EFFECT OF HYPERGLYCEMIA ON LEUKOCYTE MIGRATION AND DIFFERENTIATION IN A NOVEL THREE-DIMENSIONAL TISSUE MODEL

By

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CHAPTER I

INTRODUCTION AND REVIEW OF RELEVANT LITERATURE

1.1 The Impact of Diabetes and Vascular Complications

The National Institute of Diabetes and Digestive Kidney Diseases estimates that 20.8 million Americans (7% of population) have diabetes.[1] Oklahoma exceeds the national average with one in 10 oklahomans diagnosed as diabetic. The Native American population within the state is particularly vulnerable to diabetes. Since 1990, the prevalence of those diagnosed with diabetes increased 61%.[2] Inherently associated with the western world as the rich man's disease, past few years have seen an exponential rise in diabetic cases in developing countries as well.[3] Changes in lifestyle [4] and food habits are often implicated to be the main reasons of this exponential growth. New York Times in June 2006 reported a total number of diabetics around the world to be 230 million, a number which was 30 million 20 years back.[5] The World Health Organization on their website project the number to reach 366 million by 2030.[6]

Diabetes by itself is the fourth leading cause of death in most of the developed world and sixth in the USA.[1] But complications associated with it prove diabetes epidemic [7] far more dangerous. Common complications associated with diabetes are hyperglycemia, atherosclerosis, myocardial infarction (heart attack), ischemia, thrombosis, high blood pressure, blindness, stroke, and limb amputation. Diabetes is

often thought to be associated with an increased risk for atherosclerosis [8, 9] and also linked with many other vascular diseases [10].

Atherosclerosis is a chronic inflammatory immunological disease characterized by formation of lipid laden plaques or lesions in the intima region of blood vessels. Plaque is made up of deposits of fatty substances, cholesterol, cellular waste products, calcium, and fibrin, and can develop in arteries. These lesions are formed as a result of very specific cellular and molecular responses to various endogenous risk factors for atherosclerosis and antigenic stimuli.[11]

As these plaques grow larger in size, they tend to block the blood flow, thereby causing further cardiovascular complications. In atherosclerosis, plaque formation within blood vessels leads to ischemia (restriction in blood supply) that can cut or reduce blood supply to different tissues. For example, in coronary artery disease, atherosclerosis narrows the coronary arteries, which are the arteries supplying blood to the heart muscle. With more narrowing, the risk of a heart attack (myocardial infarction), which occurs when a coronary artery is blocked completely, is increased. Figure 1 shows how plaque formation and related phenomena, such as thrombosis, can affect the blood vessel(s) and lead to complications, such as myocardial infarction. When an atheroma is formed in an artery, it starts accumulating lipids and narrowing down the cross section of the vessel, as shown in figure 1. This lipid filled plaque may become vulnerable and rupture, leading to thrombosis and a myocardial infarction.

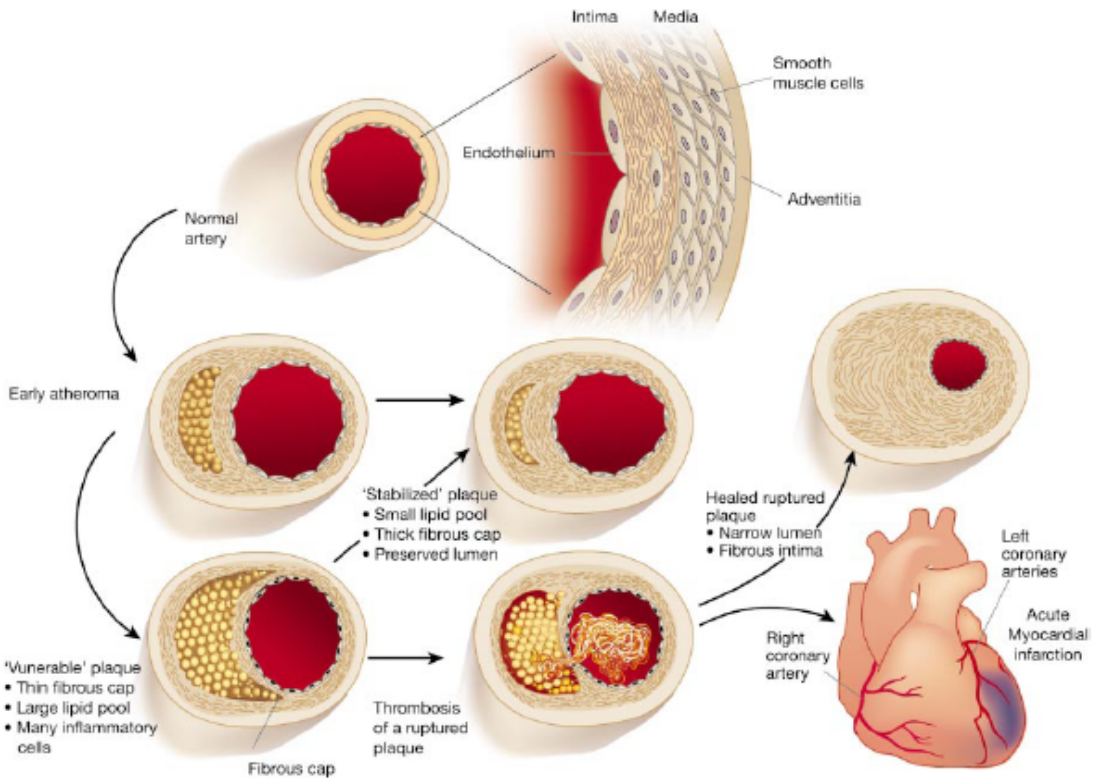


Figure 1. Development of an atherosclerotic plaque in a blood vessel.[12]

Continuous, prolonged ischemia can also reduce the blood supply to tissues leading to tissue death. This problem is common in limbs, which can result in limb amputations.[13] In the atherosclerotic narrowed down arteries, a blood clot (thrombus) may form inside a brain artery, which can cut off the blood supply to part of the brain and cause a thrombotic stroke. The ischemic damage depends on the size of the obstruction, its location, and the duration of the lesion formation.[14] Plaques can also rupture and block the coronary arteries, thereby causing thrombosis and hence responsible for myocardial infarction in another way.

Primary cause of morbidity and mortality in patients with diabetes is due to cardiovascular diseases (CVD) that affects both the micro- and macrovasculature.[15] Since diabetes is often thought to be associated with an increased and accelerated risk for

atherosclerosis [8, 9], patients with diabetes have a much higher risk of diseases like myocardial infarction, stroke and limb amputation. Epidemiological studies have shown that diabetics are at 1.5- to 3-fold greater risk of atherosclerotic disease and a two-fold higher risk of myocardial infarction. Diabetic women are five-fold more prone to coronary artery disease than the general population.[16] The treatment costs attributable to diabetes are enormous and continue to grow. A 2002 study showed that in U.S. alone, the direct and indirect costs attributed to diabetes was $132 billion.[17] To look at the share of CVD complications in these costs, a study conducted in one managed healthcare system revealed that more than 25% of the excess costs of diabetes were due to cardiovascular disease complications, including atherosclerosis.[18] Over the past two decades, progress has been made in defining the cellular and molecular interactions involved that connect diabetes to the development of atherosclerosis plaque. The following describes the most recent understanding of how the two are related. Atherosclerosis is considered a chronic disease triggered by endothelial injury and sustained by inflammation. All forms of diabetes are characterized by chronic hyperglycemia. Hyperglycemia has been shown to be an inflammatory mediator that can affect both endothelial and immune cells. Therefore, it is logical to investigate possible links between hyperglycemia and both endothelial and immune cell function. In order to understand these possible links, a discussion of hyperglycemia, endothelial cells and immune cells will follow.

1.2 Chronic Hyperglycemia Associated with Diabetes

Hyperglycemia is a condition in which the blood glucose level is elevated, or an excessive amount of glucose circulates in the blood plasma. Glucose levels in the blood can vary within the same day, depending on food intake and body metabolism. Because of this, there is no single way to express glucose concentration in blood. Fasting blood sugar (or fasting blood glucose) is the most common term used to describe blood glucose levels. Fasting blood glucose is defined as the concentration of glucose in blood at least eight hours after food has been eaten. In general, the normal blood glucose range for most fasting adults (normoglycemia) is about 80 to 120 mg/dL or 4 to 7 mmol/L. A subject with a consistent glucose level above 126 mg/dL or 7 mmol/L is considered to have hyperglycemia, whereas a consistent level below 70 mg/dL or 4 mmol/L is taken as hypoglycemic case.

Normally, blood glucose level does not remain constant throughout the day, but varies as we take in food and as it circulates to different cells.[19] The glucose concentration in blood is regulated by the secretion and action of the hormone insulin. An insufficient production/secretion or an ineffective action of this hormone makes the glucose concentration rise in the blood, as in the case of diabetes mellitus. Insulin regulates uptake of glucose from the blood into the cells for the use as a fuel or for storage as glycogen in the liver.

1.3 Blood Vessels and Endothelial Cells

The vascular system is responsible for carrying blood to and from different tissues in the body. The blood vessel network in itself is vast and if placed end to end, would

cover 50,000 miles.[20] It is a closed circular system, with the heart pumping the blood through the blood vessels. Blood vessels are grouped as arteries, veins and capillaries, and vary by size. Usually, the diameter of the veins is the largest (20 µm to 13 mm), followed by the arteries (10 µm to 3 mm) and capillaries being the smallest (5µm to 10 µm) [21]. The only exception is the aorta, which has an average diameter of 25 mm, making it the largest blood vessel (and artery) in the circulatory system.

Arteries and veins consist of a coat of longitudinal smooth muscle (tunica externa), which surrounds an inner coat of circular smooth muscle (tunica media). The inner surface is lined with a single layer of endothelial cells, the tunica intima. Figure 2 shows this layered structure of a typical blood vessel.

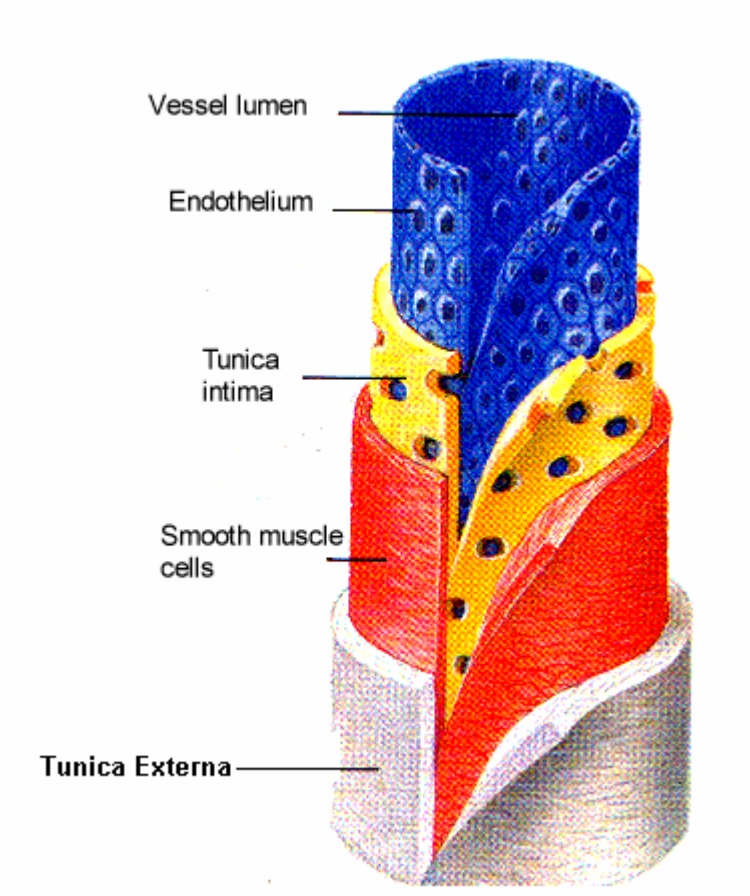


Figure 2. Layered structure of a typical blood vessel [22].

All blood vessels at their inner surface are lined with specialized cells called endothelial cells. This thin layer of confluent endothelial cells is called an endothelium and acts like a selective barrier between the blood stream and the tissues. The endothelial cells are not flat but protruding at the center and tapering towards the edges. When completely confluent, the endothelial cell layer has a characteristic cobblestone layer look. The endothelial cells spread over the whole circulatory system from the smallest capillary to the heart. The endothelial cells serve as the regulators of transport through the endothelium [23] and through the blood vessel wall. Vascular endothelium, due to their position between the blood and tissue, are able to sense changes in hemodynamic conditions and biochemical stimuli and are designed to respond to these fluctuations by regulating the cytokine microenvironment, upregulating adhesion molecules on cell surfaces, angiogenesis, etc.[24] There exist tight junctions between any two adjoining endothelial cells, the permeability of which are modulated by a number of stimuli.[23] Vascular endothelium is surrounded by a fibrous basement membrane after which lies the extracellular matrix (ECM) of the tissues. ECM composition, just like most other biological parameters, varies with time and local conditions. The ECM is predominantly composed of collagen, elastin, laminin, fibronectin, and heparan-sulphate proteoglycans with collagen being the most abundant.[25-27] As the blood cells flow through the blood vessels, they interact with the surface characteristics of the endothelium. Before describing these interactions, it is important to know a little about the blood, its composition and function.

1.4 Blood and Leukocytes

Table 1 gives an overview of the physical properties of blood. Blood is the thick, red fluid that provides oxygen, nourishment, and protection to the cells and carries away waste products. There is approximately five liters of blood in an average adult human. The whole blood consists of two major components: the plasma and the cellular components. Plasma makes up about 55% of the whole blood and the remaining 45% being the blood cells. Plasma is a straw colored liquid found in blood and lymph. It is about 90% water with the remaining being nutrients, gases and waste products.[28] The blood cells are found floating in the plasma along with certain other constituents of the blood like amino proteins, vitamins, and waste materials.

Table 1. Physical Properties of Human Blood [29].

Property	***Value***
Whole Blood pH	7.35-7.4
Viscosity (37^0C)	3.0 cP (at high shear stress)
Specific gravity ($25/4^0$C)	1.056
Whole Blood Volume	~78 ml/kg body weight
RBC Count	4.8-5.4 $*10^9$/ml whole blood
RBC average life span	120 days
WBC Count	~$7.4*10^6$/ml whole blood
WBC Diameter	7-20 microns
Platelets Count	~$2.8*10^8$ ml whole blood
Platelets Diameter	~2-5 microns

Blood cells are often characterized as red blood cells (RBCs), white blood cells (WBCs) and platelets. Red blood cells, also known as erythrocytes, make up more than 90% of the blood cells, thus approximately 40% of whole blood volume. Their function is to transport oxygen to the cells and carbon dioxide away from cells. Thus, red blood cells also play a big role in regulation of blood acidity.[21]

White blood cells, also called the leukocytes, are immune cells important for the body's immune response to foreign invaders and antigens. White blood cells have a life cycle from a few days to a few weeks in supporting environment. White blood cells can be divided into the following five groups: neutrophils, monocytes, lymphocytes, eosinophils, and basophils. Table 2 describes the individual functions of each type of WBCs.

Table 2. The Types of White Blood Cells.

Leukocyte Type	**Percentage of total WBCs**	**Function**	**Granularity**
Neutrophils	40-75%	Fight disease by phagocytosis; very important against bacterial/fungal infection.	Granular
Monocytes	~6%	Fight disease by phagocytosis; develop into macrophages or dendritic cells(DCs) on entering tissues; Largest of WBCs	Agranular
Lymphocytes -B cells -T cells -NK cells	~30%	Produce antibodies and chemicals that are active in regulating disease, allergies, and controlling tumors.	Agranular
Eosinophils	~3%	Capable of phagocytic activity and release chemicals during inflammation; their number increases greatly with parasitic infections or allergic reactions.	Granular
Basophils	~1%	Target allergic reactions.	Granular

1.5 Interactions Between the Blood Endothelium and the Leukocytes

Leukocytes are involved in body's immune response and travel to all the tissues of the body. These cells tend to migrate through the endothelium into the interstitial tissue (intima) from the lumen; this process is called transendothelial migration. Constitutive migration of monocytes from the blood is important to ensure the replenishment of the macrophage numbers within the tissue. But compared to the unstimulated/non inflamed state, this migration is enhanced drastically whenever they sense a local inflammation.[30-33] Inflammation could be caused by pathogens, infection, damage to the tissues, or a combination of those, and is the body's response to fight back these attacks. Figure 3 shows the cross section of a blood vessel and the physiology of leukocytes in the case of inflammation.

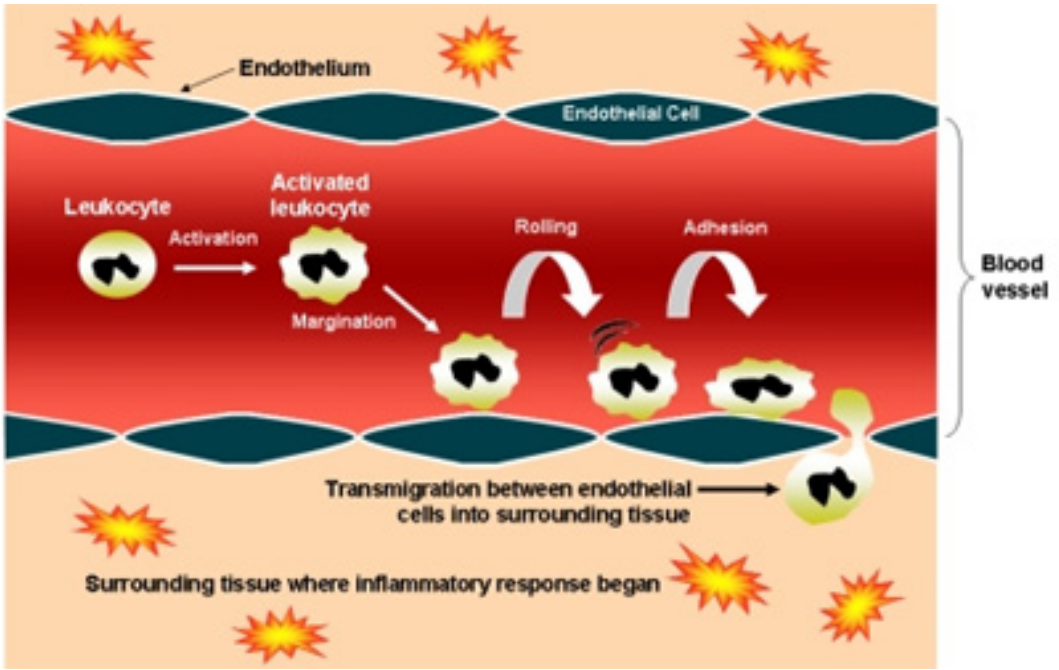


Figure 3. Cross sectional view of a blood vessel describing leukocyte rolling, adhesion, diapedesis, and transendothelial migration in response to various stimuli within the tissue.[34, 35]

Leukocytes inspect the tissues for potential bacterial, antigenic, tissue injury or other possible "inflammatory signs" and respond by phagocytosis of possible cell debris and pathogens. Transendothelial migration of leukocytes is regulated by the local microenvironment of the endothelium and the cell phenotype, in a process where cell adhesion molecules present on both the leukocytes and endothelial cells play an important role.[25, 36, 37] The process of the leukocyte interaction with the endothelium is known as the leukocyte cell adhesion cascade and is a multistep orchestrated process. Inflammation affects each of these steps from up-regulation of adhesion molecules on local endothelium to subsequent migration and possible complications. The whole process is still not completely clear, but is believed to be responsible for the local recruitment of leukocytes to sites of inflammatory challenge. Inflammation results in endothelial activation, which is considered as a crucial step in the initiation of atherosclerosis process, as well as in the development of advanced atherosclerosis. Stimulation of endothelial cells with pro-inflammatory cytokines has been found to increase the expression of adhesion molecules such as intracellular cell adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1).[38] Amongst other agents and conditions, high glucose (hyperglycemia condition) has been found to be a proinflammatory signal and contribute to the inflammatory response.[39, 40] The inflammatory response evoked by glucose is associated with an upregulated leukocyte-endothelium interaction, potentially achieved by upregulated cell adhesion molecule (CAM) expressions.

