Data represents mean values $\pm$ SEM, n=3, *p<0.0001 compared to electropolished and as received surfaces at the same time point.

4.3 Cell proliferation

Although, CyQuant cell proliferation assay showed a lower absorbance (directly related to cell count) for micro-grooved surface on all four time points compared to the electropolished as well as as received surface, the differences in absorbance and thus cell proliferation were not statistically significant (figure 8).

Figure 8: CyQuant Cell proliferation assay. Data represents mean values $\pm$ SD; n=2

However, DAPI cell counting showed a statistically significant difference in cell count per field of view between electropolished and micro-grooved surface on on day 1 (p-value 0.0068), day 3 (p-value 0.0288) and day 4 (p-value 0.0249). Although average cell count on micro-grooved surface on day2 was lower than that on electropolished surface, the difference was not found to be statistically significant (p-value 0.3744). Micro-grooved surfaces showed approximately 44%, 11.5%, 30.5%, and 20% lower cell count per field of view when compared to electropolished surface on day 1, 2, 3, and 4 respectively. Differences in cell count between as received surface and micro-grooved surface were only significant on day 1 (p-value 0.0163) and day 4 (p-value 0.0309),

where micro-grooved surface showed 54% and 19% lower cell counts per field of view when compared to the as received surface on day 1 and 4 respectively. Lastly, differences in cell count between as received and electropolished surface were not significant on any of the four time points (figure 9).

Figure 9: DAPI Cell Count per field of view. Data represents mean values $\pm$ SD; n=3; *p<0.05 compared to electropolished surface at the same time point; #p<0.05 compared to as received surface at the same time point; **p<0.05 compared to respective surface types on day1.

4.4 Cell based ELISA assay

The cell based ELISA assay developed for this application resulted in a number directly proportional to smooth muscle α -actin content per cell (figure 10). The results did not show any large differences in α -actin content between the three surface types on day 1 (p-value 0.5077) and day 2 (p-value 0.7271). As the cells started to reach confluence, α -actin content per cell on micro-grooved surface started to increase over that on the electropolished surface with micro-grooved surface demonstrating on an average approximately 15% higher α -actin content per cell compared to the electropolished surface on day 3. The difference, however, was found to be statistically insignificant (p-value 0.2521). On day 4, micro-grooved surface showed approximately 63% higher α -actin content when compared to electropolished surface (p-value 0.0318).

Additionally, while α -actin content per cell on the micro-grooved surface on day 1 and day 4 was statistically constant, it decreased 32% between day 4 and day 1 on the electropolished surface. Although for micro-grooved surface, α -actin content per cell increased by approximately 20% from day 1 to day 4, the difference was not statistically significant (p-value 0.2453). Lastly, differences in α -actin content on as received surfaces were not significantly different from other surface types, and did not change on a time scale.

Figure 10: Effect of surface topography on smooth muscle α -actin expression – Cell based ELISA assay; n=3; *p<0.05 compared to electropolished surface on the same time point; #p<0.05 compared to same surface type on day1

4.5 Immunofluorescent staining

Immunofluorescent staining also demonstrated that SMCs patterned in the micro-grooves appeared elongated and had actin filaments oriented along the direction of the channels (figure 11). Differences in the intensity of smooth muscle α -actin, however, were not apparent as with the ELISA assay.

E: Electropolished; A: As received; M: Micro-grooved; 1-4: Day1-Day4

Figure 11: Effect of topography on α -actin expression – Immunofluorescent staining;
 Green: Smooth muscle α -actin; Blue: Cell nuclei; Magnification: 400X; Bar represents
 100 μ m

CHAPTER FIVE

DISCUSSION

This study was aimed at evaluating the response of rat aortic smooth muscle cells while in contact with 316L stainless steel of three different topographies, as an attempt to translate the design criteria previously utilized for improving smooth muscle cell functions on polymers to the most regularly used metal for stent applications. For potential applications as tissue engineered vascular grafts, topography of a variety of polymers has been shown to affect smooth muscle cell response ranging from cell alignment to one of the most critical requirement of these grafts– cellular phenotype. Topography as a tool to modulate vascular cell functions, has not been exploited as well for endovascular stent applications with only one study reported thus far evaluating the potential of topography for one of the less commonly used stent material – CoCrMo [60]. This study, therefore, reports the valuable effects of micro-grooves on one of the most commonly used stent material – 316L stainless steel – on the much sought after smooth muscle cell phenotype.

