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Roles of Hemodynamic Forces in Vascular Cell Differentiation

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Abstract—The pulsatile nature of blood flow is a key stimulus for the modulation of vascular cell differentiation. Within the vascular media, physiologic stress is manifested as cyclic strain, while in the lumen, cells are subjected to shear stress. These two respective biomechanical forces influence the phenotype and degree of differentiation or proliferation of smooth muscle cells and endothelial cells within the human vasculature. Elucidation of the effect of these mechanical forces on cellular differentiation has led to a surge of research into this area because of the implications for both the treatment of atherosclerotic disease and the future of vascular tissue engineering. The use of mechanical force to directly control vascular cell differentiation may be utilized as an invaluable engineering tool in the future. However, an understanding of the role of hemodynamics in vascular cell differentiation and proliferation is critical before application can be realized. Thus, this review will provide a current perspective on the latest research and controversy behind the role of hemodynamic forces for vascular cell differentiation and phenotype modulation. Furthermore, this review will illustrate the application of hemodynamic force for vascular tissue engineering and explicate future directions for research.

Keywords—Hemodynamic forces, Shear stress, Cyclic strain, Cell differentiation, Endothelial cell, Smooth muscle cell, Tissue engineering.

INTRODUCTION

The *in vivo* environment of a blood vessel is constantly subjected to and influenced by biomechanical forces inherently present due to the pulsatile nature of blood flow. Within the vascular media, physiologic stress is manifested as a tensile strain which is perpendicular to the lumen of the blood vessel and cyclic in nature, while in the lumen, cells are subjected to a frictional force at the apical surface produced by blood flow.[5,51] These two respective hemodynamic forces, cyclic strain and shear stress, modulate vascular remodeling, development, stability, and disease within the human body, and in the past 10 years, elucidation of the effect of these mechanical forces on cellular differentiation has led to a surge of research into this area. The ability to manipulate the differentiation of vascular (endothelial and smooth muscle) cells would have enormous implications for both the treatment of atherosclerotic vascular disease and the future of vascular tissue engineering. Additionally, knowledge of this subject matter is vital to a number of cardiovascular and surgical research-related fields. While a number of current reviews focus on the influence of mechanical forces upon gene expression and signaling (see reviews[24,31]), there is no recent overview of the role of both cyclic strain and shear stress upon smooth muscle and endothelial cell differentiation. Thus, this review will provide a current perspective on the latest research and controversy behind the role of hemodynamic forces for vascular cell differentiation and will probe into applications and possible future directions for research within this area.

CYCLIC STRAIN AND CELL DIFFERENTIATION

Smooth muscle cell (SMC) differentiation within the vasculature is dependent upon a number of environmental factors, including the mechanical forces present within the vessel wall. Various models have been proposed and utilized to emulate the strain experienced by SMCs within the vascular media, but the majority of research (including our group) involves the use of the Flexercell Stress Unit (Flexercell Corp., USA) whereby SMCs are cultured in six-well deformable elastomer-bottom plates which are subjected to repeated, computer-programmed cycles of tension and relaxation. Though some models that will be mentioned below engage the use of a static culture system of stretch,[2,58] our group and others employ the Flexercell Unit. This model provides a physiologic representation of the *in situ* environment of repetitive mechanical strain produced by pulsatile blood flow which a vascular SMC would experience,[6,42] and thus, cyclic strain will be the focus here.

SMC Markers and Phenotypes

The promotion of a differentiated vascular SMC (VSMC) through the utilization of cyclic strain was first

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realized by a number of landmark studies in the mid 1990's. Birukov *et al*. demonstrated that cyclic strain produced a serum-independent increase in the differentiated SMC marker h-caldesmon,[6] whereas Reusch *et al*. showed that mechanical strain increased smooth muscle myosin heavy chain (SM–MHC) isoforms SM-1 and SM-2 indicative of a differentiated phenotype and decreased nonmuscle myosin A (usually indicative of a de-differentiated phenotype).[42] In addition, Smith *et al*. demonstrated that strain increased differentiated SMC markers myosin light chain kinase (MLCK) and desmin.[48] More recently, experiments have utilized both SM–MHC (marker of fully differentiated smooth muscle) and α-actin (marker for both mature and immature smooth muscle) to determine the extent of VSMC differentiation when exposed to hemodynamic stress.[52] Other SMC markers that have been tested for with cyclic strain applications and are indicative of a differentiated SMC phenotype include calponin, SM22,[14] α and β tropomyosins,[26] and metavinculin.[15]