1.5.1 Cell Adhesion Molecules

Cell adhesion molecules are proteins located on the cell surface and are involved with the binding to other cells or with the extracellular matrix (ECM). The adhesion molecules are classified as four types, based on their structural and biochemical characteristics, as integrins, cadherins, selectins and members of the immunoglobulin superfamily. The selectins and members of the immunoglobulin superfamily have shown key roles in atherosclerosis.

Selectin family of cell adhesion molecules are the ones that are involved in the recruitment of leukocytes in the event of an injury or inflammation. There are several types of selectins, including the P-, E-, and L-selectins. E-selectin or endothelial-leukocyte adhesion molecule-1 (ELAM-1) has been identified as participating in the rolling and adhesion of monocytes and neutrophils.[14, 25, 41-43] It is synthesized during inflammation only and hence will not be expressed on unstimulated endothelial cells, but only on stimulated conditions like in atherosclerotic lesions or after being stimulated by cytokines.[14, 41, 44] L-selectin is another important CAM, expressed at the tips of microfolds on granulocytes, monocytes, and a vast array of circulating lymphocytes.[45] However, L-selectin cannot by itself assume the rolling task at normal velocities *in vivo* [45] (happening within the living body), thereby needs the participation of either P- or E-selectin.

Immunoglobulin superfamily is a very important class of cell adhesion molecules. It is characterized by an immunoglobulin subunit and is involved in antigen recognition.[14] Some common CAMs under this category are ICAM-1, VCAM-1 and platelet endothelial cell adhesion molecule (PECAM-1). ICAM-1 is a transmembrane

glycoprotein immunoglobulin continuously present in lower concentrations in the membranes of leukocytes and endothelial cells. In the event of stimulation by inflammation or by cytokines IL-1 and TNF-α, the concentration greatly increases and is expressed by vascular endothelial cells, macrophages and lymphocytes. Another immunoglobulin is VCAM-1. In atherosclerotic plaques, VCAM-1 expression is upregulated possibly by TNF-α and IL-1, while in unstimulated HUVECs, it is minimally expressed.[46] PECAM-1, a transmembrane protein, is expressed at the junctions of the ECs [37, 43] and also to a lower degree at the surface of immune cells like macrophages, neutrophils, monocytes, lymphocytes, etc. [37]. PECAM-1 is crucial in the process of leukocyte transmigration through intercellular junctions of HUVECs. Pretreating either the leukocyte or the endothelial cells with a PECAM-1 blocking antibody is shown to reduce the transendothelial migration by 70-90%.[47] The leukocytes are seen to be trapped precisely over the intercellular HUVECs junctions, after they are treated with a blocking antibody, thereby blocking the transmigration.[47, 48]

1.5.2 Movement of Leukocytes into the Tissue in Inflammation: Leukocyte Adhesion Cascade

On sensing an inflammatory condition, the leukocytes are quickly recruited to fight the infection/antigen. In order to fight, the leukocytes need to migrate within the intima. This movement of the leukocyte into the intima is called the cell adhesion cascade and it is governed by many factors, with some known and some unknown. This cascade consists of the endothelial cells and leukocytes interacting in response to an inflammatory condition. The process consists of different stages like leukocyte rolling,

leukocyte adhesion and then firm adhesion, followed ultimately by transendothelial migration. Figure 4 shows the leukocyte adhesion cascade.

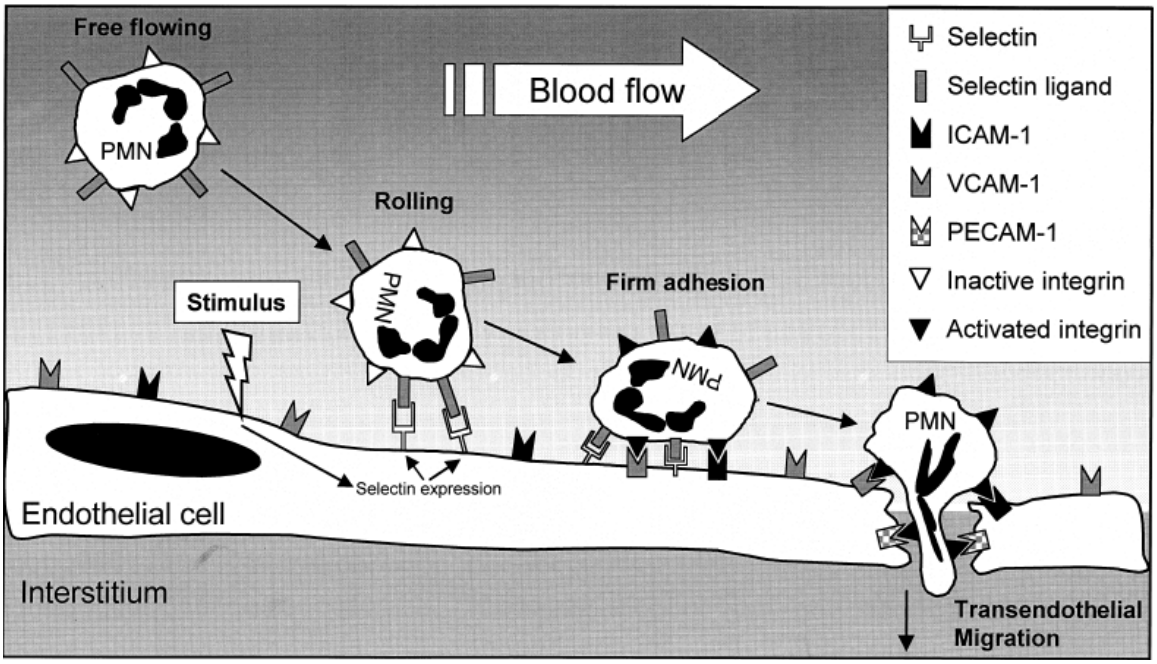


Figure 4. Schematic of the multistep paradigm of leukocyte recruitment.[49]

Rolling is seen to begin as the selectin molecules present in leukocytes and endothelial cells constantly form and break bonds.[41, 50] The selectin molecules thus decelerate the moving leukocytes until they come to a stop and adhere on to the endothelial junctions. This step of adhesion is seen to be motivated by the signals from the endothelial cells and involves selectins and immunoglobulin family CAMs like VCAM-1 and ICAM-1. The final step of the leukocyte adhesion cascade is the transendothelial migration of the leukocytes and is believed to involve ICAM-1 expression by the endothelial cells as ICAM-1 is found on both the apical and basal surfaces of the endothelial cells.[51]

1.5.3 Monocyte Differentiation and Reverse-Transmigration Across the Endothelium

One type of the leukocytes, called monocytes, actively accumulate within the intima and some of these differentiate further into macrophages and dendritic cells. These macrophages are responsible for the clearance of unphysiologic proteins, debris, and lipids through phagocytosis. Macrophages are also involved in the secretion of growth factors, cytokines, and chemoattractants, which facilitate further recruitment of monocytes. Some of the migrated monocytes that differentiate into dendritic cells move from the tissues into the draining lymphatics to present antigen in the lymph nodes, through a process called reverse-transmigration. In reverse-transmigration, cells move across the endothelium from the basolateral to the apical direction.[52] Molecular mechanisms behind migration of DCs to the lymphatics are not very well understood.[25] Migration from the tissues into lymph nodes requires passage across the endothelium of the afferent lymphatic vessel [53], however there is little information on how DCs cross this barrier *in vivo*. Recently though, p-glycoprotein (PgP/MDR-1), a membrane transporter molecule, has been identified as playing an important role in attaining reverse-transmigration [54]. Antibodies against p-glycoprotein were found to impair reverse-transmigration by DCs *in vitro* (outside the living body).[55]

1.5.4 The Effect of Flow on Endothelial Cell Behavior

Blood in the vessels follow a pulsatile non-Newtonian flow. The vessels branch out into tapering daughter vessels as they extend into a wider network. There is an increase in cross sectional area due to branching. The blood vessels are curved, and

blood vessel curvature has a profound effect on flow.[23] As a result o f this curvature and varying cross-section, adverse pressure gradients are produced that cause flow reversal and the velocity profile in these Y shaped branching regions is skewed.[23] Such regions are called shoulder regions. Figure 5 shows the velocity profile associated with such a region. The lines closer to the Y shaped region appear to have a far more disturbed velocity profile as compared to the ones far away from it. The last two lines situated just before and after the Y shaped region, show the most disturbed velocity profile. These regions are like the arterial curvature and branch points. Atherosclerotic lesions have been found to develop in these regions of arterial curvature and branch points.[56] The flow in these regions is disturbed and non uniform. Also the expression of certain proinflammatory genes (cell markers) implicated in atherogenesis (formation of atheromatous lesions in the intima) was found to be increased.[56]

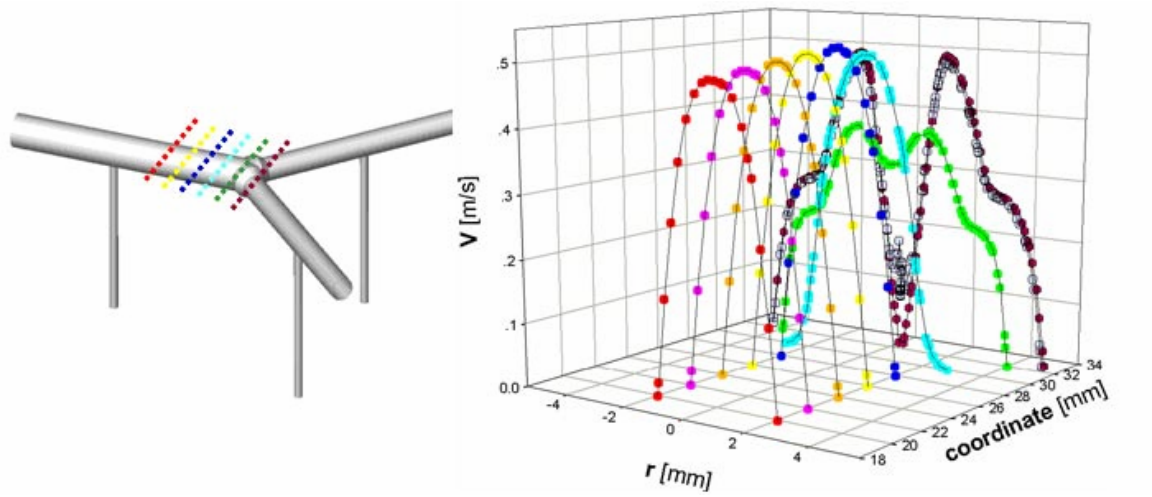


Figure 5. Velocity profile associated with a shoulder region in a blood vessel.[57]

Away from vessel branches, endothelial cells are elongated. Figure 6 describes the blood flow in an atherosclerosis-prone (AP) and atherosclerosis-resistant(AR) region of the endothelium.[58] The endothelial cells in the AP region appear rounder and do not

have one distinct orientation, unlike the AR region endothelial cells, which are all oriented towards the blood flow direction. There are a significant number of leukocytes attached to the surface of endothelium in AP areas, as against the AR regions. These type of AP regions exist around the shoulder region where the flow is found to be disturbed, while the AR regions are the ones with the normal blood flow.

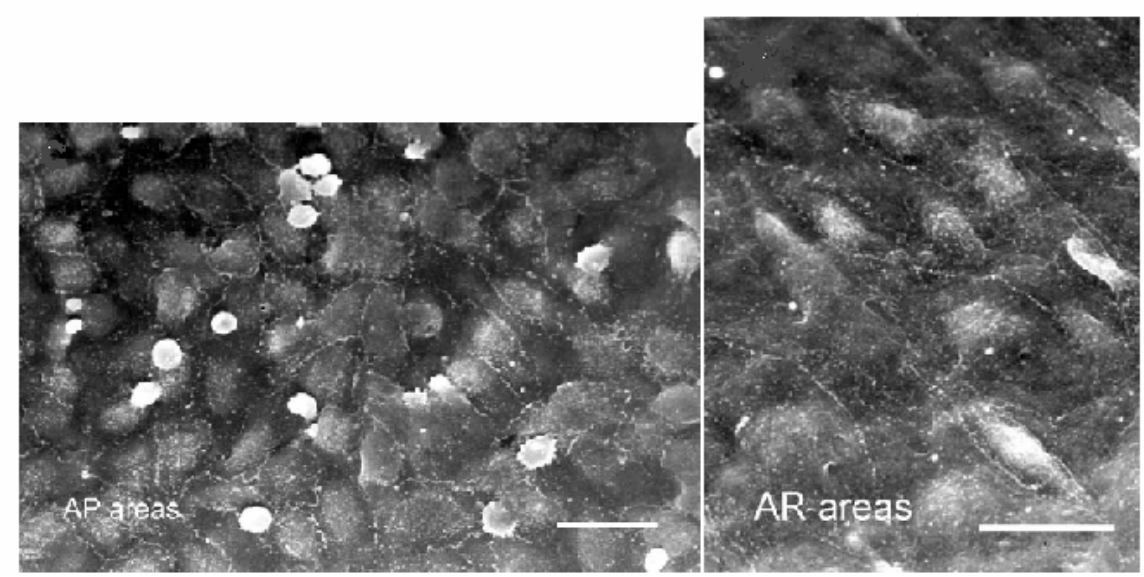


Figure 6. Endothelial cell spread morphology describing an atherosclerosis prone (AP) region and an atherosclerosis resistant (AR) region in an *in vivo* human aorta model. Scale bars: 15 µm.[58]

1.6 Link between Hyperglycemia and Atherosclerosis

In atherosclerosis, the inflammatory response is mediated by functional changes in a host of different cell types, including endothelial cells, T lymphocytes, monocyte-derived macrophages and smooth muscle cells.[59] Activation of these cells leads to the complex array of inflammatory events consisting of the release of hydrolases, cytokines,

chemokines, adhesion molecules, and growth factors, together with lipid accumulation and proliferation of smooth muscle cells and fibroblasts. Leukocytes move into the site of inflammation within the vascular intima by way of adhesion molecules, such as VCAM-1. Monocytes differentiate into macrophage or dendritic cells (DCs), depending on certain factors. These factors depend on the microenvironment surrounding the cells [60], but this phenomenon is not completely clear. When activated, macrophages also express multiple scavenger receptors that facilitate internalization of modified lipoproteins leading to the development of cholesterol-laden foam cells and plaque formation in arteries [61], as shown in Figure 7.

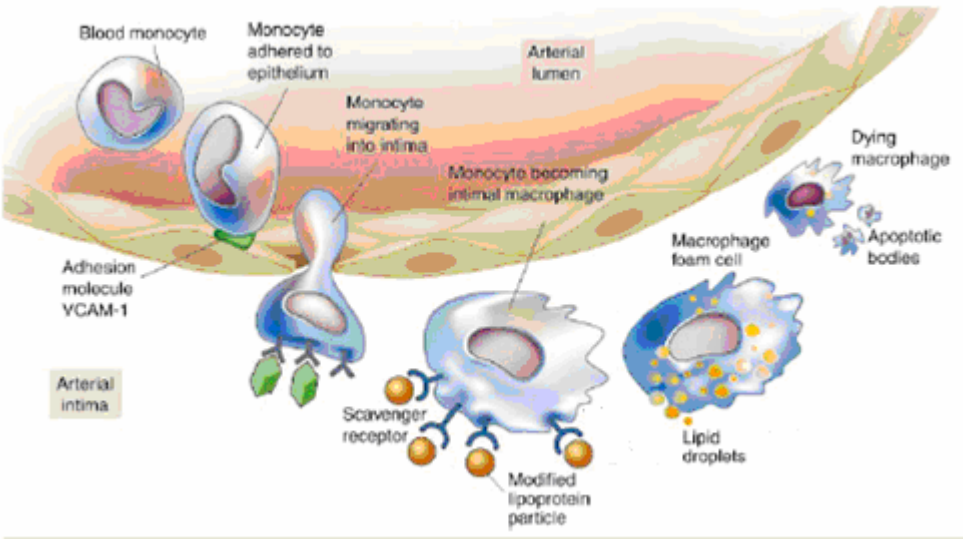


Figure 7. Recruitment of leukocytes to the nascent atherosclerotic plaque.[12]

Since hyperglycemia is found to be a proinflammatory signal [39, 40], it could actually be upregulating the cell adhesion molecules on endothelium and leukocytes [62]. Leukocyte adhesion, rolling and migration is enhanced drastically whenever they sense a local inflammation.[30-33] Thus, the initial stages of atherosclerosis could actually be related to an increased expression of the cell adhesion molecules that allow more

monocytes to come into contact with endothelial cells leading to an increase in migration into the tissue and differentiation to macrophages. *In vivo* studies done on a rat model revealed that leukocyte rolling and migration through the endothelial cells was increased when subjected to hyperglycemia.[40] Also , an associated increase in the expression of certain CAMs on ECs was observed after exposure to hyperglycemia in this *in vivo* rat model.[40]

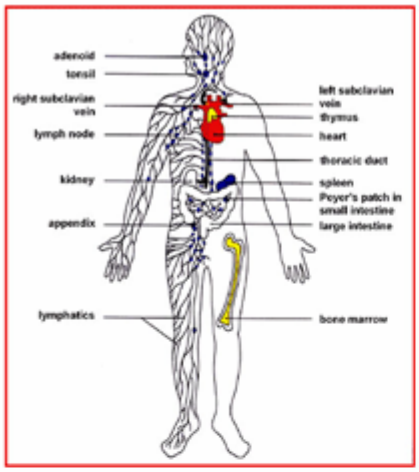
It was important to know whether the effects were due to hyperglycemia or some other factors associated with glucose, such as hyperosmolarity. Some studies have therefore investigated the effect hyperosmolarity has on hyperglycemia-atherosclerosis associated phenomena. To check this, a hyperosmolar control, mannitol, was employed in various studies to see if the presence of an equally hyperosmolar group produces a similar activation of the endothelial cells. Mannitol was found to give no significant difference within the tested concentration and incubation time ranges for monocyte binding [63], leukocyte adhesion [64-66], VCAM-1 expression [67], ICAM-1 expression [65], monocyte migration [68, 69], and endothelial cell apoptosis [70, 71].