Over-proliferation, migration and an overall phenotypic shift of smooth muscle cells following stent deployment has been deemed to be one major cause of restenosis [17,20-23]. Preserving the contractile phenotype of smooth muscle cells, therefore, represents one of the two key strategies aimed at limiting restenosis along with promoting re-endothelialization of stent struts and inter-strut space. Our results indicate that in

comparison to the current industry standard (electropolishing), micro-grooves on 316L stainless steel promotes elongated morphology, limits proliferation, and promotes α -actin expression in smooth muscle cells. Since endothelium is denuded following stent deployment in most cases and stent struts are in direct contact with medial smooth muscle cells, topographical modification of outer stent surface presents a powerful intrinsic tool to control adverse smooth muscle cell response that leads to restenosis.

Near parallel micron level grooves in stainless steel were produced by metallographic grinding, while electropolishing produced a relatively smooth surface finish. Non-contact profilometry indicated that the average surface roughness of as received surface type was about twice that of the electropolished surface, while that of the micro-grooved surface was more than 20 times compared to the electropolished surface. SEM images also showed the difference in topography between the surface types, while EDS confirmed not only that the material acquired for this study was similar to that of Palmaz stent, i.e. 316L stainless steel, but also that our surface modification process did not produce any variations in material composition. Thus, all the differences in cell behavior reported in this study are attributable to the differences in topography alone.

Cell-shape has been shown to be an important determinant of cell growth, alignment, migration, and differentiation for a variety of cell types including vascular smooth muscle cells. Recent studies have shown that micro-grooves restrict SMC

spreading in one direction resulting in a more elongated morphology [58,65,67]. Our results indicated the same trend with elongation and alignment along the grooves of depth $\sim 13\text{ }\mu\text{m}$ taking place as early as 24 hours. Thakar and collaborators observed a similar elongation of SMCs with patterned collagen strips of width 20-30 μm [58]. It is important to point out the slight dip in cellular elongation on all three surface types from day 3 to day 4. At the current cell seeding density, cells reached confluence on day 4, and we postulate that it is this confluence that caused the observed decrease in cell elongation.

Since limiting SMC over-proliferation has been a major objective in improving stent performance, our results have significant implications for stent applications. Micro-grooves did limit SMC proliferation when compared to electropolished surface type. Many researchers have observed this effect with a variety of polymers, with Thakar et al. demonstrating that elongated cell morphology alone can produce this effect irrespective of the cell spreading area [63]. Choudhary and collaborators observed an increase in SMC proliferation due to nanometer range roughness on CoCrMo with an overall increase in the ratio of endothelial to smooth muscle cells [60]. However, in a stented artery where there are less endothelial cells compared to smooth muscle cells, the beneficial effects of using nanometer scale roughness on a rather uncommon stent material with similar cell seeding densities for both cell types, would remain questionable.

The phenotype of SMCs in blood vessels plays an important role in vascular remodeling under physiological and pathological conditions. For example, during atherosclerosis and restenosis, SMCs convert from a quiescent contractile phenotype to a proliferative synthetic phenotype [21-23]. We postulated that by changing substratum topography, we could control SMC phenotype with our aim being keeping these cells in their native contractile phenotype. We selected smooth muscle α -actin as a marker of contractile phenotype, and observed higher α -actin content per cell compared to the electropolished surface when the cells reached confluence. Amongst the studies reported by others in relation to response of SMCs to micropatterning, the inability of micro-grooves to bring about any changes in cell phenotype [58,63] has been reported, while there are only a few studies reporting some degree of success in controlling SMC phenotype with micropatterning alone [66,67]. Other studies did not focus on the phenotype of cells with an aim to control cell proliferation/adhesion and/or orientation [60,62,65,76]. Within the first category, Thakar et al. demonstrated that cell shape controlled cell proliferation for SMCs, but it was insufficient in bringing about significant changes in α -actin gene expression [63]. However, this study was done with PDMS (micro-grooves of width 10 μ m), and protein level changes in α -actin were not studied. In another study, Thakar et al. observed decreased SMC proliferation in response to micro-patterned collagen strips, but these micro-patterned strips were not enough to promote contractile phenotype [58]. However, data obtained by these authors cannot be directly compared to the present results, because not only this study involved a different substrate,

