EFFECT OF ADVANCED GLYCATION END PRODUCTS ON REGULATION OF PLACENTAL GROWTH FACTOR

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EFFECT OF ADVANCED GLYCATION END PRODUCTS ON REGULATION OF PLACENTAL GROWTH FACTOR

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Abstract:

Cardiovascular disease affects nearly half (121.5 million) of the adult US population. In many of these patients, atherosclerotic lesions lead to coronary and peripheral artery disease, causing ischemia of the heart and limbs. Arteriogenesis, a natural compensatory mechanism, restores blood flow to ischemic sites. Placental growth factor (PLGF) is a key arteriogenic growth factor. Interestingly, studies have shown that PLGF is downregulated in people with type II diabetes, potentially contributing to impaired collateral artery growth. However, how diabetes affects PLGF expression is not well defined. Advanced glycation end products (AGE) and inflammatory cytokines such as tumor necrosis factor-α (TNFα) have been implicated in vascular pathology. We therefore hypothesized that AGE-TNFα signaling would impair PLGF expression in endothelial (EC) and/or skeletal muscle cells (SKMC). To test this hypothesis, human coronary artery EC and SKMC were treated with AGE, bovine serum albumin (BSA, as a control for AGE), or TNFα. AGE had no direct effect on PLGF in EC and SKMC. However, TNFα reduced PLGF in EC ($P<0.001$) but had no effect on PLGF levels in human and mouse SKMC. In order to determine whether AGE might influence PLGF in EC or SKMC through a macrophage-mediated effect, RAW 264.7 murine macrophages was treated with AGE or BSA. RAW media was then transferred to murine endothelial cells (EOMA). Treatment with both RAW-BSA and RAW-AGE media significantly reduced PLGF levels in EOMA cells, compared with control RAW media ($P<0.001$). Interestingly, ELISA analysis showed that TNFα was increased in RAW-AGE media, but not in RAW-BSA media or RAW-control media ($P<0.0001$). Analysis of RAW media using a multiplex mouse cytokine assay confirmed that TNFα was increased in RAW-AGE media compared to RAW-BSA and RAW-control media ($P<0.007$). We conclude that AGE may act to reduce PLGF expression in EC via a macrophage-mediated mechanism involving induction of inflammatory cytokine secretion. These new insights have the potential to lead to future therapies to improve collateral growth in patients with type II diabetes.

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LIST OF ABBREVIATION

CVD	Cardiovascular diseases
CAD	Coronary artery disease
PAD	Peripheral artery disease
EC	Endothelial cells
MI	Myocardial infarction
PLGF	Placental growth factor
VEGF-A	Vascular endothelial growth factor-A
AGE	Advanced glycation end products
LDL	Low density lipoprotein
VEGFR	Vascular endothelial growth factor receptor
SMC	Smooth muscle cells
VSMC	Vascular smooth muscle cells
QF	Quadriceps femoris
G-P-S	Gastrocnemius/plantaris/soleus
ECM	Extracellular membrane/matrix
NO	Nitric oxide
MAPK	Mitogen activated protein kinase
ICAM-1	Intracellular adhesion molecule
VCAM-1	Vascular cell adhesion molecule
NF-κB	Nuclear factor-kappa B
JNK	Jun amino-terminal kinases
ERK	Extracellular
RAGE	Receptor for advanced glycation end products
MCP-1	Monocyte chemoattractant protein-1
TNFα	Tumor necrosis factor-α
FGF	Fibroblast growth factor
CCL	Chemokine C-C motif ligand
CD	Cluster of differentiation
DM	Diabetes mellitus
NADPH	Nicotinamide adenine dinucleotide phosphate hydrogen
ALI	Acute limb ischemia
PECAM-1	Platelet endothelial cell adhesion molecule-1
IL	Interleukin
LPS	Lipopolysaccharide
IFN-γ	Interferon-gamma
GLUT	Glucose transporters
BM-MNC	Bone marrow mono nuclear cells
EPC	Endothelial projenitor cells
EOMA	Mouse hemangioendothelioma endothelial cells

HCAEC	Human coronary artery endothelial cell
SKMC	Skeletal muscle cells
HSKMC/MSKMC	Human skeletal muscle cell/Mouse skeletal muscle cell

CHAPTER I

REVIEW OF LITERATURE

Overview of the Cardiovascular System

The cardiovascular system is made up of the heart and the circulatory system. The heart serves as a pump that ejects blood out to the organs, tissues and cells of the body. The blood being pumped contains oxygen and nutrients that is then delivered to every cell. Carbon dioxide and waste product made by those cells are in turn removed by the blood for excretion [1].