There have been studies to characterize atherosclerotic lesions by investigating different CAM expressions, such as ICAM-1 and VCAM-1.[72] PECAM-1 expression has been found to be elevated with severe atherosclerosis and PECAM-1 expression has been detected in atherosclerotic plaques.[73] Similar upregulation of VCAM-1 expression on HUVECs and on atherosclerotic plaques was observed for *in vivo* atherosclerotic studies.[46, 74] The expression of ICAM-1 was also found upregulated significantly in the earlier stages of atherosclerosis when the vascular endothelium was still intact.[75]

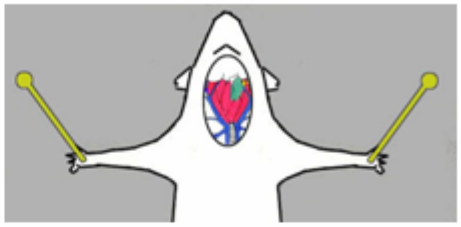
Atherosclerosis has many inflammation-mediated risk factors like hyperglycemia, abdominal obesity, tobacco smoking, dyslipidemia, bacterial and viral infection, hyperhomocysteinemia.[56, 76] In some of the *in vivo* animal studies, hyperglycemia has been shown to enhance atherosclerotic plaque growth [8], but the exact cellular mechanism is not clear. The common trends associated with both hyperglycemia and atherosclerosis-like enhanced leukocyte adhesion and CAM expressions add up to the possibility of a link between the two.

1.7 Comparison of Biological Model Systems

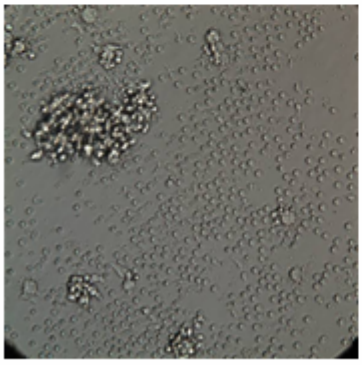
Studying the transient and complex conditions associated with biological systems often involves the use of complex experimental systems. Much work has been done to develop better *in vivo* (happening within the living body) and *in vitro* (outside the living body) systems to study such conditions. Figure 8 gives an overview of systems available for studying biological phenomena including the human models, animal models, two-dimensional (2D) cell culture models or three-dimensional (3D) tissue-engineered models.



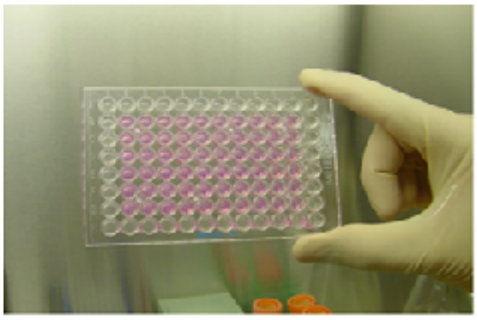
Human Models



Animal Models



2D Cell culture systems



3D Human Tissue-Engineered Constructs

Figure 8. Different Systems Available to Study Normal and Pathological Physiological Mechanisms.

1.7.1 Advantages and Disadvantages of the Model Systems

The human model is the best model to test various mechanisms, as it contains all the right "parts" and provides the exact microenvironment directed towards human physiology testing; however, they have some major drawbacks. They are very difficult to

control, owing to their complexity. Due to the presence of a multi-variable environment, it is difficult to study one variable or one system at a time, or isolate the contribution of one single variable to the overall effect. Human models are prone to high variability. This is acceptable when used as a predictor for total population analysis, but this is a problem when trying to study the effect of one single variable at a time, while keeping all others constant. The biggest drawback of the human model is that it cannot be used to study anything that will have potential health hazards to the model.

Animal models, like human models, offer complexity, but are better in the respect that the variability can be more easily controlled by using bred animals for research. Also, under the right government regulations, they can be used to study potential health hazards to the model. The problem with animal models is that they do not always translate to the human model, and need further validation. Animal models can also have the complexity associated with isolating the contribution of one single variable from the overall effect.

Two-dimensional cell culture models may not have the same level of complexity as the human and animal models, but they are widely used because of many advantages associated with them. They are relatively inexpensive and easy to create and maintain. It is also far easier to control the variables in cell culture models. Human cells, can be used in 20 cell culture models, which helps in attaining a very similar microenvironment like the one found *in vivo*. Due to this relatively less complexity, it is easier to look at the individual contribution(s) of any single variable, while keeping all other variables constant.

A 3D tissue engineered model takes the 2D systems from the bare minimum to the next level of complexity, while still maintaining the advantages of being inexpensive, easier to control, and easier to build. Also, the 3D models are suspected to behave closer to the *in vivo* conditions than the less complex 2D models. This behavior is often owing to the extra dimension involved, which influences signaling and certain other cellular functions. A 3D model thus appears to describe real system biological phenomena more closely than its 2D counterpart.

1.7.2 *In vivo* Versus *In vitro* Model Systems

The ideal approach for experimental science is to have control over all the variables in order to study the effect of individual variables or combination of variables on the system. When it comes to studying biological phenomena *in vivo*, such models can have some disadvantages associated with them. It becomes difficult to find homogenous group of samples *in vivo* (or subjects in clinical studies). Also, it becomes even more difficult to maintain the homogeneity of the samples as an experiment progresses, due to the lack of ability to modulate these *in vivo* systems. Thus to study the effect of any single factor is difficult, time consuming and more costly in the *in vivo* systems. As a remedy to this, the importance of *in vitro* systems is realized. *In vitro* systems can be difficult to design [25], but once designed are easy to be replicated. Also, it is easier to maintain homogenous conditions all throughout an experiment. Unlike an *in vivo* system like in an animal/human model, where more than one complex physiological, immunological and extra-cellular matrix interactions interfere with each

other's effects, *in vitro* systems can be used to look at the change in one specific parameter.[77]

Also, cost becomes an important factor when comparing *in vivo* with *in vitro* models. Prescription drugs are expensive by design, as they cost a lot to develop but are relatively cheaper to make.[78] According to the pharmaceutical industry's trade group, Pharmaceutical Research and Manufacturers of America (PhRMA), developing a single new drug and bringing it to market takes on average 12-15 years and costs $500 million.[79] Figure 9 represents the main areas and their associated costs involved with bringing a drug to the market. If drug testing had fewer numbers of trials and quicker approvals, they would have less expenses involved, with getting a drug to the market. The two main areas that a better testing model can positively impact are the basic pre-clinical research used to identify candidates and efficacy trials. In-vitro tests are necessary to help determine initial dose-response data, as well as to evaluate potential susceptibility and/or resistance of specific pathogens.[80] The employment of appropriate *in vivo* animal testing is vital prior to clinical evaluations. Due to this reason *in vitro* tests are conducted prior to running *in vivo* tests. Along with helping to reduce costs, the development of better *in vitro* models can also possibly reduce the amount of testing that would need to be performed on animals and humans.

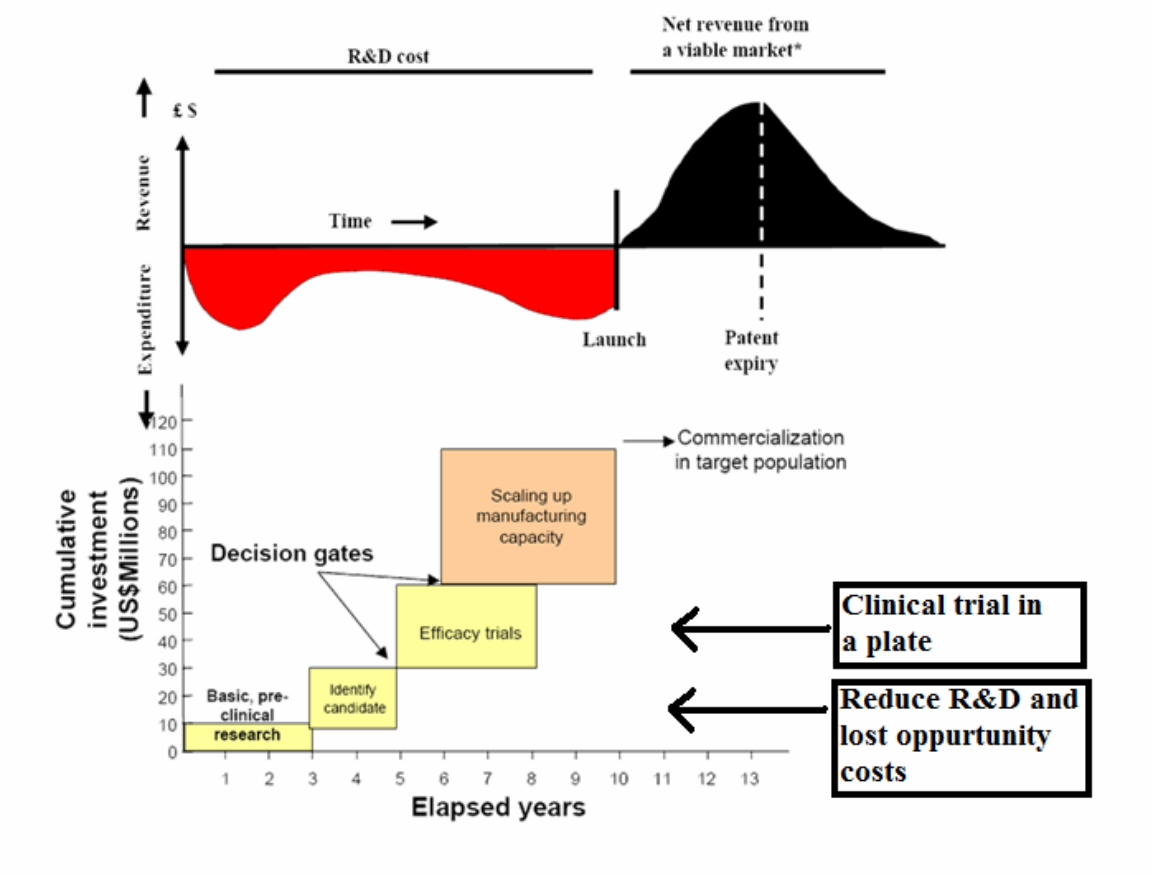


Figure 9. Steps and costs associated with bringing a new drug to market. The advantages of using an *in vitro* model are highlighted at the decision gates.

1.7.3 Two-Dimensional Versus Three-Dimensional Model Systems

Tissue culture and cell biology research frequently depends on being able to create *in vitro* environments that can mimic the *in vivo* conditions. Using artificial substrates such as plastic or glass are likely to distort findings by forcing cells to adjust to artificially flat and rigid surfaces.[81] The current understanding of cell-matrix interactions *in vitro* is primarily based on planar 2D tissue culture substrates. Relatively, little is known about cell-matrix adhesive structures formed in 3D matrices of living tissues. *In vivo* cells attach to surrounding 3D mesh-like fibers rather than to 2D coated

surfaces. In 2D coated cell culture surfaces, this attaching and further spreading is hindered or morphed, due to changes in the biophysical properties like a hard surface beneath. Due to these different biophysical properties involved, signaling and certain other cellular functions have been shown to differ in 3D compared to 2D planar systems.[82] The differentiation of monocytes was studied on 3D collagen gels and was found to have a rapid differentiation rate (1.5 times faster) than compared to 2D collagen-coated plastic layer.[83] Importance of a 3D ECM is already recognized for certain types of cells, like epithelial cells, where 3D environments promote normal epithelial polarity and differentiation.[84] Another example is culturing fibroblast cells on flat substrates induces an artificial polarity between the lower and upper surfaces of these otherwise nonpolar cells. The morphology and migration of these cells differs from 2D planar surfaces than when suspended in 3D collagen gels.[81, 85] Cells also have been shown to have an enhanced migration rate (~1.5 times) in 3D models than 2D planar protein coated surfaces.[86] The time taken by cells to reach their characteristic *in vivo* morphology is considerably lesser in 3D collagen gels.[86] Thus, it is quite certain that a 3D model would be able to describe biological phenomena more closely as a real system than its 2D counterpart.

1.8 Present Studies on Atherosclerosis and Hyperglycemia in Different Systems

1.8.1 *In vivo* Models

Studies done on an *in vivo* rat model revealed that leukocyte rolling and migration through the endothelial cells was increased when subjected to hyperglycemia.[40] Also, an associated increase in the expression of certain CAMs on EC was observed after exposure to hyperglycemia in this *in vivo* rat model, thereby reinforcing the possible relation between an increased leukocyte adhesion and enhanced CAM expressions.[40] Clinical observations and epidemiological studies clearly indicate that atherosclerosis is accelerated in diabetic patients.[87] Since, diabetes is characterized by hyperglycemia, a possible link between atherosclerosis and hyperglycemia has been sought. Studies on human artery segments from diabetic and non-diabetic patients have been used to identify any difference in CAM expressions of ICAM-1,VCAM-1 and E-selectin.[72] These studies have clearly shown an enhanced VCAM-1 expression on endothelial cells. Other studies have shown an enhanced expression of ICAM-1 and VCAM-1 on atherosclerotic lesions found in aortas.[72]

1.8.2 Two-Dimensional Solid Surface Models

In vitro studies using 2D models imitating a blood endothelium are common as well.[88] Common *in vitro* models employed to study the blood endothelium includes culture of endothelial cells on flat plastic surfaces.[25] Endothelial cells were selected from different sources within the body, like blood brain barrier [89], umbilical cord [68, 90], and valves [91] depending on the specific application of the model. The cells are cultured on plastics coated with thin layers of common ECM proteins like laminin,

fibronectin, or collagen.[83, 89] A 2D ligand coated surface would involve different cell interactions, associated signaling, and gene expression regulating differentiation into different phenotypes than a complex 3D architecture.[92] Also, such 2D studies only investigate rolling (for a flow system) or adhesion of monocytes on endothelial cells, and do study the transendothelial migration of monocytes.

1.8.3 Two-Dimensional Culture Inserts

In an attempt to study transendothelial migration of monocytes through the endothelium, models were created by using tissue culture inserts in multiwell plates.[93] These are also called within Transwell® chambers. Figure 10 shows an example of tissue culture inserts used within multiwell plates (or a Transwell® chamber). These models have two chambers separated with a porous membrane. A cell suspension is put in the upper chamber and migration across the membrane to the lower membrane is studied. The membrane may or may not have a confluent cell layer on its surface. For the case of monocyte migration across an endothelium, endothelial cells are grown to confluency on the membrane and monocytes are added to the top chamber to investigate migration across the endothelium. A small sample can be drawn out from the lower chamber at different time points, and the number of these cells can be counted. These inserts have polyethylene terephthalate (PET) membranes, which give the membranes the advantage of being mechanically durable and curl free. Other advantages associated with these types of models are that owing to their transparent/translucent nature, they can be used directly for light microscopy, electron microscopy and immunofluorescence.

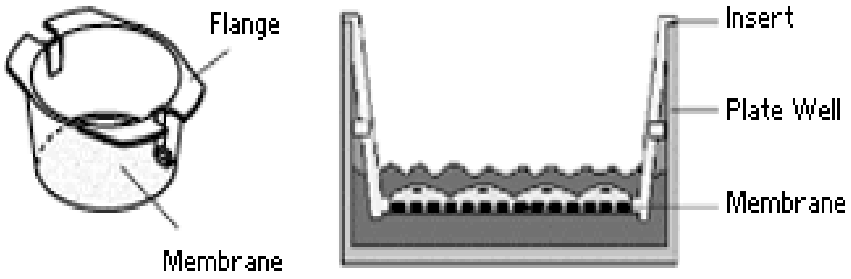


Figure 10. Multiwell plates with tissue culture inserts are commonly used to study cell migration.[94]

Missing in these models are the interactions between cells and the extra-cellular matrix (ECM). Mechanical properties of the ECM are critical in regulating cellular signaling.[95] Integrins, which are known to bind endothelial cells to the subendothelial matrix through the cytoskeletons [14], have an effect on signal transduction (a process by which a cell transforms one kind of signal or stimulus into another). Artificial substrates such as plastic or glass distort findings by forcing cells to adjust artificially to flat and rigid surfaces.[81] Another drawback of using the tissue culture inserts was that once the cells migrated across the endothelium, they were committed to the bottom of the well plate and did not have the ability to freely migrate within the subendothelium intima. The ability to study diseases like atherosclerosis, by including potential atherosclerosis promoting lipid in media, was not possible due to the absence of an ECM to make up a subendothelium intima in the model.

1.9 Development of a Better Model System: The Three-Dimensional Tissue Model

Unfortunately, the 2D models cannot capture the trans-endothelial migration at any given time point, and monocyte differentiation cannot be investigated. Monocytes differentiate into either macrophages or dendritic cells depending on a number of known and unknown, and possibly interrelated factors. Also, the possibility of studying hyperlipidemia, and thereby expecting plaque formation, is also not feasible in 2D models. A need exists for developing 3D tissue models that can closely mimic the physiology and transient conditions associated with blood endothelium and leukocyte trafficking and differentiation. Such a model would be a significant research tool in studying different conditions associated with inflammation and their possible roles towards atherosclerosis and other complications. Ultimately, the successful development of a 3D *in vitro* tissue model will provide a better testing model of new targets for preventive and therapeutic interventions of atherosclerosis.

PROJECT OBJECTIVES:

1. Develop a 3D model that can mimic the physiology of cells following a hyperglycemic episode.
2. Analyze the changes in the blood endothelium following a hyperglycemic episode.
3. Determine the effect of hyperglycemia on leukocyte migration across the endothelium.
4. Determine the effect of hyperglycemia on monocyte differentiation into either dendritic cells or macrophages.

CHAPTER II

MATERIALS AND METHODS

2.1 Reagents

Bovine type I collagen was purchased from INAMED Biomaterials (Fremont, CA); collagenase was purchased from Roche (Basel, Switzerland); Ficoll-Paque^{TM} PLUS was purchased from GE Healthcare (Uppsala, Sweden); fetal bovine serum (FBS) and trypan blue solution were purchased from Hyclone (Logan, UT); bovine serum albumin (BSA), D-mannitol, and glucose assay reagent were purchased from Sigma-Aldrich (St. Louis, MO); D-glucose (dextrose), methanol and triton X-100 were purchased from Fisher Scientific (Pittsburgh, PA); human-fibronectin was purchased from Biomedical Technologies Inc. (Stoughton, MA); sodium azide was purchased from Acros Organics (Geel, Belgium); CellTracker^{TM} Green, dulbecco's phosphate buffered solution (DPBS), ethidium homodimer-1, media 199 (1X & 10X), penicillin-streptomycin-glutamine (PSG) solution and trypsin-EDTA solution were purchased from Invitrogen Corporation (Carlsbad, CA); mouse anti-human fluorochrome-conjugated antibodies CD3, CD19, CD14, CD56, CD83, CD206 (macrophage mannose receptor), CD208 (DC-LAMP), CD31 (PECAM-1), CD106 (VCAM-1), PgP (MDR), CD64 and appropriate isotype controls, were purchased from BD Biosciences (San Jose, CA); mouse anti-human fluorochrome conjugated CD14 and the appropriate isotype control were purchased from

BioLegend (San Diego, CA); tumor necrosis factor-α (TNF-α), mouse anti-human fluorochrome conjugated CD 54(ICAM-1), CD 62E(E-selectin) and the appropriate isotype control were purchased from R&D Systems (Minneapolis, MN).