but a large proportion of their substrate area was also unavailable for cell growth since the strips were only 2-3 cells wide.

Very recently Cao and collaborators reported that SMCs switched to a more contractile phenotype when cultured on relatively wide microchannels (80-160 μ m) only when the cells reached confluence [66]. For the micro-grooved surface, we observed only a slight increase in α -actin (~20%) content when cells reached confluence (day4) when compared to day1, with the difference in smooth muscle α -actin content per cell being significantly different from that on electropolished surface only as the cells became confluent. Furthermore, it is a common practice to clinically use electropolished stents in order to minimize thrombus formation. While it has been proven beneficial to electropolish the inner blood contacting surface of stents, as indicated by our results, electropolished surface encourages smooth muscle cell differentiation. We demonstrate in this study that SMCs on electropolished 316L stainless steel lost a significant amount of smooth muscle α -actin on day 4 compared to day 1, while micro-grooves helped preserve that α -actin and thus the contractile phenotype. Immunofluorescent imaging showed a similar trend in that many cells on electropolished surface had almost no α -actin on day 4. However, the differences in α -actin between the three surface types were not as easy to pick up as with the quantitative ELISA assay.

CHAPTER SIX

CONCLUSIONS

A model was developed to replicate vascular tissue in contact with stent material of different topographies. Topography of 316L stainless steel was modified using electropolishing and metallographic grinding, with the surface completely characterized prior to smooth muscle cell culture. Cellular morphology, proliferation, and expression of smooth muscle α -actin were described by confocal microscopy, growth curves, and ELISA assay respectively. Several conclusions can be drawn from this research:

1. Smooth muscle cells aligned along micro-grooves of depth about 13 μ m, and became elongated demonstrating morphology closer to that of contractile smooth muscle cells.
2. Micro-grooves limited smooth muscle cell proliferation when compared to electropolished and as received surface types.
3. Smooth muscle cells on micro-grooved stainless steel had higher α -actin content compared to that on electropolished surface near confluence.
4. Smooth muscle cells on electropolished surface lost a substantial amount of α -actin between days 1 and 4, while micro-grooved surface preserved the α -actin and thus the contractile phenotype of smooth muscle cells.

Results show that micro-grooves approximately 13µm deep on 316L stainless steel are able to preserve the contractile phenotype of smooth muscle cells while limiting their proliferation when compared to the current industry standard for bare metal stents – electropolishing. This research, therefore, demonstrated the potential of micro-grooves on outer stent surface as an intrinsic tool to prevent neointimal hyperplasia and thus in-stent restenosis.

CHAPTER SEVEN

RECOMMENDATIONS

1. Metallographic grinding used in this study permitted limited control over groove dimensions. Dimensions of micro-grooves should be optimized by generating precisely controlled surfaces with varying groove orientation, width, depth and pitch.
2. Blood vessels are constantly subjected to hemodynamic stresses, with the pulsatile nature of the blood flow resulting in cyclic mechanical strain in the vessel walls. Since mechanical strain has been shown to regulate vascular SMC phenotype, functions and matrix remodeling, response of SMCs to micro-grooved stainless steel while subjected to a cyclic strain stimulus should be evaluated.
3. Migration of smooth muscle cells under the influence of chemoattractants such as PDGF-BB is an important event in the progression of neointimal hyperplasia. Influence of micro-grooved topography on smooth muscle cell migration, therefore, should be studied.
4. Mechanotransductive pathways involved in the reported phenotypic shift in SMCs should be probed to gain a better understanding of cell-substratum relationship.
5. Animal studies using micro-grooved stents should be done to confirm if the in vitro findings would manifest in substantial clinical benefits.
6. Effect of material removal due to topographical modification of stent struts on the overall radial strength of stents should be evaluated.