As one can deduce from the studies above, differentiation is most often measured with markers of contractile proteins for ease and study uniformity, but the differentiated phenotype should be looked upon as more than just the presence or absence of markers. Furthermore, SMCs are not permanently or terminally differentiated. Thus, phenotype modulation by cyclic strain can also be described as a spectrum of states of differentiation, and herein, the current state of research and controversy resides.

The contractile phenotype has been associated with the differentiated, quiescent state of VSMCs seen *in vivo* and has also been maintained by cyclic strain in culture whereby histologic orientation perpendicular to the direction of stretch and the expression of contractile proteins mentioned above are observed.[6,25,42,48,49] However, if current literature is taken into account, this relative state of differentiation is anything but quiescent. Rather, studies have demonstrated that cyclic strain enhances extracellular matrix remodeling and synthesis as well as cellular proliferation in association with the SMC differentiation.[6,29,30,32,34,38,43] For example, Li *et al*. demonstrated a 1.4–1.6 fold increase in proliferation of rabbit VSMCs undergoing cyclic stretch at 5 to 15% elongation and 30 cycles/min for 24 h.[32] This is in stark contrast to other studies which have demonstrated a reduction in cellular proliferation (with augmented differentiation) due to cyclic mechanical strain.[9,19] Additionally, Hipper *et al*. verified that this reduction in cellular proliferation under conditions of strain was accompanied by a significant reduction in DNA synthesis independent of the associated extracellular matrix.[19] The essence of a quiet, differentiated state would preclude cellular proliferation and matrix synthesis, and instead, one would expect to see this sort of result with the synthetic, de-differentiated VSMC phenotype seen in static culture associated with decreased markers of SMC differentiation and proposed to be similar to those VSMCs seen within atherosclerotic lesions.[42]

Growth Factors and Extracellular Matrices

Therefore, does cyclic strain really represent a reliable, physiologic model system? One has to remember that the association between phenotype and relative differentiation cannot be explained by mechanical strain alone; a number of underlying influences such as the presence or absence of growth factors and the type of matrix that the SMCs are cultured upon must be taken into account to give a systemic picture of the milieu to which VSMCs are exposed to and influenced by. On one hand, de-differentiation and proliferation have been demonstrated with platelet-derived growth factor (PDGF) expression in association with a mechanically strained culture.[33,42,49] On the other hand, differentiation has been enhanced when transforming growth factor β1 (TGF-β) was present in culture subjected to mechanical strain.[17,49] Also, the application of cyclic strain has augmented differentiation in association with growth upon type I collagen,[28] laminin,[6,42,51,56] and pronectin.[51] However, it should be noted that studies of extracellular matrices (ECM) have utilized different neonatal[42] or adult VSMCs[6,49,52] from various species of animal subjects. As one example of the dichotomy of results, it has been shown that the proliferation and differentiation of *neonatal* rat VSMCs is highly dependent upon the matrix composition upon which they are grown, but matrix effects are negligible for differentiation of *adult* rat VSMCs and proliferation is not observed with any matrix.[51] Thus, a lack of experimental consistency has resulted in controversial experimental proliferation and differentiation outcomes and created a muddled, phenotype-based definition of differentiation. However, this lack of experimental uniformity has also facilitated a more in depth examination of the possibility for phenotypic manipulation as an engineering tool. For the creation of an engineered vascular construct, both proliferation and differentiation may be desired at different time points in the production process. For example, mechanical strain with PDGF may be utilized early in the vessel construction process to aid proliferation and complete coverage of the vessel scaffold with SMCs. Then, strain with TGF-β may be employed later in the construction process to promote SMC differentiation since this synergy has been demonstrated in the past.[49] An overall analysis of these strain-based studies also demonstrates that the ECM which VSMCs are cultured upon influences the differentiation of these respective cells, and thus, utilization of a scaffold material for vessel engineering based with type I collagen,[28] laminin,[6,42,51,56] or pronectin[51] may be advantageous.