2.2 Cell Culture

Pooled human umbilical vein endothelial cells (HUVECs) were purchased from PromoCell (Heidelberg, Germany). HUVECs were cultured in Medium 199 containing 20% FBS and 1% PSG. Culturing was carried out at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air (henceforth referred to as standard conditions). For all experiments, the cells were consistently used within five population doublings. The typical time required to grow the HUVECs to a number that can be used to create the tissue model was approximately seven days.

Human peripheral blood mononuclear cells (PBMCs) were isolated from the blood obtained from healthy donors from the Oklahoma Blood Institute (OBI) (Oklahoma City, OK) by using a Ficoll density gradient separation method. Autologous plasma was also obtained from OBI and used as the serum supplement for all experiments involving PBMCs. The glucose concentration of all plasma samples were measured to ensure normoglycemia levels.

2.3 Determination of Glucose Concentration in the Plasma Samples of Blood Donors

To measure the glucose concentration in the plasma samples, a standard enzymatic assay using glucose assay reagent was used. Glucose is phosphorylated by adenosine triphosphate in the reaction catalyzed by hexokinase. Glucose-6-phosphate is

then oxidized to 6-phosphogluconatein the presence of oxidized nicotinamide adenine dinucleotide (NAD) in a reaction catalyzed by glucose-6-phosphate dehydrogenase. During this oxidation, an equimolar amount of NAD is reduced to NADH. The consequent increase in absorbance at 340 nm is directly proportional to glucose concentration. Standard glucose solutions over a wide range (0-10 mM) were made with distilled water. One part of the standard solution is mixed with five parts of the glucose assay reagent in cuvets. These cuvets were incubated for 15 minutes at room temperature. These standards were then transferred to a 96-well plate and the optical density of each sample at a wavelength of 340 nm was measured. A standard plot showing optical density as a function of glucose concentration was prepared and showed a linear correlation. This standard plot was used to determine the glucose concentration in unknown plasma samples. Same mixing and incubation procedure were followed with the samples, followed by measuring the optical density at 340 nm. This optical density was compared with the standard glucose plot and the unknown glucose concentrations in samples were determined.

2.4 Development of 3D Tissue Model

To prepare the 3D collagen matrix, a collagen solution containing 57.1 vol% type 1 bovine collagen (3 mg/ml), 7.14 vol% 10x Medium 199, and 35.7 vol% 0.1 M sodium hydroxide was added to designated wells of a 48-well plate that would result in an approximately 500 μm thick matrix measured by microscopy analysis. The cushions were incubated at standard conditions for 60 minutes for the collagen to gel. The collagen gels were seeded with HUVECs at a density of 100,000 cells/cm^2, and the cells

were cultured at standard conditions with the same media system described previously. The 3D human tissue models were used for testing at one day after the cells became completely confluent on the collagen matrix (by microscopic examination). The typical time required for the cells to become confluent for the tissue model to be ready for testing was approximately seven days. Figure 11 shows the development of the 3D tissue model.

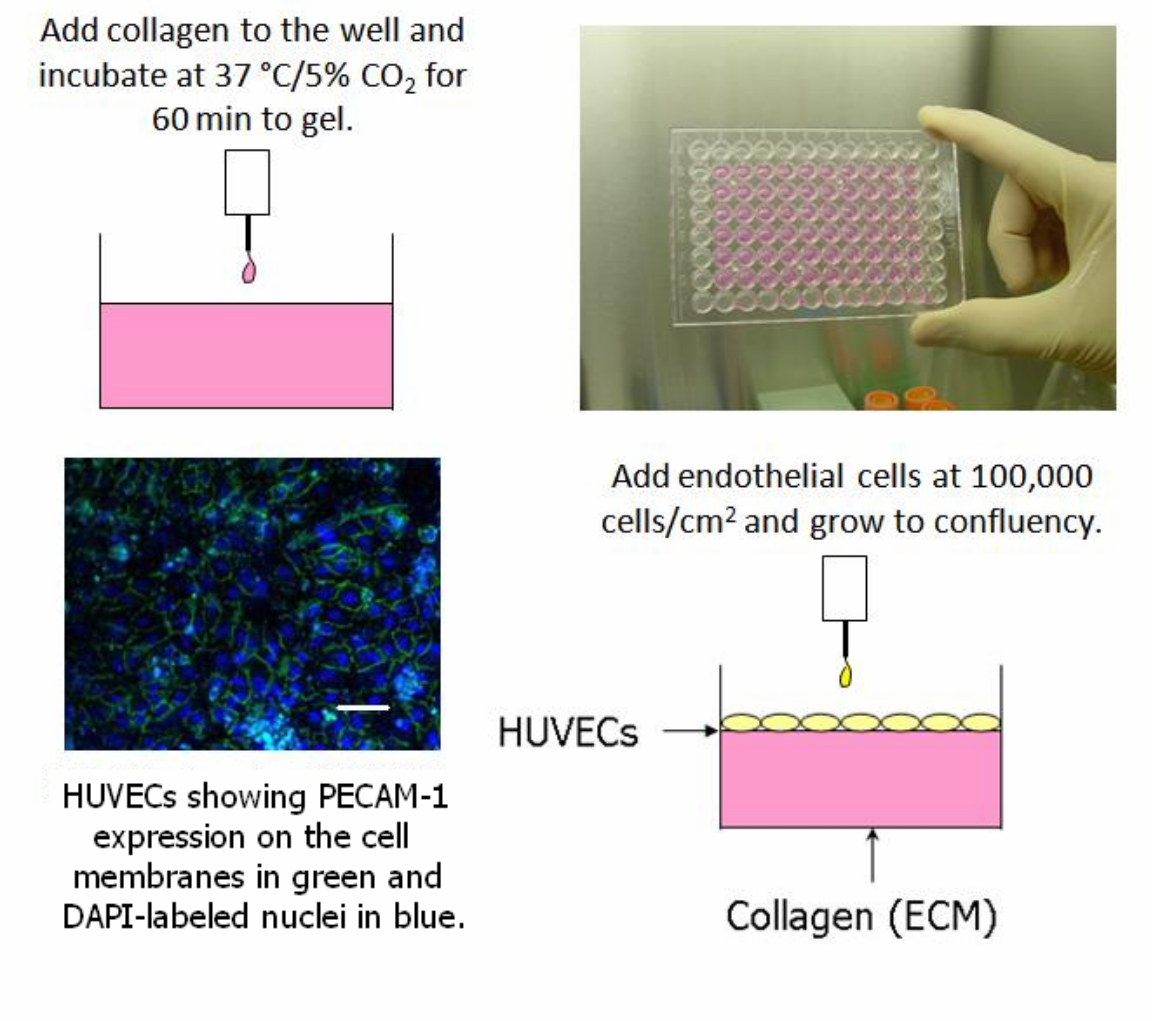


Figure 11. Development of the 3D tissue model in a 96-well plate. Scale bar: 15 µm.

2.5 Determination of the Optimal Time to Incubate the 3D Tissue Model with Glucose

The effect of hyperglycemic conditions on the endothelial cells is a transient process. For example, hyperglycemic conditions can be toxic to the cells, but depends on both glucose concentration and cell exposure time to the hyperglycemic conditions. The expression and/or regulation of various CAMs can also depend on both concentration and time. For example, a threshold glucose concentration may be needed to elicit CAM expression, but this also takes a certain amount of time for the cells to sense the stimuli and then respond, by CAM expression. Since the goal of this project was to only investigate the effect of glucose concentration, a study was performed to find the optimal cell exposure time to glucose to elicit a detectable response from the high glucose concentrations. The 3D human tissue model was developed using normal culture media (5.6 mM media M199) as described above. One day post confluency, the endothelial cells were incubated with varying glucose concentrations (5.6 mM, 15 mM, 30 mM) for 3, 9 and 24 hours. The cells were rinsed three times with DPBS to remove any accumulated cell debris and cell aggregates that could interfere with leukocyte migration. An equal number of PBMCs, 1.56 x 10^6 per cm^2, in media containing Medium 199 with 20% autologous plasma and 1% PSG were added to each sample and incubated at standard conditions for two hours to allow for initial cell migration into the tissue model. After two hours, the samples were rinsed three times with DPBS, in order to remove any cells that did not migrate across the endothelium and into the subendothelial space of the tissue model. The samples were cultured in the media containing the autologous plasma at standard conditions for 48 hours, to allow for further cell migration and differentiation.

The time points chosen for leukocyte migration and differentiation were based on previous results from a similar model used to study monocyte migration and differentiation under normal conditions [90].

The following three cell populations were studied for this model: 1) the cells that initially migrated into the subendothelium (SE) space of the tissue model after the two-hour incubation period, called the "SE-2hr" cells, 2) the cells that remained within the SE space after the 48-hour incubation period, called the "SE-48hr" cells, and 3) the cells that reverse-transmigrated (RT) back across the endothelial layer to the top of the tissue model after the 48-hour incubation period, called the "RT" cells. The individual cell populations were collected for analyses. To collect the RT cells, after the 48-hour incubation time, the cells from the top of the HUVEC layer were collected by using a micropipettor to gently transfer the cells within several washes with DPBS to collection tubes. To obtain the SE cells, the tissue models made up of collagen were digested using collagenase, and the cell suspension filtered through a 40 μm cell strainer to remove the larger HUVECs. Individual migration numbers within the three cell populations were counted. Viability of the cells was also checked by using trypan blue.

2.6 Incubation of 3D Tissue Model with Glucose

Prior to any testing, the endothelium was rinsed gently with PBS to remove any debris from the surface that can interfere with normal cell function. Media containing varying glucose concentrations [5.6, 15, 30 mM (and in some cases 45 mM)] were put on designated wells and incubated for nine hours. The effect of hyperosmolarity associated with a high glucose concentration was also investigated by using a hyperosmolar control,

mannitol. The 5.6 mM concentration is the glucose concentration in the standard Medium 199 culture media (normoglycemia) and serves as the negative control group for the experiments. The cell incubation time with glucose for all experiments was determined from the results of prior experiments and from other studies that show endothelial cells respond to cytokines such as IL-1 and TNF-α, by an increase in the expression of VCAM-1 with maximal activity reached by 6-12 hours.[96] Therefore, since it is hypothesized that high glucose concentrations will have a similar effect on HUVECs, a similar cell incubation time was chosen. After the nine-hour incubation time, the media containing the varying glucose concentrations were removed from the wells and standard culture media was used for any further analyses or tests of the samples.

2.7 HUVEC Viability Assay

Viability of the HUVECs was assessed after the nine-hour incubation period of the 3D tissue model with varying glucose concentrations. A sample of cells exposed to 1% Triton X-100 in the culture medium for the same nine-hour incubation period was used as a positive control for dead cells. At this concentration of Triton X-100, the cell membrane is permeabilized, resulting in cell death. Viability of the cells was determined by staining with CellTracker™ Green (CMFDA) and ethidium homodimer-1. Cell-permeant esterase substrates, such as CellTracker™ Green, serve as viability probes that measure both cell-membrane integrity, which is required for intracellular retention of the probe and enzymatic activity, which is required to activate the fluorescence of the probe. Viable cells were identified by a green fluorescent cytoplasm. Cell impermeant nucleic

acid stains, such as ethidium homodimer-1, were used to detect the dead-cell population. This probe was able to penetrate dead cells with compromised membranes, resulting in dead cells with red fluorescent nuclei. The fluorescently stained cells were observed by a fluorescent microscope.

2.8 Analysis of the Surface Expression of PECAM-1 and VCAM-1 on HUVECs by Confocal Microscopy

HUVECs were grown on thin collagen gels within wells in chamber slides purchased from Nunc Lab-Tek (Rochester, NY), shown in Figure 12a. The cells were seeded at the same density and grown under the same conditions as those described for the development of the 3D human tissue model. At one day post-confluency of the HUVECs, specific wells were incubated with media containing varying glucose concentrations (5.6, 15, and 30 mM) for nine hours. Some wells were also incubated for the same time period with TNF-α (100 U/ml) to serve as a positive control for VCAM-1 expression. TNF-α is known to activate HUVECs and cause an increase in the expression of certain cell adhesion molecules, such as VCAM-1. After the nine-hour incubation period, fluorochrome-conjugated antibodies were used to identify PECAM-1 and VCAM-1 cell-surface expression. Briefly, the samples were washed with DPBS and fixed in methanol at 4 °C for 10 minutes. The samples were washed with DPBS and incubated with a blocking buffer of DPBS containing 1% BSA at room temperature for 30 minutes. Appropriate dilutions of the primary antibodies and controls were added to the samples and incubated at 37 °C at standard conditions for one hour, after which the samples were washed three times with DPBS. The chamber walls were removed from

the slides containing the samples and a cover slip attached, as shown in figure 12. The samples were analyzed by confocal microscopy.

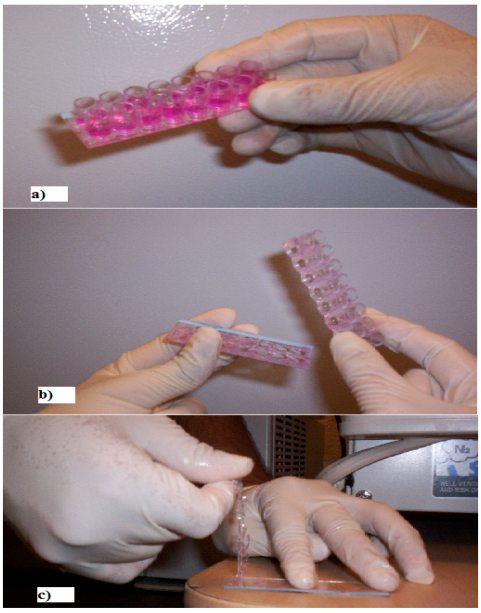


Figure 12. Lab picture showing: a) the 3D tissue models in the chamber slide, b) removing the side wells from the plate, c) removing the gasket from the slide. The samples are covered with a coverslip and ready for analysis by confocal microscopy.

2.9 Analysis of the Surface Expression of PECAM-1, VCAM-1, ICAM-1 and E-Selectin on HUVECs by Flow Cytometry

One day post-confluency of the HUVECs in the 3D tissue models, specific wells were incubated with media containing varying glucose concentrations (5.6, 15, 30, 45 mM) for nine hours. Some wells were also incubated for the same time period with TNF-α (100 U/ml) to serve as a positive control, due to TNF-α has been shown to activate HUVECs and cause an increase in the expression of certain cell adhesion molecules (CAMs). Also, to check if hyperosmolarity plays a significant role in regulating CAM expression, a 45 mM mannitol (5.6 mM Glucose + 39.4 mM Mannitol) control group was

also included. After the nine-hour incubation period, the media were removed from the samples and the collagen gels were digested with collagenase. The digested collagen and HUVECs were collected and centrifuged to isolate the cells from the collagen solution. Cells were washed in blocking buffer (1% BSA in PBS) for 10-15 minutes at 37^0C. A typical fluorochrome-conjugated antibody panel was selected to identify each type of CAM expression on the surface of the HUVECs: Cluster of differentiation 31 (CD 31) for PECAM-1 expression, CD 106 for VCAM-1 expression, CD 54 for ICAM-1 expression, and CD 62E for E-selectin expression. For immunostaining, the cells in each population were collected and rinsed with the staining buffer of DPBS containing 2% FBS and 0.09% sodium azide. Next, the cells were incubated with various antibody combinations along with the proper isotype controls for 45 minutes at 4 °C. The cells were washed with the staining buffer and analyzed by flow cytometry using FACSCalibur within 24 hours of staining. By flow cytometry analysis, the fluorescence intensity for each CAM corresponding to each glucose concentration was measured. The fluorescence intensity of each sample was normalized based on the isotype controls fluorescence intensity, and the percentage changes in fluorescence intensity corresponding to increasing glucose concentrations was determined.

2.10 Analysis of Leukocyte Migration and Monocyte Differentiation within the 3D Tissue Model

To study the effect of high glucose concentration on leukocyte migration and monocyte migration, samples were incubated with varying concentrations of glucose (5.6, 15, 30 and 45) as described in section 2.6. Control samples for the experiment include the

control group for hyperosmolarity (45 mM Mannitol) and endothelial activation (100 U/ml TNF- α): samples were rinsed three times with DPBS in order to remove any cell aggregates from the cell surface that could interfere with leukocyte migration and to remove FBS from the samples that could possibly interfere with monocyte differentiation. Figure 13 shows the next steps in the leukocyte migration and differentiation model. An equal number of PBMCs, 1.56 x 10^6 per cm^2, in media containing Medium 199 with 20% autologous plasma and 1% PSG were added to each sample and incubated at standard conditions for two hours in order to allow for initial cell migration into the tissue model. After two hours, the samples were rinsed three times with DPBS in order to remove any non migratory cells remaining on top of the endothelial cell layer of the tissue model. The samples were cultured in media containing autologous plasma at standard conditions for 48 hours, to allow for cell migration and differentiation.

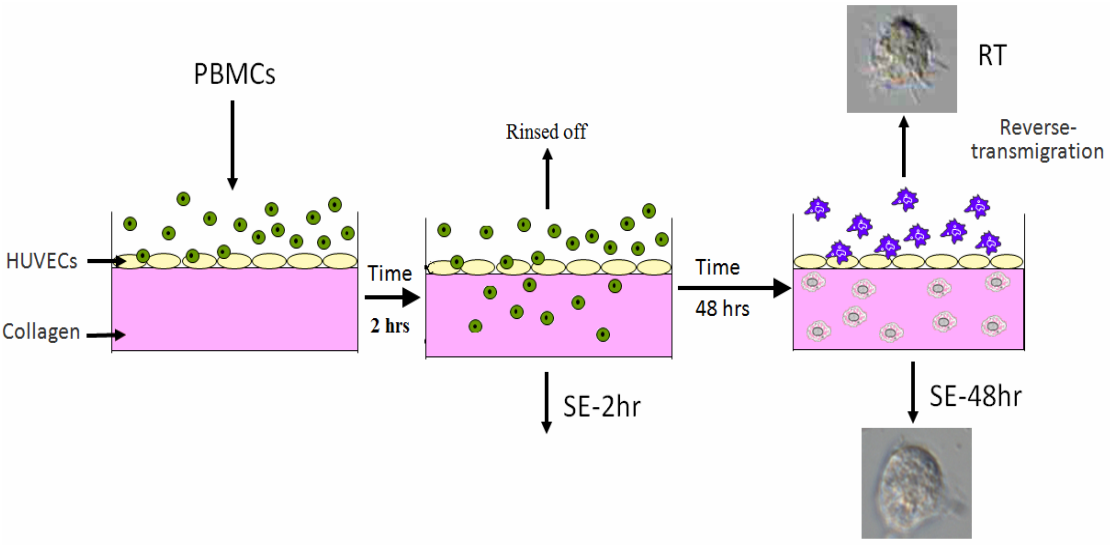


Figure 13. Leukocyte Migration and Differentiation within the 3D Tissue Model

The three cell populations that were studied for this model include the following: 1) the SE-2hr cells, 2) the SE-48hr cells, and 3) the RT cells, as described previously. The

individual cell populations were collected for analyses. Cell viability and numbers were determined for each group by using trypan blue exclusion and counting with a haemocytometer.