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APPENDICES

APPENDIX A: Response of SMCs to Hydroxyapatite (HA) coating

Introduction

Since the basic mechanisms governing the interactions between an implant in general, and stents in specific, with tissues/blood have not yet been deciphered completely, the biocompatibility and hemocompatibility of stents still remains an issue. Coating the stent surface with other materials to alter its surface characteristics without interfering with the bulk properties of the metal stent has been one of the major rational approaches to address this compatibility issue [77]. Moreover, coatings can be loaded with anti-inflammatory and/or anti-coagulant drugs to help reduce thrombosis and neointimal hyperplasia – the two major events leading to restenosis. While a majority of the coatings for endovascular stent applications are made of polymers, a number of ceramic coatings have been investigated for the aforementioned application.

HA, a naturally occurring mineral form of calcium apatite ($\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$) present in various tissues of the body, has been extensively used as implant materials for orthopedic and dental applications because of its favorable interaction with bones [78,79]. HA coating for stents is a relatively new idea, but it may be beneficial for several reasons. First, HA forms a porous coating on the stent surface that can be utilized as a unique drug delivery platform for drugs such as bisphosphonates that bind avidly to HA. Recently, Wu et al. demonstrated inhibition of SMC proliferation, adhesion, and migration by addition of 10-100 μM of Zoledronate – a type of bisphosphonate [80].

Secondly, HA coating might provide a beneficial surface for stented artery re-endothelialization as demonstrated by Okada et al. [81,82]. Additionally, reduction in the release of metallic ions including nickel from coated implants including nickel-titanium alloys and stainless steel as compared to a non-coated control has been reported for HA coating in in-vitro studies [83,84].

Although the effect of HA coating on endothelial cells has been evaluated, its effects on smooth muscle cells have not been reported. With more smooth muscle than endothelial cells remaining at the site of stenting [19], evaluating the response of smooth muscle cells to the stent/coating material becomes very critical in establishing the prospects of using HA coating for stents. The objective of this study, therefore, is from a biomaterials perspective to evaluate the response of smooth muscle cells in terms of cell morphology, proliferation, and phenotype, to HA coating as compared to the current industry standard for bare metal stents – electropolished 316L stainless steel.

Materials & methods

Surface preparation

316L stainless steel discs of diameter 1.5 cm were coated with Hydroxyapatite (Enbio, Ireland). Electropolished 316L stainless steel discs were prepared as described earlier. The metal discs were first cleaned with 100% acetone for 20 minutes in an ultrasonic cleaner. They were then placed in a 1% (by vol.) detergent solution (Liquinox brand, Alconox Inc.) ultrasonicated for another 20 minutes. The discs were then put through three 15 minute ultrasonic rinse cycles in ultra pure de-ionized water, where the water was completely exchanged after each rinse. Air-dried samples were then sterilized using standard procedures of ethylene oxide sterilization.

SEM

Scanning Electron Microscopy (SEM, Model S-3400, Hitachi, Japan) was performed to visually evaluate the metals surface characteristics. This characterization tool was utilized to both observe the topography of the coating and to guarantee that a uniform coating was present on the metal surface.

Cell culture

Using an approved protocol by the Institutional Animal Care and Use Committee, aortas were harvested from 6-10 weeks old female Sprague-Dawley rats. Adventitia and fat layers were peeled off, while endothelium was gently scraped off to leave medial layer of aorta. Rat aortic smooth muscle cells (RASMCs) were collected using

collagenase type II (Worthington Biomedical, Lakewood, NJ), Elastase (Worthington Biomedical, Lakewood, NJ), and soybean trypsin inhibitor (Worthington Biomedical, Lakewood, NJ). The cells were maintained in Dulbecco's modification of Eagle medium (DMEM, 10-013-CV; Mediatech, Herndon, VA) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (F-4135; Sigma-Aldrich, St. Louis, MO) and 1% antibiotic-antimycotic (A5955, Sigma-Aldrich, St. Louis, MO) under standard cell culture conditions (37°C, 5% CO₂ with 95% air, humidified environment). 15000 RASMCs were seeded per disc at passage 4-7, with medium replaced every second day.