The respective state of a VSMC could lie along a spectrum of differentiation from the contractile phenotype to the synthetic phenotype to the proposed osteoblast-like phenotype demonstrated by SMCs in static culture (and

inhibited by cyclic strain).[37] Regardless of the relative phenotype, markers of SMC differentiation will remain the mainstay of substantiating the mechanical influences on VSMC differentiation until a consensus about phenotype and differentiation can be reached. Thus, further research into phenotype modulation and utilization of cyclic strain for VSMC differentiation is warranted both to gain knowledge of the physiologic process behind VSMC transformation in atherosclerotic disease resulting from hemodynamic alteration and to exploit mechanical force for VSMC differentiation within a tissue engineering scope.

Stem Cells and Endothelial Cells

Perhaps one of the more current applications of mechanical force for tissue engineering involves the utilization of cyclic strain for stem cell differentiation. Recently, Hamilton *et al*. illustrated that bone marrow progenitor cells (BMPCs) subjected to 7 days of cyclic strain expressed increased levels of α-actin and questionable increases in h1-calponin compared to static controls.[18] A similar outcome has also been reported by Park *et al*. demonstrating that cyclic uniaxial (but not equiaxial) strain resulted in transiently increased levels of α-actin and SM22-α in mesenchymal stem cells.[41] Although the authors in these two studies failed to demonstrate full differentiation of BMPCs toward a differentiated SMC lineage,[18] these studies represent an exciting future direction for cyclic strain application. Because stem cells possess growth potential and proliferative capacity, these studies have implications for vascular tissue engineering utilizing cyclic strain for SMC differentiation.

Another current, exciting finding in the realm of cyclic strain and SMC differentiation involves the trans-differentiation of endothelial cells toward a SMC lineage when exposed to conditions of mechanical strain. Recent research within our group has shown that human umbilical vein endothelial cells (HUVECs) cultured at 8% stretch and 60 cycles/min for 2 days demonstrated significant expression of SMC markers α-actin and SM22-α. When compared to unstretched controls, the levels of these SMC markers were increased 5- and 2-fold, respectively.[8] Thus, cyclic strain may promote SMC differentiation as well as induce endothelial cell to SMC trans-differentiation.

Signal Transduction Pathways

As mentioned previously, a number of reviews thoroughly cover signaling (see reviews[24,40]), so the scope of discussion here will be limited to an overview of current and controversial topics involving the signaling pathways leading to expression of markers of SMC differentiation as a result of exposure to conditions of cyclic strain.

The response to mechanical strain is mediated by ECM-integrin interaction at the cell membrane. Studies by Wilson *et al*. and Kim *et al*. have demonstrated that the extent and ensuing response of proliferation or differentiation to strain is mediated by specific cellular integrins which are involved in adhesion to the ECM.[29,56] For example, soluble fibronectin, the integrin binding peptide GRGDTP, and antibodies to both $\alpha_v\beta_5$ and β_3 integrins have been shown to reduce the proliferative response of vascular SMCs to cyclic strain by blocking the interaction between cellular integrins and the ECM.[56] Thus, future engineering approaches may utilize these integrin-binding peptides or antibodies to influence SMC phenotype within an engineered vessel according to whether the construct necessitates a synthetic, proliferative SMC phenotype or a more quiescent and differentiated contractile phenotype. Additional approaches may target focal contact components within the cell signaling pathway such as vinculin and paxillin which are redistributed to the integrin adhesion complex (also due to the applied mechanical strain) where it is postulated that they provide cytoskeleton linkage or participate in cell signaling.[11] From here, two different signaling pathways have been proposed which are not mutually exclusive of one another. The first model pathway, recently explored by Zeidan *et al*. and Albinsson *et al*., illustrated that stretch increased Rho-associated kinase and actin polymerization with a resulting decrease in G-actin and significant increases in markers of SMC differentiation such as SM22α, desmin, and tropomyosin.[2,58] The decrease in G-actin is postulated to aid nuclear translocation of a myocardin-related transcription factor which acts upon the promoter of SMC differentiation markers. The second model pathway involves activation of the MAP kinase cascade. Stretch has been recognized to result in activation of both JNK and p38 MAP kinases with subsequent induction of SM-α-actin expression,[51] but conflicting results have been reported for the importance of ERK1/2 activation.[51,58] Ultimately, both of these two model pathways either complement or activate serum response factor (SRF), a transcription factor which binds the promoter region and regulates expression of nearly all smooth muscle specific differentiation marker genes.[7] Future research in cyclic strain application will undoubtedly lead to the elucidation and clarification of this molecular pathway leading to SMC differentiation.