The phenotype of the cells for the three cell populations collected was determined by immunostaining and flow cytometry. A typical antibody panel was selected to identify the cell types within the PBMC population, including CD3 for T-cells, CD19 for B-cells, CD14 for monocytes, and CD56 for natural killer cells. To detect macrophages or dendritic cells and their possible maturation states in the cell populations, the following antibodies were used: CD64, CD83, CD206, and CD208. To determine the potential role of a cell surface molecule in reverse transmigration, the transmembrane protein MDR-1 was studied. Studies have shown that this transmembrane protein is important for reverse transmigration of the cells.[90] For immunostaining, the cells in each population were collected and rinsed with the staining buffer of DPBS containing 2% FBS and 0.09% sodium azide. Next, the cells were incubated with various antibody combinations, along with the proper isotype controls for 45 minutes at 4 °C. The cells were washed with the staining buffer and stored at 4 °C until ready for analysis by flow cytometry within 24 hours of staining.

2.11 Statistical Analysis

Statistical analyses were performed by use of analysis of variance (ANOVA) followed by a student's t-test. A value of $P < 0.1$ was considered significant. For the flow cytometry data, samples were normalized based on the 5.6 mM glucose concentration control group, and the percent increases determined.

CHAPTER III

RESULTS AND DISCUSSION

3.1 Determination of the Optimal Time to Incubate the 3D Tissue Model with Glucose

A time to incubate the 3D tissue model with glucose was to be determined, that could elicit a detectable response from the high glucose concentrations. In order to determine the optimal incubation time for the 3D tissue model with glucose, three different incubation times (3, 9 and 24 hours) were chosen and the corresponding number of leukocytes migrating within the 3D tissue model was determined.

Figures 14 and 15 show the total number of cells migrating based on the three incubation times for SE-2 hr and RT cell types, respectively. The number of cells migrating for the SE-48 hr population was also determined, but showed no significant difference among the times tested (data not shown). Multiple cell counts were performed and an average standard deviation of 10% was determined. The number of cells migrating in the SE-2 hr population was significantly greater for the nine-hour incubation time, over the three and 24 hour. A similar effect was seen for RT cells with a significant increase in the number of cells migrating for the nine-hour incubation time point. Incubation of the 3D tissue model for 3, 9 and 24 hours gave significantly different overall cell migration numbers, confirming that glucose incubation time has a role in

leukocyte trafficking across the endothelium. It is important to note that these numbers show total cell migration and are not a measure of individual cell types. Cell classification of the total cell migration population was determined and will be discussed in the next sections. Based on these results, it was decided to conduct all future experiments with an incubation time of nine hours.

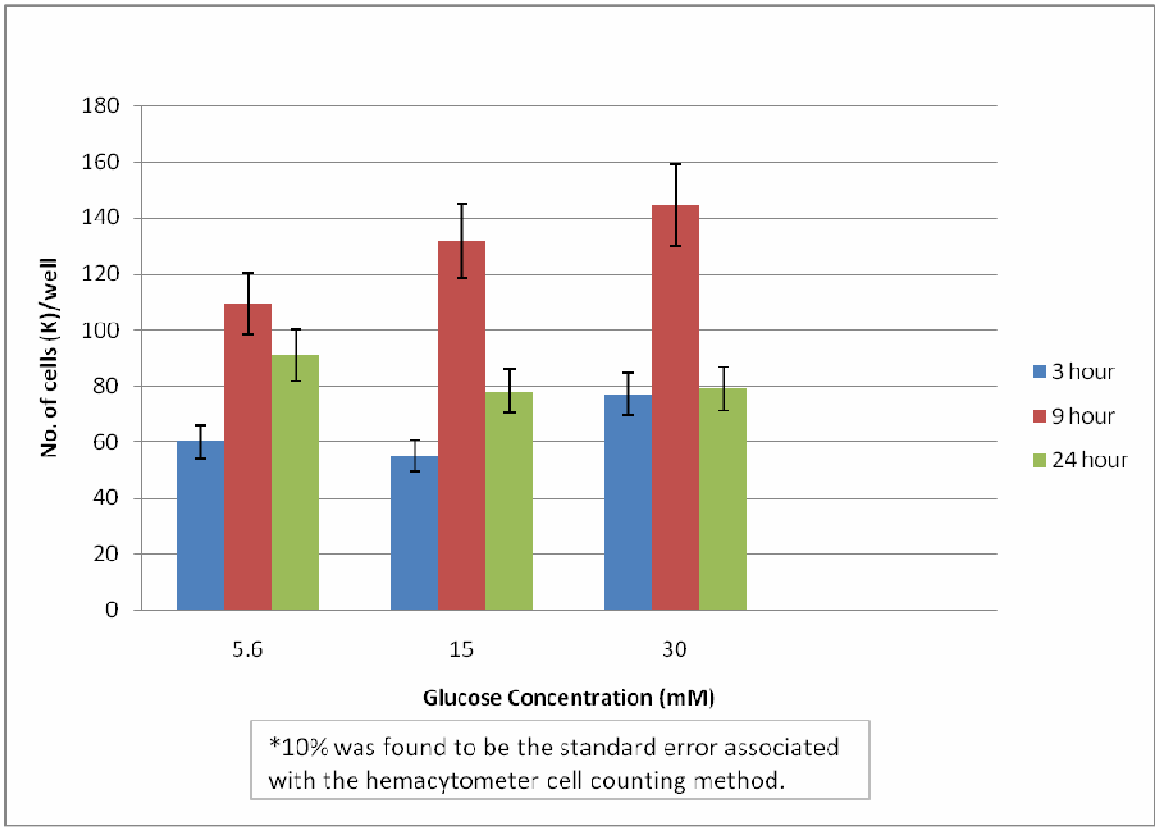


Figure 14. Effect of incubation time of the 3D tissue model with glucose on SE-2hr total cell migration numbers. (Shown are average values with s.d. from n=3).

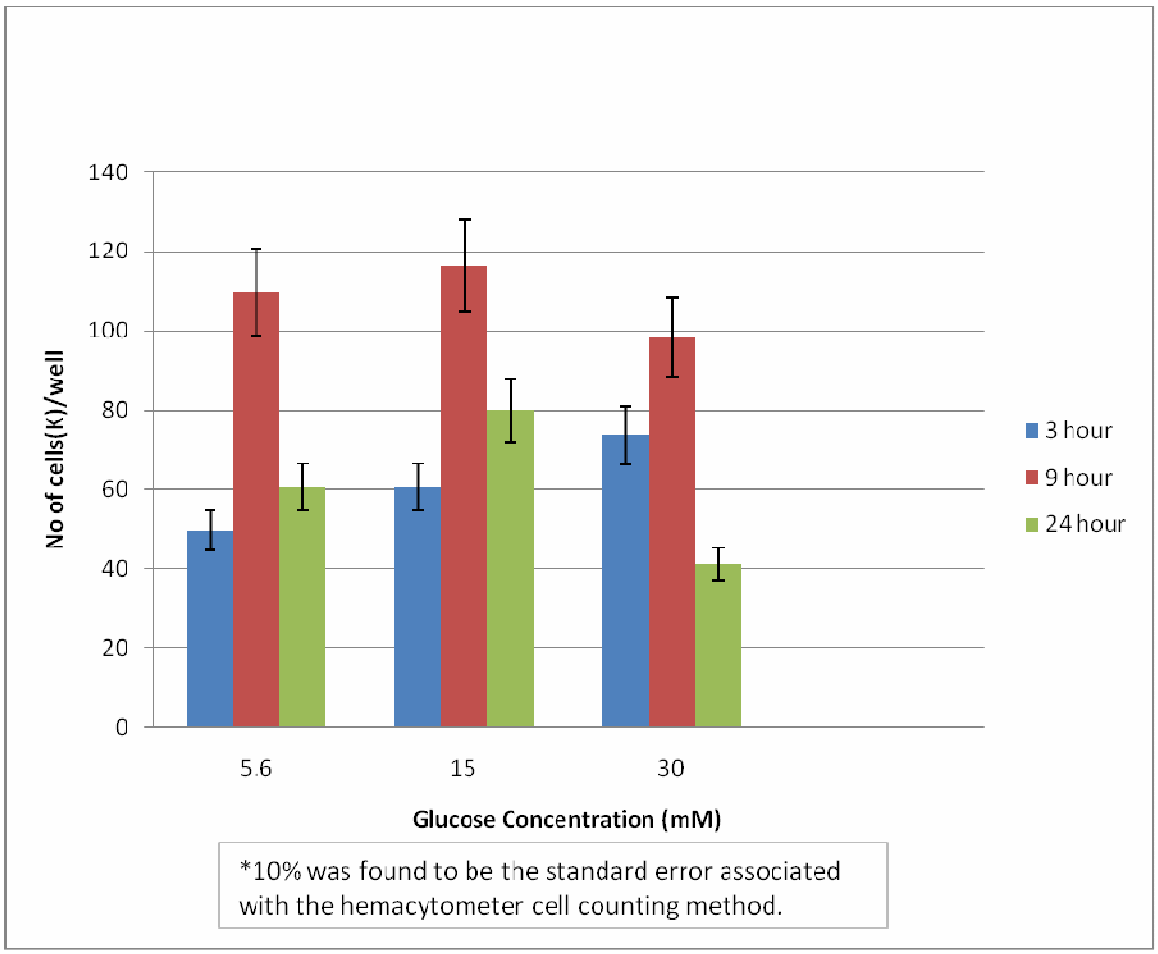


Figure 15. Effect of incubation time of the 3D tissue model with glucose on RT total cell migration numbers. (Shown are average values with s.d. from n=3).

3.2 Determination of Glucose Concentration in the Plasma Samples of Blood Donors

The blood samples used to obtain the PBMCs and plasma for the experiments were checked for certain levels of factors that may interfere with the study of cell migration and differentiation in response to hyperglycemia, such as plasma glucose and cholesterol levels. It was important to make sure that the leukocytes isolated from the blood samples were not facing any abnormal condition before testing. Cholesterol limits were checked in the clinical reports that accompanied the blood samples. They were all

found to be within the normal cholesterol level i.e. within 200 mg/dl (data not shown). A glucose assay was used to determine the plasma glucose concentration of each blood sample. All the blood samples were found to have glucose levels either less than or within the normal glucose range of 4 mM to 7 mM i.e. none were hyperglycemic. Thus, the leukocytes were attained from normal physiological condition prior to facing experimental conditions.

3.3 HUVEC Viability Assay

Viability was determined as a measure of fluorescence associated with the fluorophores CellTracker^{TM} Green and ethidium homodimer-1. CellTracker^{TM} Green is a stain for live cells, giving it a bright green color, and ethidium homodimer-1 stains the nucleus of the dead cells red. Figure 16 shows the fluorescent images of the samples that were used to determine HUVEC viability after exposure to varying glucose concentrations for the nine-hour incubation time. The images were taken at 100X magnification. Fluorescent intensity of the samples was measured using imaging software called ImageJ. There was no significant difference in fluorescence intensity between the negative control sample containing 5.6 mM glucose and the samples with the elevated glucose concentrations of 15 and 30 mM for the nine-hour incubation time. There was however, a significant difference among the positive control for this analysis, the Triton X-100 group, and the other groups. Thus, for the incubation period of nine hours, it was concluded that cell viability is not affected at the hyperglycemic conditions tested.

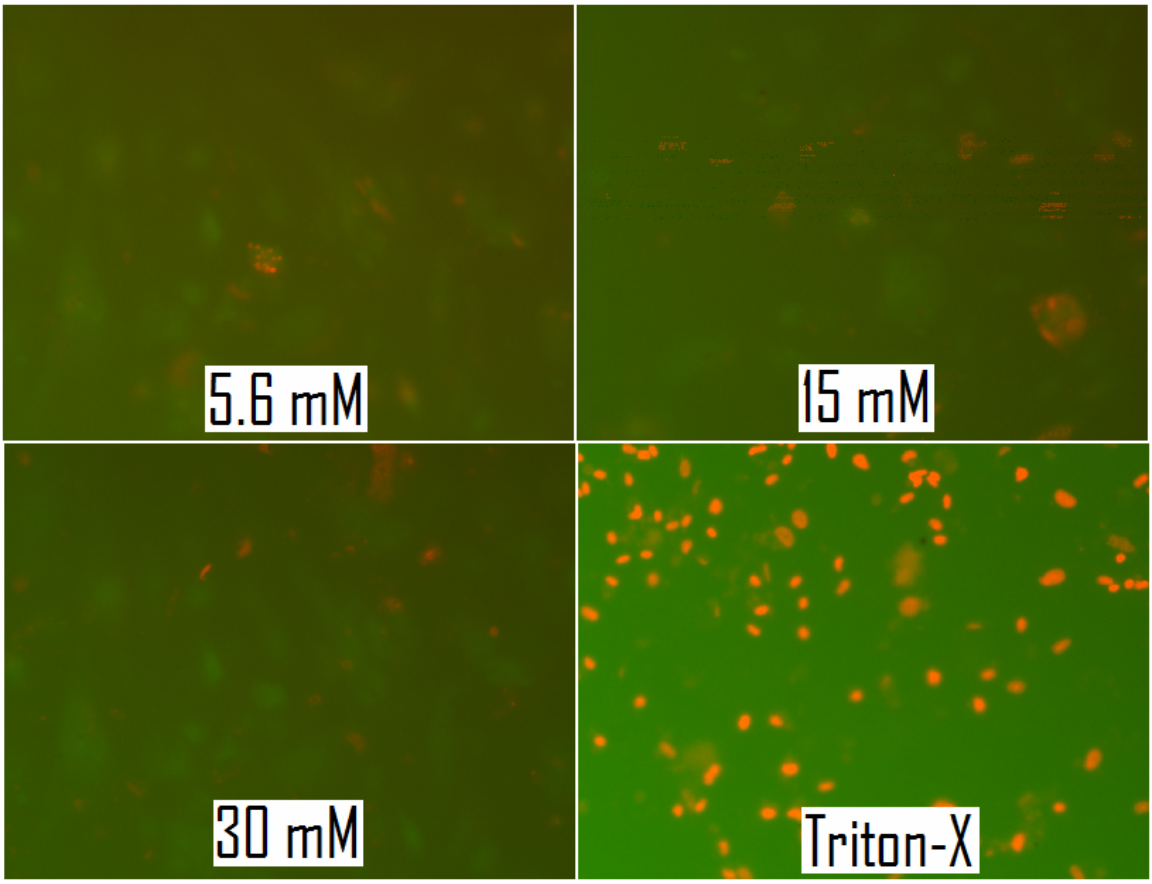


Figure 16. Effect of high glucose on HUVEC cell viability.

3.4 Analysis of the Surface Expression of PECAM-1 and VCAM-1 on HUVECs by Confocal Microscopy

Many *in vitro* and *in vivo* studies have shown the surface expression of the cell adhesion molecules PECAM-1 [47, 74], and VCAM-1 [74] on HUVECs in the event of inflammation. Since hyperglycemia is thought to be an inflammatory mediator, CAM expressions were investigated at hyperglycemic conditions. The 3D tissue model samples in the chamber slides that were exposed to different glucose concentrations were stained with PECAM-1 and VCAM-1 antibodies. The expression of these CAMs was

analyzed by using confocal microscopy, to provide a qualitative analysis of the change in CAM expression.

On visual inspection using confocal microscopy, it was found that there was no significant difference in PECAM-1 expression for any of the groups that were incubated with varying glucose concentrations (data not shown). All the groups showed expression of PECAM-1 along the cell-cell boundaries, thereby confirming the role of PECAM-1 in leukocyte trafficking within the 3D tissue model. This suggests that even though PECAM-1 is important in leukocyte trafficking, it is not affected by an increase in glucose concentration for this incubation period.

Figure 17 shows the effect of varying glucose concentrations on VCAM-1 expression for the HUVECs. There is no change in VCAM-1 expression for the samples exposed to a 15 mM glucose concentration compared to the negative control group that was exposed to a 5.6 mM glucose concentration. However, an increase was observed in VCAM-1 expression for the samples exposed to 30 mM glucose concentration compared to the negative control group. Thus, this analysis shows that after attaining a threshold value (approximately 30 mM), VCAM-1 is expressed on the endothelial cells. The positive control TNF-α also showed an increase in VCAM-1 expression, even greater than that for the 30 mM glucose samples.

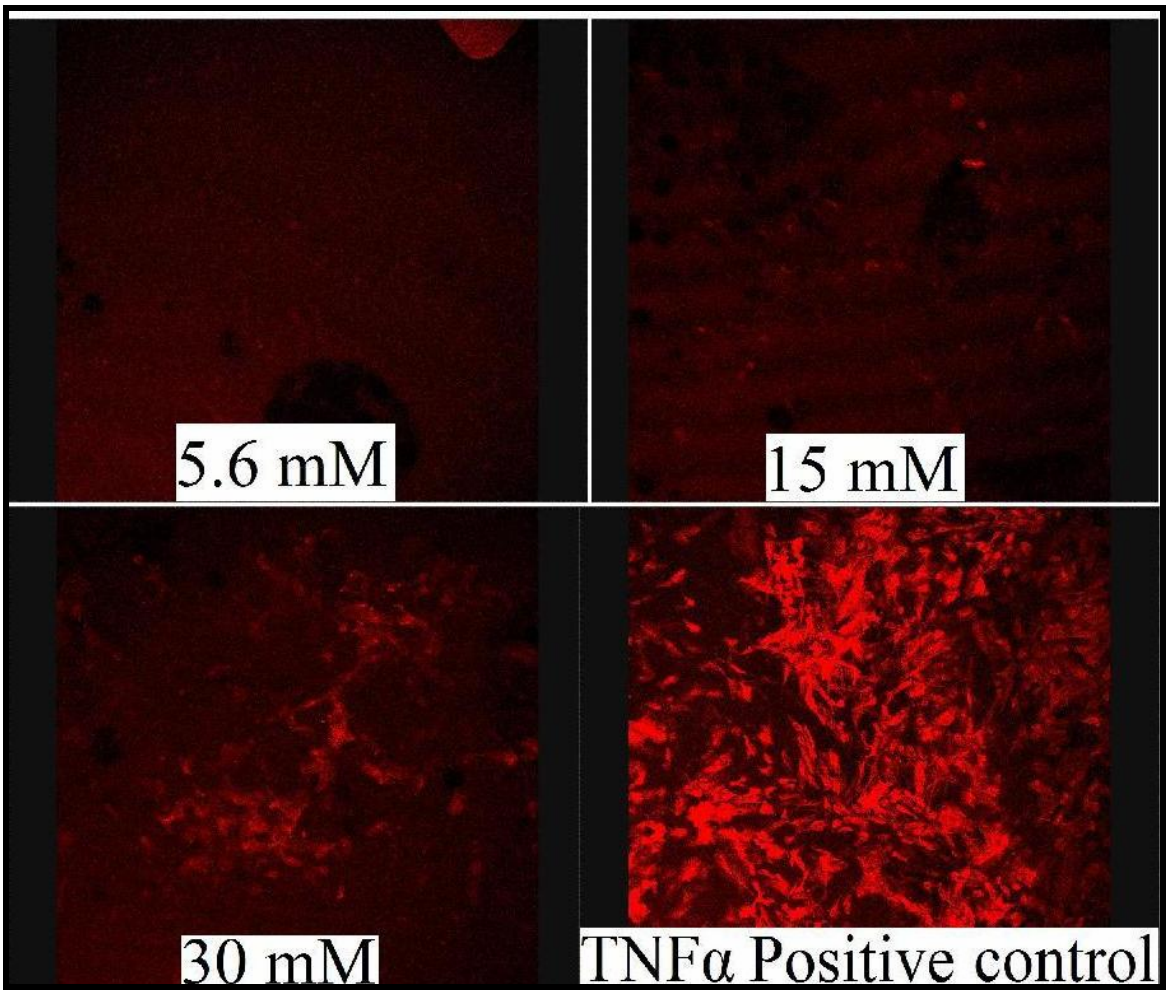


Figure 17. Effect of high glucose concentration on VCAM expression on HUVECs in the 3D Tissue model

3.5 Analysis of the Surface Expression of PECAM-1, VCAM-1, ICAM-1 and E-Selectin on HUVECs by Flow Cytometry

After a qualitative investigation of CAM expression (PECAM-1 and VCAM-1) on the HUVECs with immunostaining and confocal microscopy, the next step was to use flow cytometry for a more quantitative analysis. Flow cytometry was used to quantitatively measure the expression of each CAM in response to the various glucose concentrations tested. Additional CAMs E-Selectin, ICAM-1, were included that may

have a potential role in leukocyte trafficking in the 3D tissue model. Since it appears that 30 mM is the threshold for CAM expression, an even greater concentration of glucose (45 mM) was used to investigate if the expression continues to increase with increasing glucose concentration. The fluorescence intensity of each sample was compared to the isotype control fluorescence intensity corresponding to increasing glucose concentrations.