Microscopy for F-actin and cell nuclei

At 48 hours, the cells were fixed in a 2% paraformaldehyde solution for 20 minutes, incubated with glycine for 5 minutes, followed by permeabilization with 0.1% TritonX-100. Cytoskeletal F-actin and cell nuclei were stained using rhodamine-phalloidin (Invitrogen, R415) and 4',6-diamidino-2-phenylindole (DAPI) (Molecular Probes, D-1306, Eugene, OR) for one hour and 10 minutes respectively. Samples were thoroughly washed with PBS and refrigerated after covering with Vectashield Mounting medium (Vector Laboratories, Burlingame, CA) until imaged (Nikon LV-UDM, Nikon Instruments Inc., Melville, NY).

Live/Dead assay

At 48 hours, non adherent cells were removed by gently rinsing with PBS. Live/Dead assay reagent (Invitrogen Corporation, Carlsbad, CA) that consisted of 4 μ M

EthD-1 and 2 μ m Calcein solution in PBS, was then directly added to adherent cells for 45 minutes in dark. Images were then taken using fluorescent imaging with live cells represented by green color and dead cells with red (Nikon AZ-100, Nikon Instruments Inc., Melville, NY).

CyQuant Cell Proliferation assay

At days 1, 2, 3, and 4, Cell Titer 96 AQueous ONE Solution Cell Proliferation Assay (MTS) (Promega Corporation, G-3580, Madison, WI) was used to evaluate cell proliferation. This was done by adding 60 μ l of the AQueous One assay solution to each metal disc immersed in 300 μ l of serum free DMEM. After incubating for 2hrs, the supernatant was collected and 100 μ l was dispensed into three wells of a 96 well plate for each of the individual metal discs. The absorbance was then measured at 490 nm (Beckman Instruments, Inc., Model# DU[®] 640B, Fullerton, CA).

Smooth muscle α -actin expression

α -actin expression in smooth muscle cells grown on stainless steel discs of varying topography was analyzed using a modified cell-based ELISA assay [75]. Briefly, at indicated times, the media was removed, and the cells were fixed in cold methanol. They were then incubated with a blocking solution that consisted of 40mg/ml bovine serum albumin in PBS, 6% FBS, and 0.05% Triton-X for 45 minutes at room temperature. Primary mouse anti- α -actin antibody diluted 1:200 in the blocking solution was then added to the metal discs for 2 hours at room temperature followed by a 2hr

treatment with biotin conjugated bovine anti-mouse IgG also diluted 1:200 in the blocking solution. After extensive washing, the cells were incubated with streptavidin-conjugated alkaline phosphatase for 45 minutes at 37°C. Chromogenic substrate (3 mM p-nitrophenyl phosphate, 0.05 M Na₂CO₃, and 0.05 mM MgCl₂) was then applied to produce color, and the absorbance was then read at 405 nm with a microplate reader (Beckman Instruments, Inc., Model# DU[®] 640B, Fullerton, CA). The cells were then DAPI stained and imaged to facilitate cell counting. Lastly, absorbance due to non-specific binding was subtracted from that of the experimental groups, with the net absorbance normalized against cell count on each disc.

Statistical Analysis

Data was evaluated using an ANOVA statistical analysis paired with Tukey analysis. This was done using SAS statistical analysis software (SAS Institute Inc., Cary, NC) with $p < 0.05$ indicating a significant difference.

Results

SEM

Figure 12 shows representative SEM images of HA coating in topography and 3D mode. The coating was evenly present throughout the disc with minimal cracks visible. Furthermore, HA coating presents a rougher surface when compared to electropolished 316L stainless steel (figure 4b).

Figure 12: SEM images of HA coating in (a) topography mode; and (b) 3D mode

Cell morphology

Figure 13 shows SMC morphology on HA coating and electropolished 316L stainless steel at 48 hours. The cells on HA coating were spread out and had numerous processes compared to bare metal surface.