SHEAR STRESS AND CELL DIFFERENTIATION

Fluid flow over the endothelial cell (EC) surface is one environmental stimulus which may act to induce cellular differentiation, and the effect of shear stress upon EC differentiation can be hypothesized to work in two different stages. The first stage of EC differentiation involves differentiation from an immature, dedifferentiated cell phenotype cultured in static conditions to a mature endothelial cell phenotype similar to that found within the endothelium *in vivo* which is exposed to physiologic mechanical shear force. The second stage of EC differentiation involves

the modulation of differentiated ECs *in vivo* toward either the pro-inflammatory, adhesive phenotype associated with oscillatory flow or the anti-inflammatory, protective, and quiescent phenotype associated with normal endothelium and laminar flow.

Immature and Mature Endothelial Cells

Yamamoto *et al*. recently demonstrated that shear stress accelerated the differentiation of endothelial progenitor cells (EPCs) toward fully mature endothelial cells. This study illustrated that when compared to EPCs cultured under static conditions, EPCs exposed to laminar shear stress exhibited increased expression of the mature EC-specific markers KDR, Flt-1, and VE-cadherin.[57] Furthermore, shear exposed cells displayed morphologic elongation and histologic orientation with long axes in the direction of flow as seen with a mature endothelium. Current work within our group has revealed that expression of mature EC markers CD31, vWF, and VE-cadherin were significantly increased 757-, 108-, and 23-fold, respectively, in a murine embryonic mesenchymal progenitor cell line cultured under conditions of shear stress.[53] Additionally, shear stress resulted in altered cell morphology and increased the ability of acetylated LDL uptake (seen in mature ECs). Thus, shear stress may promote a differentiated EC phenotype compared to static controls.

Other markers of differentiation have been utilized in additional studies to distinguish and perceive the change from de-differentiated cells in static culture to a differentiated EC phenotype. Besides elongation and orientation in the direction of flow, shear has been recognized histologically to promote increased EC adherence[4] and cellular hypertrophy.[39] Further markers of cellular differentiation which have been employed to test differentiation due to shear also include VEGFR-2, Tie-2, and the ability of shear conditioned ECs to form capillary tubes when seeded in collagen gels.[27,57]

As with SMC differentiation and cyclic strain, one must remember that a spectrum of EC differentiation is possible due to different ECM and growth factor-related influences. In particular, attention has recently turned towards basic fibroblast growth factor (bFGF) which is released from the cytoplasm of ECs upon shear stress exposure and promotes cellular differentiation.[16,47] This has been demonstrated by Gloe *et al*. who showed that capillary tube formation by shear exposed ECs was completely inhibited by bFGF receptor neutralization.[16] Also, vascular endothelial growth factor (VEGF) has been shown to be necessary for progenitor to EC differentiation in static culture,[44] and undoubtedly, the existence of VEGF in a mechanical shear environment will only further enhance EC differentiation. Thus, addition of bFGF or VEGF to a shear exposed culture may be utilized as a means to directly control cell differentiation within a vascular tissue engineering context.

Mature Endothelial Cell Phenotype

Endothelial phenotypic modulation has received attention lately because of the implications of oscillatory flow and the resulting proposed inflammatory phenotype seen as a link and gateway for atherosclerotic disease.[10,55] This inflammatory phenotype is prevented by antioxidant and anti-inflammatory gene expression which is induced by laminar flow and will be discussed below.