Figure 18 shows the fluorescence intensity of fluorescently-labeled PECAM-1, ICAM-1, E-Selectin and VCAM-1 on the HUVECs in response to an increase in glucose concentration and to TNF-α. PECAM-1 was expressed in all the samples, but there was not a considerable difference in the expression that would implicate a dependence on glucose concentration for PECAM-1 expression. This was in agreement with the qualitative confocal microscopy method where PECAM-1 expression was not affected by increasing glucose concentration. There was no detectable ICAM-1 expression in any of the samples, except for the positive control. There was no detectable VCAM-1 expression for the samples exposed to the lower glucose concentrations of 5.6 and 15 mM, but was significantly enhanced for the higher glucose concentrations of 30 mM and 45 mM. Thus, both visual inspection by confocal microscopy (described previously) and flow cytometry analysis show that the cells express VCAM-1 once a high glucose concentration is reached and then increases proportionally with concentration. The positive control, TNF-α, also showed a VCAM-1 expression quite greater than that of the glucose samples. There was no detectable E-Selectin expression for the samples exposed to the various glucose concentrations or to TNF-α.

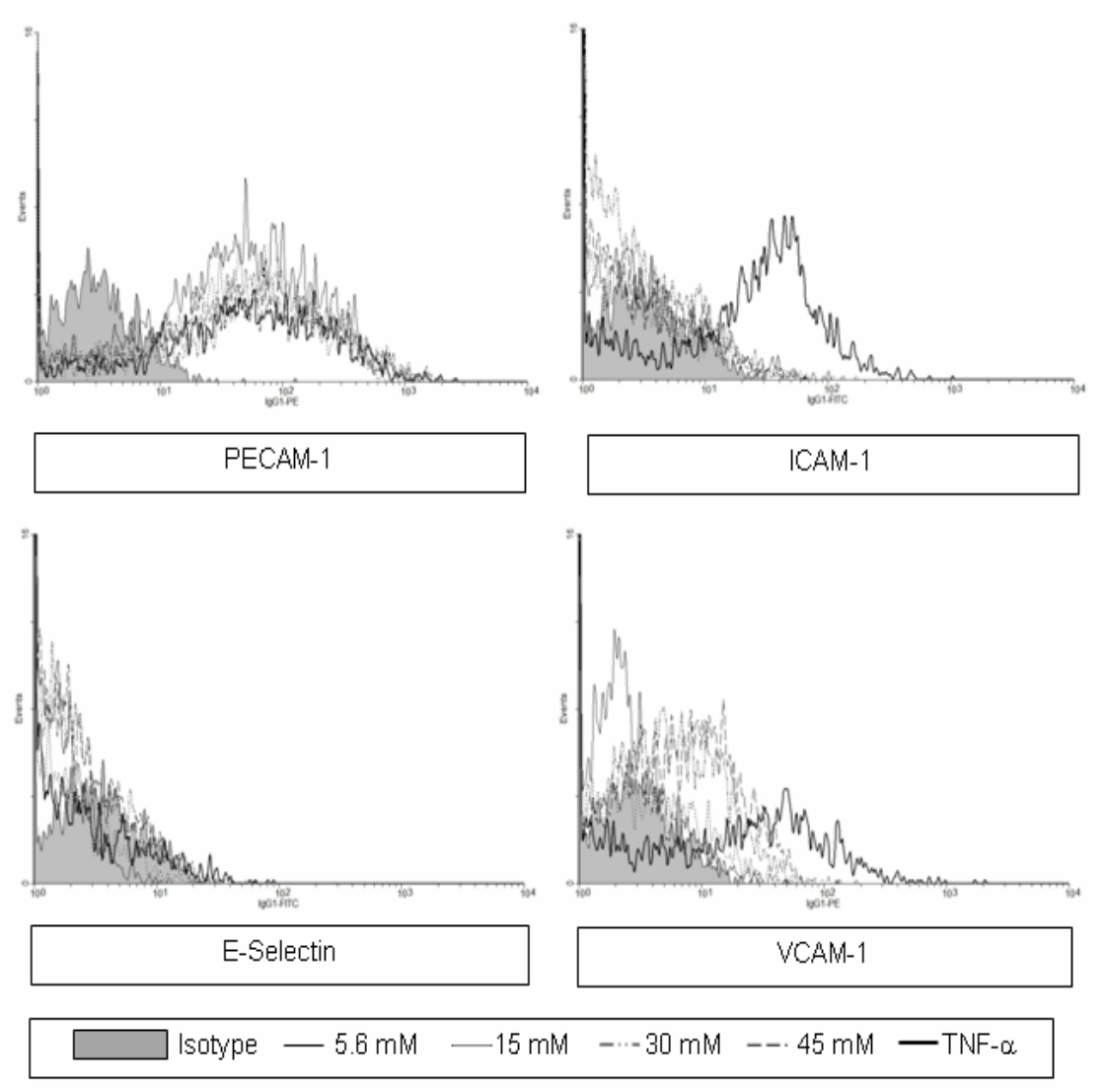


Figure 18. Fluorescence intensity of fluorescently-labeled PECAM-1, ICAM-1, VCAM-1 and E-Selectin on the HUVECs in response to an increase in glucose concentration and to TNF-α.

Another experiment was used to check for the hyperosmolarity effect that could be responsible for the changes in CAM expressions on the HUVECs. Figure 19 shows the histogram of the relative fluorescent intensities corresponding to measured CAM expression in response to increasing glucose concentrations and a high concentration of mannitol. PECAM-1 was expressed in all the samples, but there was not a significant difference in the expression between mannitol and samples incubated with glucose. As before, ICAM-1 expression was not found in any of the samples, except for the samples exposed to TNF-α. Thus, there was no effect of a high glucose or high mannitol concentration on ICAM-1 expression. There was no VCAM-1 expression on the HUVECs exposed to a high mannitol concentration, thus showing that mannitol does not cause any significant change to VCAM-1 expression. There was a problem with the staining procedure for the sample exposed to the 45 mM glucose concentration for the VCAM-1 analysis, which resulted in no data for this sample. The HUVECs exposed to TNF-α showed an upregulation of VCAM-1 expression. There was no detectable E-Selectin expression for any of the samples exposed to either glucose or the hyperosmolarity control mannitol. Thus, the conclusion is that the hyperosmolarity control mannitol did not cause any significant change in any of the CAM expressions compared to the 5.6 mM negative control.

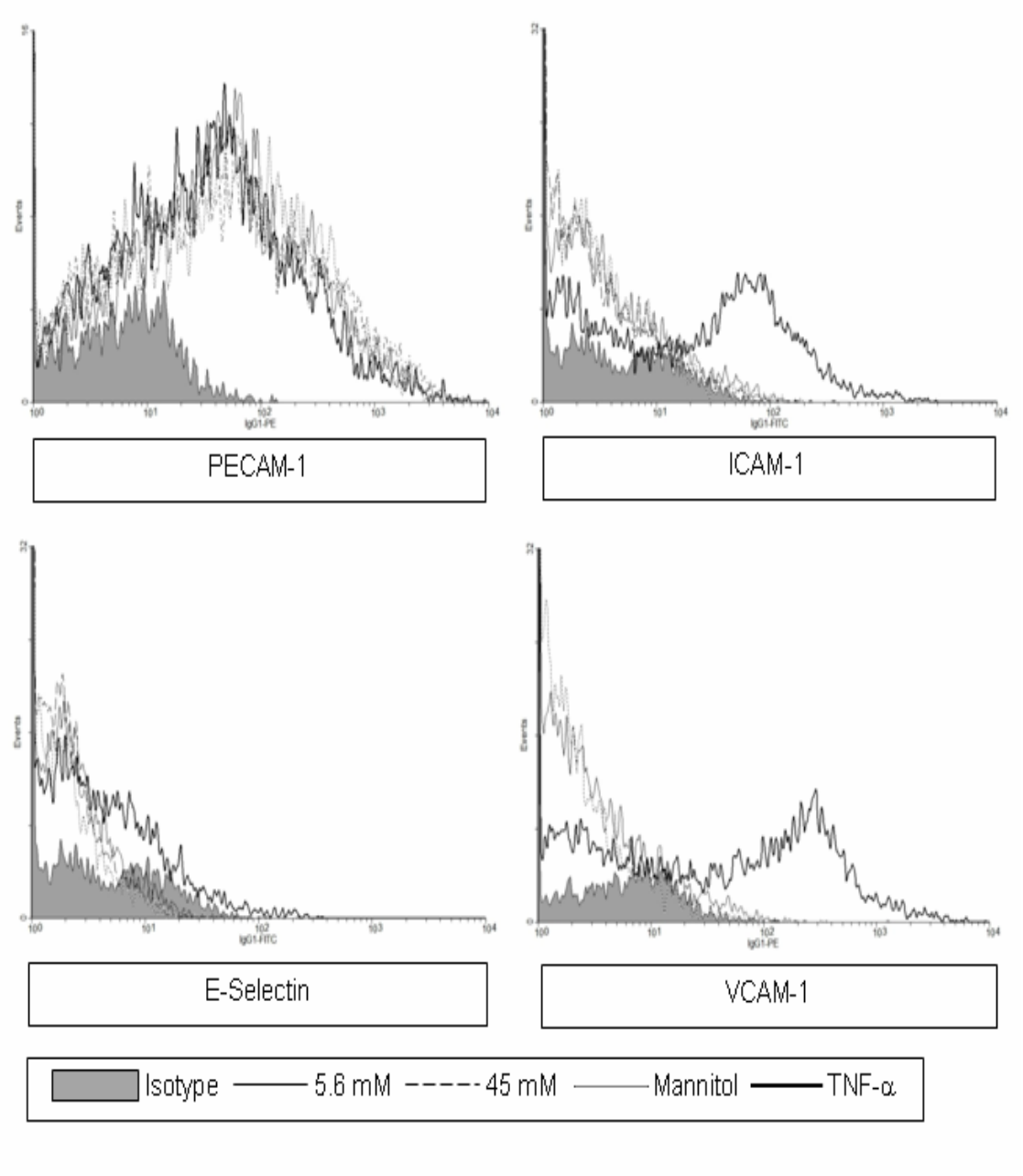


Figure 19. Fluorescent intensity of fluorescently-labeled PECAM-1, ICAM-1, VCAM-1 and E-Selectin expression on the HUVECs in response to a high glucose concentration and a high mannitol concentration.

3.6 Analysis of Leukocyte Migration and Monocyte Differentiation

3.6.1 Overall Leukocyte Migration

This study investigated leukocyte trafficking into the 3D tissue model in response to varying glucose concentrations. To account for the possible role of hyperosmolarity associated with glucose on the 3D tissue model, a hyperosmolarity control, mannitol, was used as well. Upon incubation of the 3D tissue model with different glucose concentrations for the nine hour incubation time, the three groups of cells were collected and counted: 1) SE-2 hr: cells that migrate into the subendothelium region of the tissue model after a 2-hour incubation period; 2) SE-48 hr: cells that remain within the subendothelium after an additional 48-hour incubation period; and 3) RT: cells that reverse-transmigrate across the endothelium after the 48-hour incubation period.

Figure 20 shows the total cell migration numbers in all these three groups in response to increasing glucose concentration. The SE-2hr group shows that there was an increase in initial cell migration into the tissue model, proportional to increasing glucose concentration. The SE-48 hr group also shows a significant increase in the number of cells remaining within the subendothelium after 48 hours, proportional to increasing glucose concentration, with the model exposed to 30 mM glucose concentration showing a significant increase compared to the 5.6 mM control group. There was no significant difference in cell migration numbers for the RT group in response to increasing glucose concentration. Multiple cell counts were done using a hemacytometer with the standard deviation associated with each count at approximately 10%. After counting, immunofluorescence was used to label certain cell markers and flow cytometry analysis

was performed on each group of cells, to count and classify each of the leukocyte species present in each.

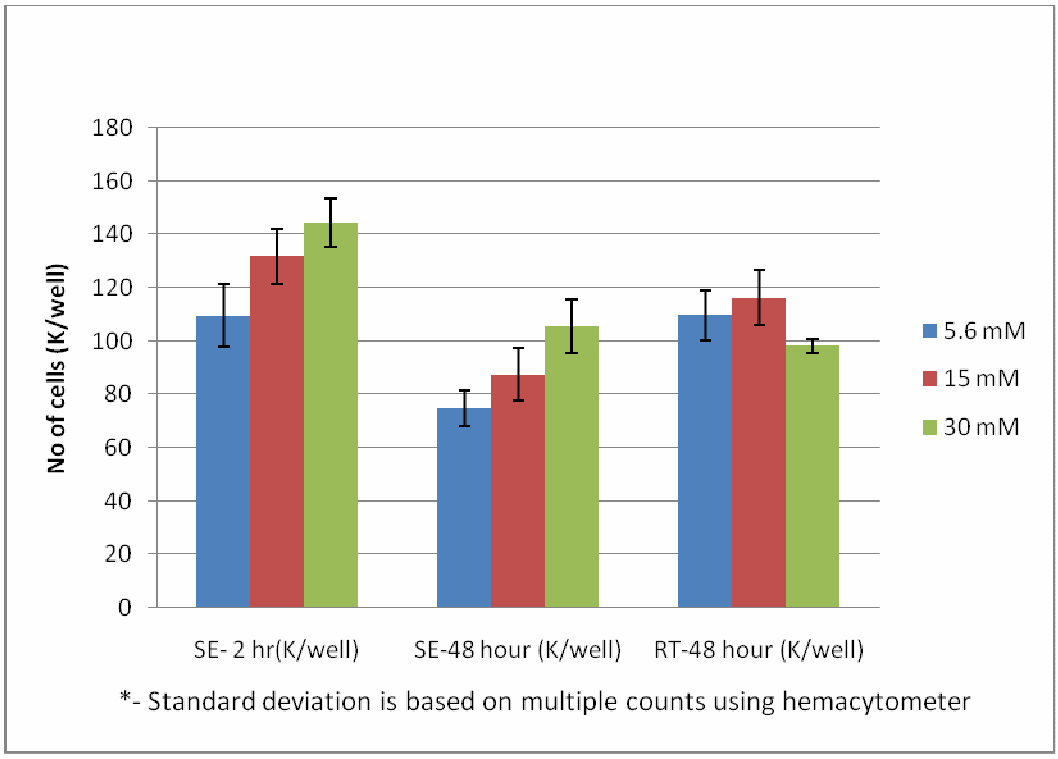


Figure 20. Cell migration in the 3D tissue model in response to incubation with various concentrations of glucose. (Shown are average values with s.d. from n=3).

Flow cytometry analysis was performed to determine the individual leukocyte species present in the pool for each of SE-2hr, RT, and SE-48hr group of cells: CD3+ (T cells), CD14+ (Monocytes), CD83+/CD206- (Immature DCs), CD83-/CD206+ (Macrophages), CD83+/CD208+ (Mature DCs), CD64+(Monocytes or macrophages). Initial studies with the 3D tissue model showed no CD19+ (B cells) or CD56+ (natural killer cells) cells in any of the cell populations (data not shown).

Flow cytometry analysis also was used to measure MDR-1 expression on the leukocytes. MDR-1 is a well known transporter molecule, often studied for its possible mediatory role in efflux and influx of chemotherapeutic agents and cell transportation.

Though it is known that these molecules are expressed by leukocytes and endothelial cells under activation, the complete physiological functions of the molecules are poorly defined. A recent study [90] showed a possible role of MDR-1 in basal to apical transendothelial migration of leukocytes. This role of MDR-1 was investigated for in the 3D tissue model.

3.6.2 Leukocyte Migration and Monocyte Differentiation: SE-2 hr Cells

Figure 21 describes the percent increase in cell migration of certain cell markers with an increase in glucose concentration. The numbers are normalized with respect to the 5.6 mM glucose concentration control group. Within the SE-2 hr group, an increase in glucose concentration was found to increase CD14+ (monocyte) migration into the intima, along with an increase in CD83+/CD206- (immature DCs) population. A significant increase in CD3+ (T cell) population with a higher glucose was also observed for the SE-2 hr group. There was an increase of approximately 40% when exposed to 15 mM, and 100 % when exposed to 30 mM compared to the control group of 5.6 mM. This was a very significant development showing an enhanced initial migration of T cells, when exposed to an inflammatory condition (in this case, hyperglycemia). For the CD14+ monocyte population, a glucose increase to 15 mM showed a 30% increase, and a further glucose increase to 30 mM, resulted in a 55% increase in monocyte migration compared to the control group of 5.6 mM. The CD 83+/206- population dd not show a significant increase at lower glucose concentrations, but showed a 70% increase compared to the 5.6 mM control group when exposed to a high 30 mM glucose. This

finding indicates the possible differentiation of monocytes into immature DCs; however, this combination of markers can also be found on the monocytes as well.