Figure 13: Representative micrographs of F-actin (Red) and cell nuclei (Blue) stained SMCs Magnification: 100X; Bar represents 100µm

Live/Dead assay

Figure 14 shows Live/Dead assay images at 48 hours. More dead cells were observed on HA coated samples compared to bare metal surface samples.

Figure 14: Representative Live/Dead assay images for (a) HA coating, and (b) Electropolished 316L stainless steel; Live and dead cells are shown in green and red respectively; Magnification: 10X; Bar represents 1000µm

Cell proliferation

CyQuant cell proliferation assay showed lesser cell count on HA coating on all four time points when compared to the electropolished surface. On an average, HA coating showed approximately 27.5%, 21%, 34% and 32% lower absorbance (directly related to cell count) when compared to electropolished surface on day 1, 2, 3, and 4 respectively (figure 15).

Figure 15: CyQuant Cell proliferation assay. Data represents mean values $\pm$ SD; n=3;

*p<0.05 compared to electropolished metal surface at the same time point.

Cell based ELISA assay

Results from ELISA assay indicate significantly lower smooth muscle α -actin content per cell compared to the electropolished surface on all four time points. SMCs on HA coating had on an average 69%, 59%, 62%, and 49.5% lower smooth muscle α -actin content per cell compared to electropolished surface on days 1, 2, 3, and 4 respectively (figure 16).

Figure 16: Effect of HA coating on smooth muscle α -actin expression; n=3; *p<0.05 compared to electropolished metal surface at the same time point.

Discussion

Hydroxyapatite (HA) in orthopedic applications has been shown to have a favorable biologic response, as it does not induce local or systemic toxicity, inflammation, or foreign body response while promoting integration with bone [79]. Response of osteoblasts, and mesenchymal stem cells to HA have been studied extensively [85-89], but to the author's best knowledge, this study was the first attempt to elucidate the response of vascular smooth muscle cells to hydroxyapatite for potential applications as a stent coating material. Cellular morphology, proliferation, and phenotype were studied as an indicator of neointimal hyperplasia presenting a scenario, where the finite amount of loaded drug have been released, while the coating itself remains tightly appositioned against the artery wall.

SEM images (figure 12) show the topography and integrity of coating on the metal surface. This coating presented smooth muscle cells with a rougher surface when compared to the electropolished bare metal surface. However, since the chemistry of the two surface types studied here are different, differences in cell response cannot be attributed to topography alone. Conversely, this study was aimed at evaluating smooth muscle response to the coating, without probing in to the causative factors.

Blocking over-proliferation of medial smooth muscle cells presents a very potent way to limit neointimal hyperplasia. This study demonstrates the merits of hydroxyapatite coating in limiting smooth muscle cell proliferation over electropolished 316L stainless

steel by upto 34% (figure 15). Varying trends in osteoblast proliferation to HA have been reported in the literature primarily based on the coating process. For example, while a sintered dense HA disc resulted in lower osteoblast cell count compared to plastic control [90], a coincident microblasting [85] and electrochemical coating process [86] resulted in greater cell proliferation compared to their respective control surfaces. Studies with fibroblasts have yielded varying results in terms of proliferation too [91]. An overall trend pertaining to effects of HA on cellular proliferation remains challenging to determine, because of more than one variable being included in the study. Roughness [86], wettability [92], micro-structure [87], and chemical structure [87] of HA coating have all been shown to affect cellular behavior separately. Furthermore, live/dead assay images (figure 14) showed considerably higher number of dead cells on the HA coating when compared to bare metal surface. But, given the problem of over-proliferation of smooth muscle cells during restenosis, these results might as well manifest in to a reduction in neointimal hyperplasia. These results, therefore, must be interpreted carefully, and are subject to further investigation. Despite the mentioned discrepancies in quantitative cellular response to HA coating, it has almost always been shown to promote cell spreading with cell types including osteoblasts and fibroblasts possessing many processes when cultured directly on HA [86,87]. A similar response with rat aortic smooth muscle cells was observed here (figure 13). This cell morphology was significantly different on electropolished surface where the cells were less spread with no processes.