A subject of controversy in the area of EC differentiation and phenotype involves the associated proliferation of ECs in response to shear stress. Selected research has demonstrated that laminar shear stress promotes EC proliferation,[3,57] while others have offered reports that shear inhibits cellular proliferation.[1] First, it should be noted that most of these experiments vary in the conditions, confluence, and origins of endothelial cells utilized, and thus, different resulting proliferative responses may be due to environmental conditions. A further look at the experimental settings of these studies reveals that the presence or lack of proliferation may be correlated to the level of shear to which cells are exposed or the relative maturity of the cells utilized. For example, Yamamoto *et al*. demonstrates EPC proliferation upon shear stress exposure, but these progenitor cells are cultured in only 0.1 to 2.5 dyn/cm^2 of shear.[57] On the other hand, Akimoto *et al*. determined that more relatively mature populations of bovine aortic endothelial cells (BAECs) are not growth inhibited at 1 dyn/cm^2, and growth inhibition due to laminar flow was only observed starting at levels of 5 dyn/cm^2 of shear.[1] Also, recent gene expression studies have illustrated a downregulation of genes associated with cellular proliferation when ECs were exposed to 10 dyn/cm^2 of laminar flow.[55] One would intuitively expect a fully differentiated endothelium to exude a quiescent, non-proliferative nature associated with laminar flow, and thus, in the continuum of EC differentiation, the protective, quiescent phenotype is likely anti-inflammatory and anti-proliferative. Thus, future vascular tissue engineering may utilize this knowledge and exploit the use of higher levels of shear to create a more mature engineered construct.

Signaling and Gene Expression

For full coverage of the molecular mechanisms and signaling within ECs in response to shear stress, readers should be directed to the recent, excellent reviews by Lehoux and Tedgui[31] and Wasserman and Topper.[54] Briefly, laminar shear stimulates specific integrin–ECM mediated signaling cascades as seen with SMCs and cyclic strain.[16] Diverse pathways lead to the upregulation of transcription factors such as Hath6 for genes regulating EC differentiation,[55] gut-enriched Kruppel-like factors (GKLF) and lung Kruppel-like zinc finger transcription factor (LKLF) for genes regulating cellular quiescence,[13,45] and antioxidant response elements (ARE) for genes involved in protection from oxidative stress and inflammation.[10] Gene expression, overall,

is highlighted by upregulation of genes associated with anti-proliferative, anti-inflammatory, anti-oxidant, and differentiative properties, and by downregulation of genes associated with cell cycle progression and cellular proliferation.[55] Consequently, laminar flow confers a quiescent and protective phenotype normally seen *in vivo*.

VASCULAR TISSUE ENGINEERING

In the search for a stable small diameter vascular graft, researchers have engineered blood vessels *in vitro* under physiologic conditions of shear stress and strain in an effort to prevent new graft failure. Hemodynamic forces have been utilized to promote vascular cell differentiation within these engineered constructs to create vessels far superior to those cultured under static conditions. As mentioned above, a period of exposure to shear stress *in vitro* after EC seeding promotes EC differentiation with resulting greater cellular adherence and a more homogeneous, confluent graft surface compared to static controls.[4,21,27] For example, Hoerstrup *et al*. illustrated this advantage with a novel pulse duplicator system whereby grafts exposed to 21 days of shear exhibited greater EC confluence and organization compared to grafts cultured under static conditions.[21] The integrity and retention of the EC monolayer within the vascular graft resulting from a period of shear preconditioning will reduce *in vivo* graft thrombosis and subsequent neointimal hyperplasia, two common causes of graft failure after implantation.[12,46] A study by Kaushal *et al*. remarkably demonstrated that EC progenitor-seeded grafts exposed to 2 days of shear preconditioning (starting at 1 dyn/cm^2 and gradually increased to 25 dyn/cm^2) remained patent for up to 130 days *in vivo*.[27] Furthermore, the lumen of all shear preconditioned grafts in this study were confirmed to be smooth and without thrombus formation, as compared to control grafts which occluded with thrombus soon after implantation.[27]

Furthermore, by preconditioning a vessel *in vitro*, SMCs within the engineered media are exposed to cyclic strain, and their ensuing differentiation results in greater contractility, increased ECM production, increased cellular density, amplified maturity, and more histologic organization compared to SMCs seen within grafts cultured under static conditions.[23,35,36] As an example of the benefit of utilizing pulsatile conditions for tissue maturation and cellular differentiation, Niklason *et al*. showed that SMCs cultured in small caliber arterial conduits under pulsatile conditions retained a more differentiated function and stained more heavily for markers of SMC differentiation than static controls.[35] Thus, SMCs cultured under pulsatile-like conditions display a mature contractile phenotype similar to native artery and more suitable for implantation.