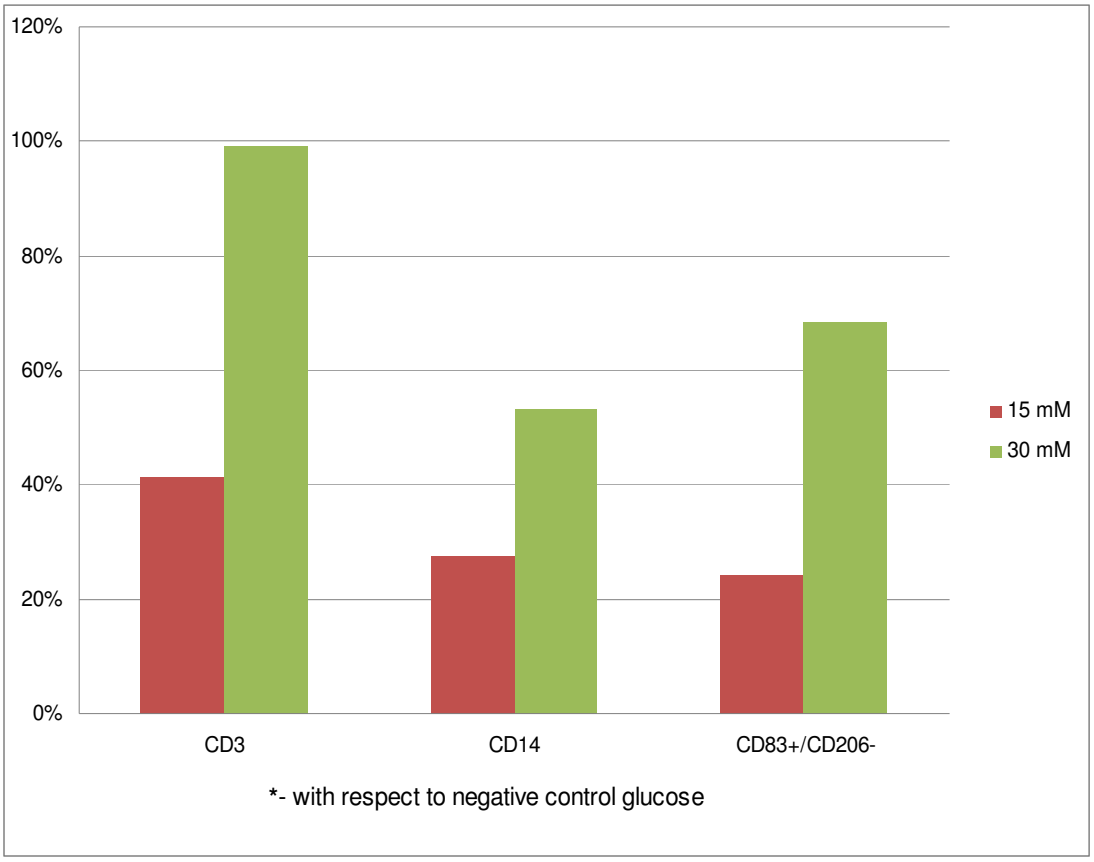


Figure 21. Average percent increase in certain cell markers for SE 2 hour cells after the 3D tissue model was exposed to high glucose concentrations. (n=2)

The 3D tissue model was also tested with a higher concentration of glucose (45 mM) and a mannitol control. In this test, the expression of MDR-1 and CD64+ (monocyte or macrophage population) was also investigated for the groups of cells collected from the model. Figure 22 shows the percent increase for each cell marker and MDR-1 expression, in response to increasing glucose concentration. The numbers are normalized with respect to the control group of 5.6 mM glucose.

An increase in glucose concentration from 5.6 mM to 15 mM resulted in approximately 45%, 35% and 65% increase in CD3+ (T cell), CD14+ (monocyte) and CD64+ (monocytes or macrophages) populations, respectively. The expression of MDR-1 at 15 mM increased by approximately 20%, compared to the 5.6 mM control group.

Compared to the 5.6 mM glucose concentration, exposure to the 30 mM glucose concentration resulted in approximately 100%, 60%, 65% and 60% increase in CD3+ (T cell), CD14+ (monocyte), CD83+/CD206- (immature DCs) and CD64+ (monocytes or macrophages) populations, respectively. At this glucose concentration, MDR-1 expression was also increased by 125%, compared to the control group 5.6 mM glucose.

An increase in glucose concentration from 5.6 mM to 45 mM resulted in approximately 140%, 75%, 85% and 45% increase in CD3+ (T cell), CD14+ (monocyte), CD83+/CD206- (immature DCs) and CD64+ (monocytes or macrophages) populations, respectively. Exposure to 45 mM resulted in a 280% increase in MDR-1 expression, compared to the 5.6 mM control group.

For MDR-1, mannitol was found to have an effect close to that measured for the 15 mM glucose concentration. Also, the effect of mannitol on all cell population migration numbers was less than the effect produced by the 30 mM glucose concentration. This reflects a relatively small role that hyperosmolarity has on initial migration of these cell populations into the subendothelial space. Thus, the role of mannitol was not completely negligible, but definitely not comparable to the effect from the high glucose concentration.

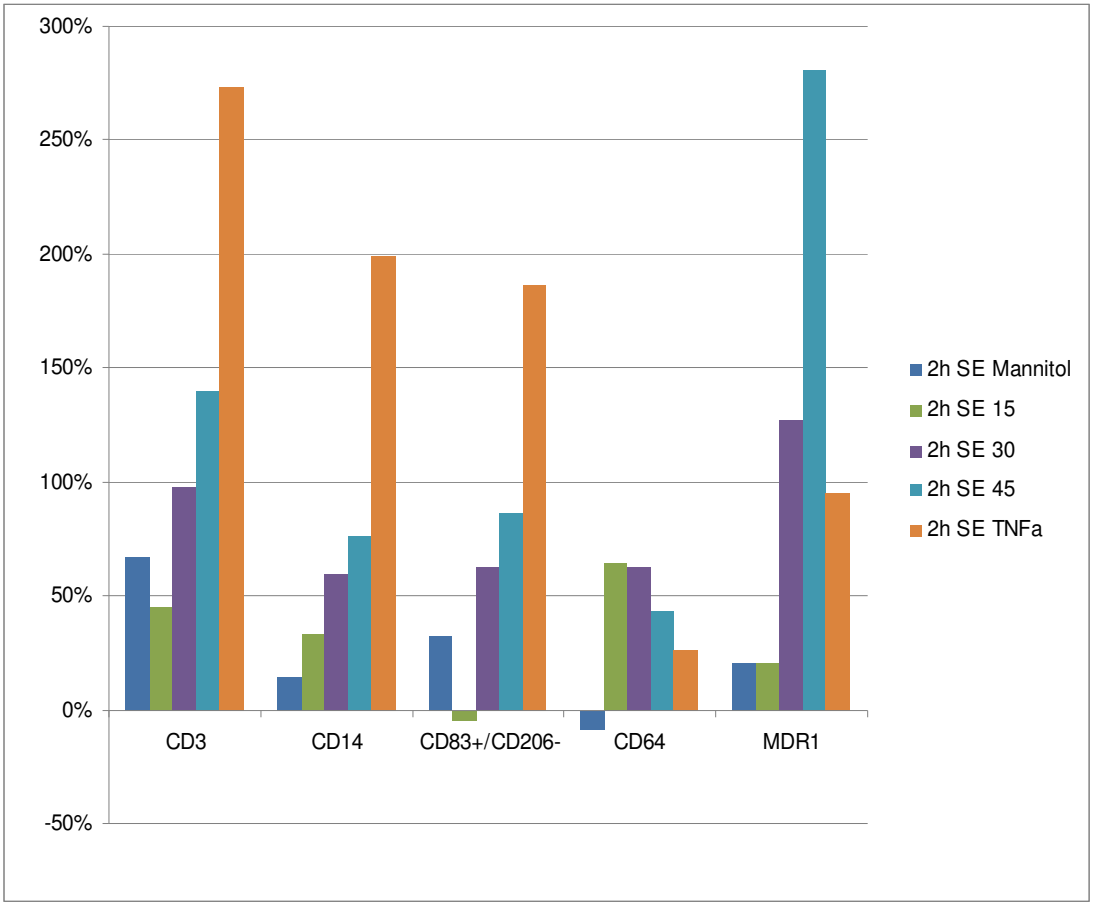


Figure 22. Percent increase in certain cell markers for SE 2 hr cells exposed to high glucose concentrations and the hyperosmolarity control, mannitol.

3.6.3 Leukocyte Migration and Monocyte Differentiation: RT Cells

Figure 23 describes the percent increase of certain populations from the cells that reverse transmigrated from the tissue model in response to an increase in glucose concentration. The numbers are normalized with respect to the 5.6 mM control. Within the RT group, an increase in glucose concentration was found to increase CD3+ (T cell) and CD83+/CD206- (immature DCs) cells. For the CD3+ T cell population, there was observed an increase of approximately 40% when exposed to 15 mM, and 110 % when exposed to 30 mM compared to 5.6 mM glucose concentration. There was a significant

increase in CD83+/206- immature dendritic cell population of 25% for 15 mM and 50% increase for 30 mM compared to the control group. These numbers indicates a higher degree of immature dendritic cells found in the reverse transmigrating cells, when exposed to higher glucose concentrations. It is believed that these are indeed immature dendritic cells, since CD14 was no longer expressed. This could possibly be tied to the fact that more monocytes migrated into the tissue model; therefore, more will reverse-transmigrate back out and differentiate into dendritic cells. This possibly shows that a higher glucose concentration, serves as a more severe injury condition, and promotes more iDCs to be formed.

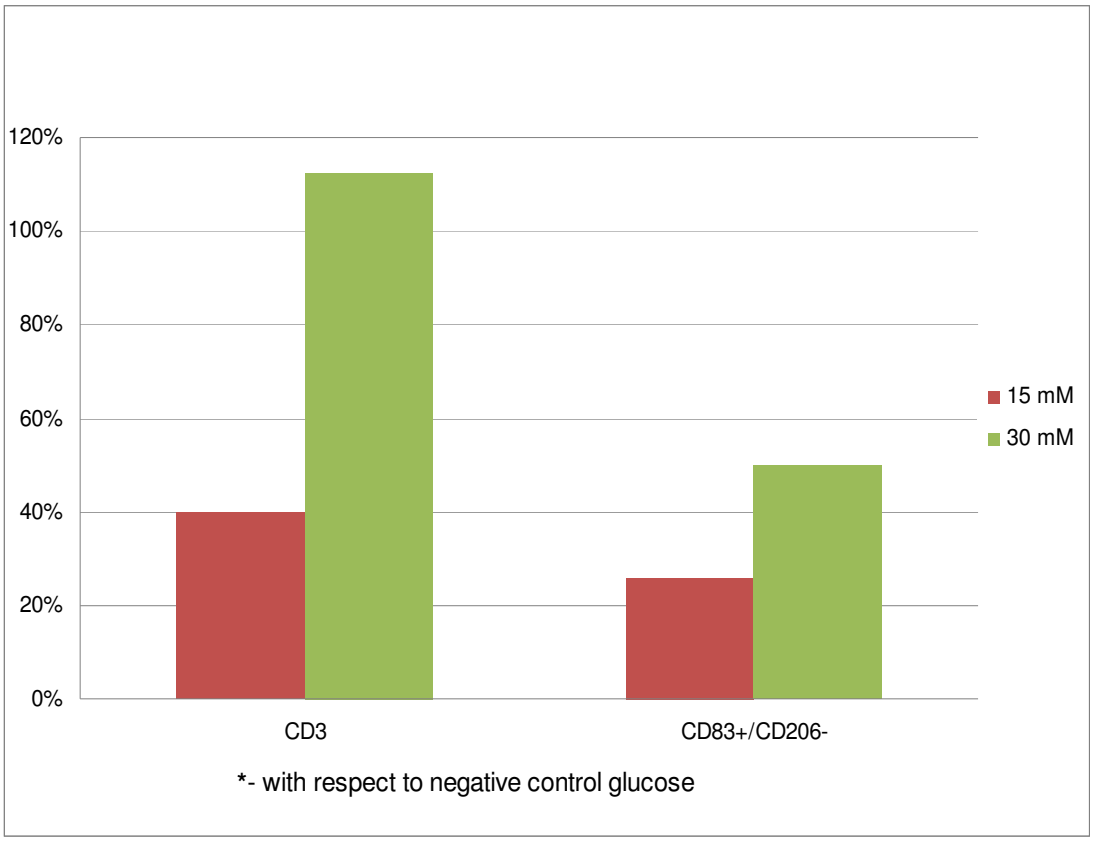


Figure 23. Average percent increase in certain cell markers for RT cells after the 3D tissue model was exposed to high glucose concentrations. (n=2).

The 3D tissue model was also tested with a higher concentration of glucose (45 mM) and a hyperosmolarity control, mannitol. The expression of MDR-1 and CD64+ (monocyte or macrophage population) was investigated in response to an increase in glucose concentration. Figure 24 shows the percent increase for each cell marker and MDR-1 expression, in response to increasing glucose concentration. The numbers are normalized with respect to the control group, 5.6 mM glucose.

An increase in glucose concentration from 5.6 mM to 15 mM resulted in approximately 60%, 30% and 50% increase in CD3+ (T cell), CD83+/206- (immature dendritic cells) and CD64+ (monocytes or macrophages) populations, respectively. The expression of MDR-1 for the 15 mM glucose sample increased by approximately 20%, as compared to the 5.6 mM control group.

Compared to the 5.6 mM control group, the 30 mM glucose concentration group resulted in approximately 130%, 55%, and 50% increase in CD3+ (T cell), CD83+/CD206- (immature DCs) and CD64+ (monocytes or macrophages) populations, respectively. At this glucose concentration, MDR-1 expression was also increased by 10%, compared to the 5.6 mM glucose control group.

An increase in glucose concentration from 5.6 mM to 45 mM resulted in approximately 225%, 85%, and 175% increase in CD3+ (T cell), CD83+/CD206- (immature DCs) and CD64+ (monocytes or macrophages) populations, respectively. Exposure to the 45 mM glucose concentration resulted in an 80% increase in MDR-1 expression, compared to the control group.

Mannitol was found to have an effect similar to that of the 5~15 mM glucose concentration groups. This shows a relatively small role of hyperosmolarity in the

reverse-transmigration of these cell populations. Thus, the role of mannitol was not completely negligible, but not as significant as the response to the high glucose concentration.

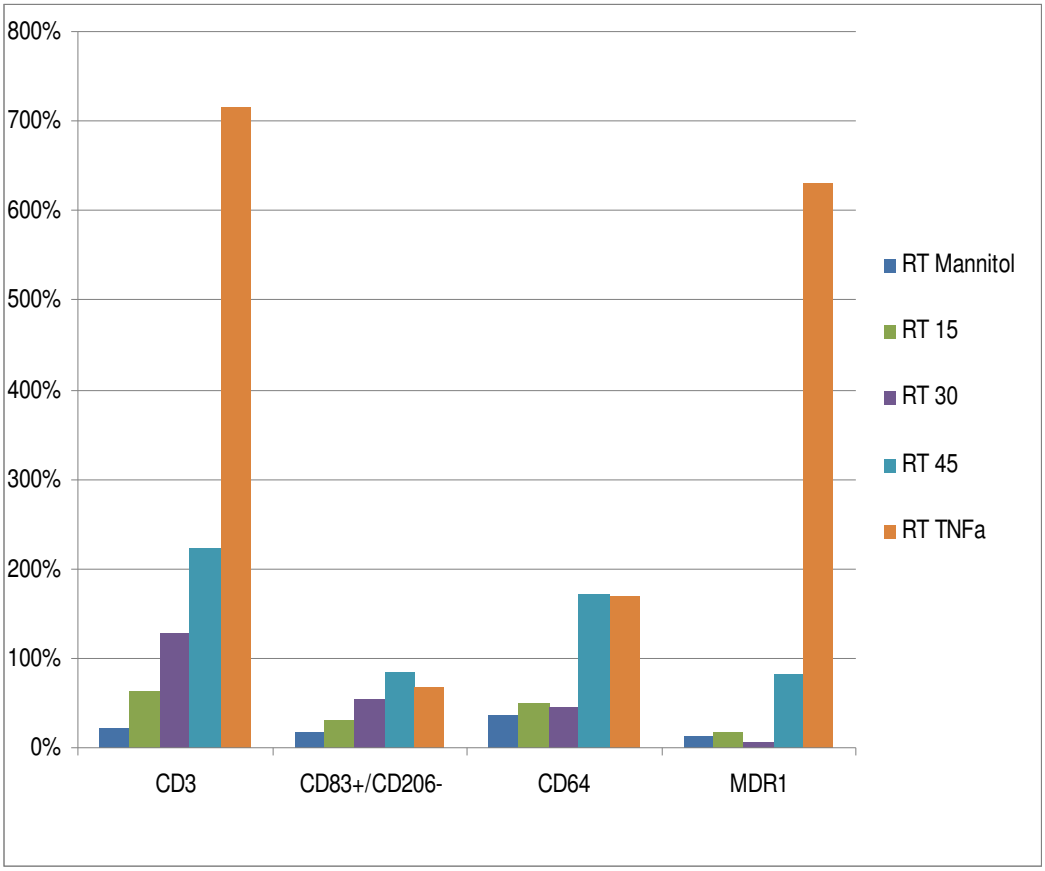


Figure 24. Percent increase in certain cell markers for RT cells exposed to high glucose concentrations and the hyperosmolarity control, mannitol.

3.6.4 Leukocyte Migration and Monocyte Differentiation: SE-48 hr Cells

Individual cell populations found in the SE-48 hr group of cells were also examined. Figure 25 describes the percent increase in the number of cells for certain cell markers associated with an increase in glucose concentration. The numbers are normalized with respect to the 5.6 mM glucose concentration control. An increase in

glucose concentration was found to increase CD3+ (T cell), CD14+ (monocyte) and CD83-/CD206+ (macrophages) populations remaining within the subendothelial space after 48 hours. For the CD3+ (T cell) population, there was an increase of approximately 70% for the 15 mM group, and 225 % for the 30 mM group compared to the 5.6 mM glucose concentration control group. For the CD14+ monocyte population present within the subendothelial space after 48 hours, an increase to 15 mM glucose concentration resulted in an increase of approximately 15%. A further increase to 30 mM glucose concentration resulted in a 25% increase. The CD 83-/206+ macrophages cell population showed a significant increase compared to the control group of approximately 20% for the 15 mM group. This was further increased to 45% for the 30 mM glucose group. This was different compared to the SE-2 hr group, where there were a negligible number of macrophages. For the SE-2 hour cell group, monocytes may not have been able to differentiate into macrophages within the first two hours. This finding indicates the possible differentiation of monocytes into macrophages within the 3D tissue model for the 2~48 hour time frame.