Perhaps, one of the most important cellular events in the progression of in-stent restenosis is the phenotypic shift of smooth muscle cells. SMCs convert from a quiescent contractile phenotype to a proliferative synthetic phenotype while migrating towards vessel lumen [21-23]. While low SMC proliferation, as observed here, is a trait of contractile cells, we observed significantly less smooth muscle α -actin content per cell on HA coating relative to electropolished surface (figure 16). Taken together with a somewhat triangular morphology of SMCs as seen in figure 13, lower α -actin content on HA coating is indicative of synthetic SMC phenotype. These results, therefore, raise a question regarding aptness of the established criteria for distinguishing between contractile and synthetic smooth muscle cells - morphology, proliferation, and smooth muscle α -actin as a marker of contractile phenotype. Furthermore, with the main aim of stent and/or stent coating being to maintain vessel patency, would limiting cell proliferation per se irrespective of cell phenotype, be substantial in maintaining lumen patency is a question that needs to be addressed in order to realize the full potential of hydroxyapatite as a coating material for stents.

Conclusions

1. Smooth muscle cells on HA coating were more spread and had a lot of processes when compared to those on electropolished 316L stainless steel.
2. HA coating significantly limited smooth muscle cell proliferation over electropolished bare metal surface.
3. Relative to bare metal, significantly greater number of dead cells were seen on HA coating at 48 hours.
4. Smooth muscle cells lost a substantial amount of α -actin upon culturing on HA coating when compared to bare metal surface.
5. No conclusion can be made on the phenotype of cells since the three established characteristics of either phenotype have not been met.

Recommendations

1. Keeping into consideration the osteogenic potential of hydroxyapatite, smooth muscle cell calcification should be evaluated by the expression of osteogenic markers by standard molecular biology techniques.
2. Since wettability of an implant surface is an important criterion in biological responses as it controls the adsorption of proteins followed by cell attachment on the surface, surface tension should be determined using contact angle measurements.
3. Because of the brittle nature of HA, the post-strain integrity of HA coating should be studied using electron microscopy.

4. Response of smooth muscle cells to HA coating while subjected to a cyclic strain stimulus should be evaluated to simulate hemodynamic stresses.

APPENDIX B: Cell based ELISA assay protocol

A modified cell based ELISA assay was developed with advice from Dr. JeoungSoo Lee as well as some additions based on first-hand experience:

1. Fix SMCs growing in 24-well plates with cold methanol (-20°C) for 25 minutes.
2. Rinse wells with PBS three times to remove methanol completely.
3. Block with blocking agent (2gm BSA, 3ml FBS, 25 μ TritonX-100, and 47ml PBS) for 45 minutes at room temperature.
4. Add primary antibody to smooth muscle α -actin (diluted 1:200 in blocking agent) for 2 hours at room temperature.

Note: Negative control group received blocking agent without any primary antibody for the same time.

5. Rinse with PBS three times waiting 5 minutes in each washing step to remove any unbound antibody.
6. Add biotinylated mouse IgG (diluted 1:200 in blocking agent) for 2 hours at room temperature.
7. Rinse with PBS three times waiting 5 minutes in each washing step to remove any unbound antibody.
8. Incubate with streptavidin conjugated alkaline phosphatases (diluted 1:500 in TBST at pH8) for 45 minutes at 37°C.
9. Rinse with PBS three times waiting 5 minutes in each washing step to remove any unbound enzyme.

10. Incubate with chromogenic substrate (3 mM p-nitrophenyl phosphate, 0.05 M Na_2CO_3 , and 0.05 mM MgCl_2) for 45 minutes at 37°C.
11. Transfer 100 μl of colored product from each well to a 96 well plate and measure absorbance at 405 nm.
12. Rinse cells with PBS to remove pnpp and add 0.1% DAPI for 10 minutes at room temperature.
13. Rinse with PBS briefly and take DAPI images for cell counting.
14. Subtract absorbance of respective negative controls from their experimental counterparts and normalize against cell count on each disc/well.