The capacity to manipulate vascular cell phenotype with hemodynamic force is an important potential in working toward the ultimate goal of a fully engineered vessel. As mentioned above, different aspects of the biomechanical environment combined with selective use of growth factors and extracellular matrices can be exploited to influence cellular differentiation. As a theoretical example using the studies and information cited throughout this manuscript, one could reason that a pronectin-based scaffold[51] could be seeded with SMCs and allowed to mature with strain and utilization of TGF-β.[49] Afterwards, ECs may be seeded into the lumen of the graft, and a combination of high levels of shear[1] and bFGF[16] may be used to promote endothelial organization and maturation. Thus, hemodynamic force may play an imperative role in vascular cell differentiation within these constructs in the future.

However, certain limitations currently exist which will need to be addressed and expanded upon. The first inadequacy of placing SMCs on cyclic strain and ECs in shear stress is that these two systems are not entirely physiologic. It has been noted that, in addition to shear, ECs also experience tensile strain perpendicular to the lumen.[5] Likewise, studies have suggested that SMCs can experience some degree of shear stress which may significantly reduce SMC proliferation.[50] Thus, a more physiologic system in the future may combine some aspects of tensile strain and shear stress to more adequately attend to the overall milieu of hemodynamic forces experienced by vascular cells. Another inadequacy that will need to be dealt with is elucidation of the "cross-talk" experienced between SMCs and ECs in biomechanical culture. Endothelial cells have been shown to affect the differentiation pattern of SMCs,[20] and co-cultured SMCs have been shown to influence the EC response to shear stress.[22] For example, Imberti *et al*. utilized a co-culture model to illustrate that ECs cultured under conditions of shear with pre-strained SMCs showed a 64% reduction in proliferation compared to ECs cultured in shear without SMCs.[22] Thus, future studies employing co-culture models will open new avenues for creating a more physiologic model system and reveal new tools for phenotype modulation and tissue engineering.

CONCLUSIONS

Current research continues to focus on the role of hemodynamic force in SMC and EC differentiation. The results of the vast majority of these studies can be utilized in the movement (Table 1) toward engineering a fully implantable small diameter graft or understanding the pathophysiology behind atherosclerotic disease. Many challenges lie ahead for researchers in this arena, including further elucidation of phenotype grouping and clarification of the ideal combination of growth factors, scaffolding, and cell source necessary for differentiation. Future studies will undoubtedly deal with these issues and reveal innovative applications for shear stress and cyclic strain.

TABLE 1. Timeline of major findings for the role of hemodynamic forces in vascular cell differentiation.

Time frame	Major findings	References
Early 1990's to 1997	Cyclic strain produces an increase in markers representative of the differentiated phenotype and change in orientation due to stretch.	7–9,11,12,14
Early 1990's to 2003	Shear stress promotes EC adherence and increases markers of endothelial differentiation characteristic of the mature endothelial phenotype.	35,38,39
Mid 1990's to the present	Combination of cyclic strain and other environmental conditions such as growth factors and ECM results in SMC phenotype modulation.	2,7,8,15,16,18,21,25–27
Mid 1990's to the present	Growth factors and level of shear potentiate the effect of shear stress on endothelial cell phenotype modulation.	40–42,44–46
Late 1990's to the present	Utilization of biomechanical forces to precondition and promote cellular differentiation for vascular tissue engineering purposes.	37–39,50–55
2003 to the present	Shear stress results in the differentiation of progenitor cells along an endothelial lineage.	35,36
2004 to the present	Cyclic strain initiates differentiation along smooth muscle cell lines in progenitor cells.	29,30

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