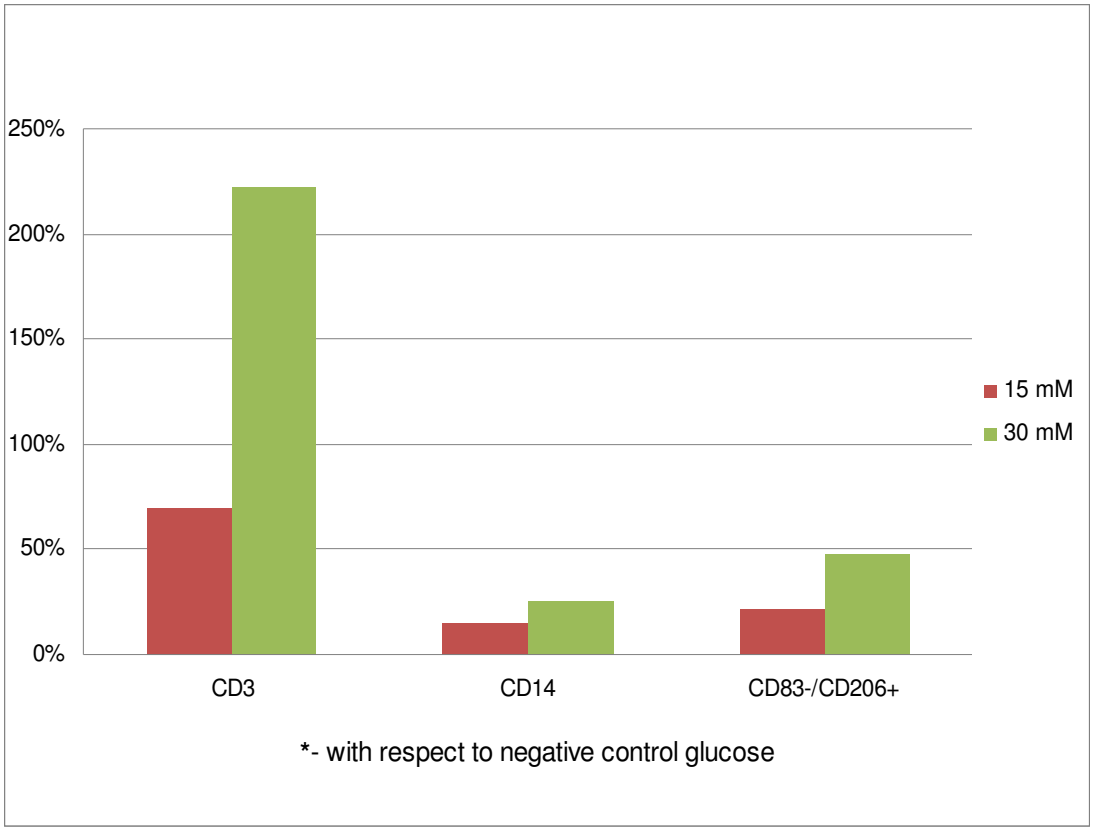


Figure 25. Average percent increase in certain cell markers for SE 48 hour cells after the 3D tissue model was exposed to high glucose concentrations. (n=2).

The 3D tissue model was also tested with a higher concentration of glucose (45 mM) and a hyperosmolarity control, mannitol, and the SE-48 hr cell group was characterized. The expression of MDR-1 and CD64+ (monocyte or macrophage population) cells were also detected in response to an increase in glucose concentration. Figure 26 shows the percent increase in number of cells for each cell marker and for MDR-1 expression, in response to increasing glucose concentration. The numbers are normalized with respect to the 5.6 mM glucose control group.

An increase in glucose concentration from 5.6 mM to 15 mM resulted in approximately 50%, 25%, 25% and 20% increase in CD3+ (T cell), CD14+ (monocyte),

CD83-/206+ (macrophages) and CD64+ (monocytes or macrophages) populations, respectively. The expression of MDR-1 at 15mM increased by approximately 35%, compared to the 5.6 mM control group.

Compared to the 5.6 mM glucose concentration group, the 30 mM glucose concentration group showed approximately 170%, 20%, 40% and 50% increase in CD3+ (T cell), CD14+ (monocyte), CD83-/CD206+ (macrophages) and CD64+ (monocytes or macrophages) populations, respectively. At this glucose concentration, MDR-1 expression also increased by 35%, compared to 5.6 mM glucose control group.

An increase in glucose concentration from 5.6 mM to 45 mM resulted in approximately 380%, 75%, 100% and 80% increase in CD3+ (T cell), CD14+ (monocyte), CD83-/CD206+ (macrophages) and CD64+ (monocytes or macrophages) cell populations, respectively. Exposure to the 45 mM glucose concentration resulted in a 60% increase in MDR-1 expression, compared to the control group.

For the mannitol group, the expression of MDR-1 was similar to that for the 15 mM glucose concentration group. The effect of mannitol on all cell migration numbers was also similar to the 15 mM glucose concentration group. This shows a relatively small role of hyperosmolarity towards cell migration and retention of these cell populations within the subendothelial space. Thus, the role of mannitol was not completely negligible, but definitely not comparable to the effect seen for the high glucose concentration groups.

The expression of CD64+ (monocytes or macrophages) was shown to increase with increasing glucose concentration. This shows an increasing number of macrophages that remain within the subendothelial space. After 48 hours, most monocytes have

already differentiated to either dendritic cells or macrophages, and the cells that are expressing CD64, are macrophages.

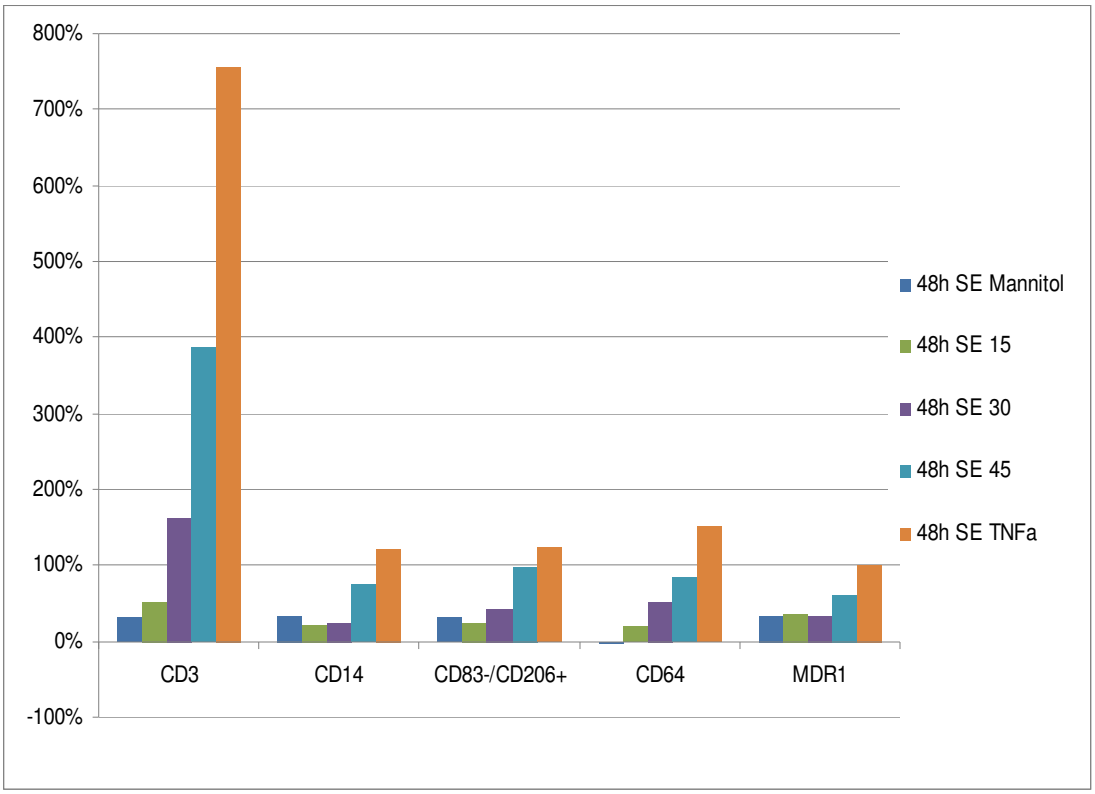


Figure 26. Percent increase in certain cell markers for SE 48 hr cells exposed to high glucose concentrations and the hyperosmolarity control, mannitol.

The number of CD83+/CD206- (immature DCs) cells in the SE-48 hr group of cells was found to be much lower compared to the RT cell group. This observation is related to the nature of the dendritic cells, which are known to migrate towards the lymphatics *in vivo*. Thus, at the end of 48 hours, more immature DCs have already reverse transmigrated across the endothelium and were collected in the RT group of cells, and few remain within the SE-48 hr group.

CHAPTER IV

CONCLUSIONS AND FUTURE RECOMMENDATIONS

The present research project used a 3D tissue model exposed to high glucose concentrations in order to investigate the effect of hyperglycemia on endothelial cell dysfunction and on immune cell trafficking and differentiation. The main conclusions of the project are:

1. A 3D tissue model was successfully created that was used to investigate the effect of hyperglycemia on endothelial cells and leukocyte migration and differentiation.
2. For the time periods tested, endothelial viability was not significantly affected in the 3D tissue model by incubation with high glucose concentrations.
3. Increasing glucose concentrations resulted in increase in VCAM-1 expression on endothelial cells, indicating that this adhesion molecule plays a possible role in leukocyte recruitment for the tissue model. An increase in glucose concentration to a threshold of 30 mM was shown to increase VCAM-1 expression on endothelial cells. The expression of PECAM-1 on the endothelial cells was not affected by increasing glucose concentrations.
4. For the initial migration of leukocytes across the endothelium and into the subendothelial space, the T cell, monocytes and immature dendritic cell populations increased with increasing glucose concentrations. There was a slight

5. increase for the 15 mM concentration and significant increases for the 30 mM and 45 mM concentrations.
6. For the group of cells undergoing reverse transmigration out of the subendothelial space at a later time point, an increase in glucose concentration resulted in an increase of monocyte and immature dendritic cell populations.
7. For the cells remaining within the subendothelial at a later time point, there was an increase of T cell, monocyte, and macrophages populations, relative to increasing glucose concentrations. The T cell population was greatly increased for the high glucose concentration of 45 mM.

The main objective of this research was to develop a 3D tissue model to simulate the blood endothelium and subendothelial space that could be used to test the effect of hyperglycemia on endothelial cells and leukocyte migration and differentiation. The lack of experimental models to study the behavior of these cells within a 3D environment that can recapitulate *in vivo* conditions was the major driving force for this project. The 3D tissue model developed for this project can be used as a powerful research tool to study various mechanisms associated with the blood endothelium, like the relationship between diabetes and atherosclerosis, or as a potential testing device for the therapeutics.

Unlike human or animals models, which are difficult to control due to their multivariant, very transient microenvironment, an *in vitro* tissue model like the one used for this research, offers better control over the system to be studied. The *in vitro* 3D tissue model can also be used to study anything that would have potential health hazards to humans and could not be studied *in vivo*.

The advantage of being 3D gives the tissue model an edge over the present 2D cell models. The 2D models can only be used to investigate leukocyte-endothelium interactions, like the leukocyte rolling and adhesion, where a subendothelial space is not required. The 2D models cannot be used to study phenomena like transendothelial migration. Membrane plates (Transwell®) have been used to study transendothelial migration, but they lack the important 3D environment that can be used to study cell-matrix interactions. These interactions are important for the leukocytes to function normally, as they would *in vivo*. Because the 2D models lack the sub-endothelial space, they cannot be used to study cell migration and differentiation within the subendothelial space, at different time points. Cell migration and differentiation within the subendothelial space can be studied in the 3D model. The novel 3D model also has the advantage of being able to arrest the leukocyte species at any time point, in order to study the kinetics of the model. This is especially useful since the end state of a blood monocyte traversing the endothelium is very difficult to characterize, due to its transient behavior that is dependent on the inflammatory stimulus and the tissue involved.

A novel 3D model was developed that can mimic the behavior of cells under both normal and pathological conditions. The model is composed of human blood endothelial cells cultured on top of a collagen matrix within a multi-well plate format. The 3D model simulates the endothelium and subendothelial space existing in a blood vessel. Peripheral blood mononuclear cells were used to study the leukocyte trafficking through the endothelium. Two time points based on Randolph's work [90] were chosen to investigate the initial migration of cells into the subendothelial space (SE2hr), the later migration of cells that are either reverse-transmigrating back out of the subendothelial space after 48

hours (RT) or remaining within the subendothelial space after 48 hours (SE48hr). It was important to characterize the tissue model before using it to test different experimental conditions. To represent the hyperglycemic conditions that exist *in vivo*, an optimal time to incubate the 3D tissue model with various concentrations of glucose was determined that would be able to evoke a sufficient activation in the endothelial cells and leukocytes. Three incubation time periods (3, 9, and 24 hours) were chosen and leukocyte migration through the endothelium was studied for these three incubation times. A nine hour incubation time resulted in enhanced leukocyte migration across the endothelium. All following experiments were based on this incubation time. Varying glucose concentrations were chosen in order to represent normoglycemia and various levels of hyperglycemia that would be seen in diabetics.

The effect of high glucose concentrations on endothelial cell behavior was studied within the 3D tissue model. Cell viability and the expression of a number of CAMs were investigated under increasing glucose concentrations. There was no significant decrease in cell viability for the incubation time of nine hours that was chosen for the model. Some studies have shown that high glucose concentrations like the ones chosen for this research does affect cell viability [70, 71], but only when exposed for longer time periods like 48 hours [97].

To determine the effect of high glucose concentrations on CAM expression on the endothelial cells, the following were detected by immunofluorescence and flow cytometry analysis: PECAM-1, VCAM-1, ICAM-1 and E-selectin. Among the CAMs tested, the expression of VCAM-1 increased for the samples exposed to a minimum threshold glucose concentration (30 mM). The expression of VCAM-1 increased in

response to even higher glucose concentration (45 mM), thereby suggesting that VCAM-1 expression is proportional to glucose concentration, after crossing a threshold glucose concentration. This suggests that enhanced leukocyte migration in response to high glucose concentration may be due to the upregulation of VCAM-1 expression.

The effect of high glucose concentration on initial leukocyte trafficking in the 3D tissue model was investigated. The initial migration of monocytes (SE2hr) into the subendothelial space was increased in response to increasing glucose concentration. Also, with an increase in glucose concentration, there was an increase in the number of immature dendritic cells migrating into the sub endothelial space. This shows glucose to be proinflammatory, resulting in an increase in leukocyte migration across the endothelium. An increase in MDR-1 expression on the leukocytes was also found, which has been found to be involved in enhancing leukocytes migration into the lymphatics *in vivo.*[54] With reference to the 3D tissue model, a cell population expressing MDR-1 would mean these cells are looking to reverse-transmigrate across the endothelium.

The effect of high glucose concentration on cells that initially migrate into the subendothelial space and then reverse-transmigrate back across the endothelium was investigated. An increase in the number of immature dendritic cells in response to an increase in glucose concentration was observed. The function of dendritic cells in the immune system is to act as antigen presenting cells by taking up antigen and presenting it to other immune cells, such as T cells, to elicit a response. To do this, they have to migrate to the lymph nodes, where the T cells are found, by way of the lymphatics. Thus, an increase in immature dendritic cells with an increase in glucose concentration suggests an increased immune response to the proinflammatory conditions. An increase in MDR-

1 expression for the reverse transmigrated cells was also found, which has been implicated in leukocyte migration towards lymphatics.[90] With reference to the 3D model, an increase in MDR-1 would result in an increase of leukocytes migrating out of the subendothelial space, by reverse transmigration across the endothelium (as if moving towards the lymphatics).

There was an increase in the number of macrophages within this group of cells with an increase in glucose concentration. Macrophages are developed from the differentiation of monocytes and are involved in foam cell formation within the subendothelium. These macrophages take up low density lipoproteins (LDL) and cell debris from the blood to form foam cells. These foam cells result in plaque formation, which is a hallmark of atherosclerosis. Thus, an increase in the number of macrophages could be an early indicator of plaque formation. The expression of MDR-1 was much less in this category of cells compared to the "SE2hr" and "RT" group of cells. Most of the leukocyte species expressing MDR-1 have already reverse transmigrated out of the subendothelial space by this time.

The present 3D tissue model has successfully been used to study the early effects of hyperglycemia on endothelial cell function and leukocyte migration and differentiation. However, to further characterize the early steps associated with plaque formation in atherosclerosis and to further develop a model that can be used to test therapeutics, future work must be directed towards:

- Similar to *in vivo* conditions for diabetics, examine the effect of cyclic episodes of hyperglycemia on the kinetics of leukocyte migration and differentiation. *In vivo*, there is a postprandial glucose rise in the blood. Slowly, this glucose is either

stored as glycogen in the liver or metabolized for energy. Thus there is cyclic increase and decrease of blood glucose. To study this phenomenon, the 3D tissue model would be exposed to cycles of low and high glucose media.

- Add lipoproteins to the model and investigate LDL uptake by the cells in the 3D tissue model. In atherosclerotic conditions, lipoproteins are phagocytosed by macrophages and start depositing along with other cellular debris as plaques. The present model could be used to investigate the early signs of foam cell formation that would lead to plaque formation. To study this phenomenon, LDL would be added along with a high glucose concentration, and then LDL uptake would be monitored in response to increase in glucose.
- Increase the complexity of the tissue model by the addition of smooth muscle cells and investigate their role in atherosclerosis. In the event of inflammation, there is the migration and proliferation of smooth muscle cells. An excessive inflammatory responses can lead to thickening of the arterial wall, thereby forming atherosclerotic lesions.[98]
- Validate the 3D tissue model by testing known inhibitors of the mechanisms associated with atherosclerosis, so that the model can be used to test new treatments of the disease.

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VITA

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Thesis: EFFECT OF HYPERGLYCEMIA ON LEUKOCYTE MIGRATION AND DIFFERENTIATION IN A NOVEL THREE-DIMENSIONAL TISSUE MODEL

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Major Field: Chemical Engineering

Scope and Method of Study: Leukocytes are known to migrate across an endothelium continuously but this migration is enhanced in case of certain inflammatory signals, such as those associated with hyperglycemia. Currently, there have not been any studies to characterize the transient behavior of leukocyte migration and differentiation in response to hyperglycemia. A 3D human vascular tissue model has been developed to investigate the effect of high glucose concentrations first, on endothelial cell behavior and second, on leukocyte trafficking and differentiation. The 3D tissue model was exposed to varying glucose concentrations of 5.6, 15, and 30 mM for an optimal incubation time of nine hours. The effect of glucose on endothelial cell viability, expression of cell adhesion molecules (CAMs) on endothelial cells, and leukocyte trafficking and differentiation within the 3D tissue model was studied.

Findings and Conclusions: Endothelial cells incubated with high glucose concentrations for nine hours showed no significant decrease in cell viability compared to cells incubated at a normal glucose concentration of 5.6 mM. Among the different CAMs tested, only VCAM-1 showed a significant change in expression in response to the high glucose concentrations. An increase in glucose concentration affected leukocyte migration and differentiation patterns within the 3D tissue model. There were significant increases in certain cell populations for the 3D tissue model exposed to 30 mM glucose, compared to the control at 5.6 mM. For the cells undergoing initial migration across the endothelium into the subendothelial space, T cell and monocyte cell populations increased by 140% and 75%, respectively. For the cells undergoing reverse-transmigration across the endothelium, the immature dendritic cell population increased by 80%. For the cells remaining within the subendothelial space, T cell and macrophage cell populations increased by 380% and 100%, respectively.

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