The process of circulation is made possible by a complex network of blood vessels. Blood rich in oxygen is taken away from the heart to the body by arteries and arterioles until it reaches the capillaries, and the oxygen depleted blood is then returned back to the heart by venules and veins. As the name implies, in the circulatory system, blood is pumped out and back to the heart in a closed circuit, flowing through all these vessels again and again [2].

There are two circulatory systems in the human body that operate in series. The systemic circulation supplies oxygen and nutrients to the body cells. It also transports disease fighting substances throughout the body, carries hormones from glands to

organs, and delivers waste to the kidneys. The pulmonary circulation delivers deoxygenated blood to the lungs where inspired O_2 is exchanged for CO_2, which can then be expirated, and returns oxygenated blood to the heart [3].

As in the fully grown adult, survival of a developing embryo depends on the circulation of blood to maintain homeostasis and the cellular environment, which accounts for the reason why the circulatory system makes its appearance early in embryogenesis and attains a functional level even before other major organ in the body. That small formed heart begins to beat as early as the fourth week after fertilization.

The heart as a pump is muscular to be able to generate the pressure force needed to circulate the blood and ensure it reaches all the tissues in the body. The heart muscle (the myocardium) therefore, in other to survive, needs a continuous supply of nutrients and oxygen and removal of its metabolic waste. Without this, heart cells soon go through irreversible changes that can lead to cell death. When the heart muscle is damaged, the heart may lose its pumping effectiveness. Depending on the severity of the damage, loss of heart function for even a few seconds can compromise life.

Blood is supplied to the myocardium by the right and left coronary arteries, which branches from the ascending aorta. Blood passes through the capillaries of the myocardium, enters a system of coronary (cardiac) veins which drains into the coronary sinus, and is returned to the right atrium.

Blood is ejected from the ventricles into the aorta and the large elastic arteries that branch off from it. The wall of an artery consist of three layers (Figure 1). The innermost

layer is called the tunica intima or tunica interna. It is made up of simple, squamous epithelium (endothelial cells) and surrounded by a connective tissue basement membrane with elastic fibers. The middle layer is the tunica media, primarily made up of smooth muscle; this is the thickest layer. It gives support for the vessel and it is also the region that changes in diameter to regulate blood flow and pressure. The outermost layer is the tunica externa or tunica adventitia. It attaches the vessel to the external environment. It is made up of connective tissue with varying amounts of elastic and collagenous fibers. The connective tissue in the tunica externa closer to the tunica media is quite dense while the tissue closer to the periphery is loose.

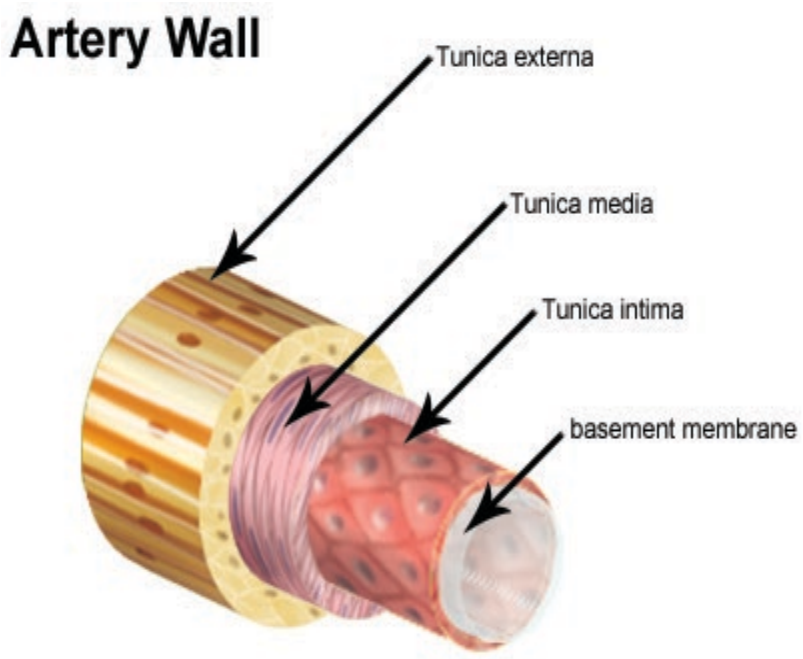

Figure 1. Structure of the artery wall. This illustration shows the component of the artery wall the innermost layer (intima) is at the bottom while the outermost layer (intima or adventitia) is at the top [1]

The capillaries, the most abundant of the blood vessels, form the connection between the arteries and the veins. Capillaries are the site at which O_2, CO_2, nutrients, and waste products are primarily exchanged between blood and tissue. Therefore, capillary distribution reflects the metabolic activity of a particular tissue. Tissues such as skeletal muscles, liver and kidney have abundant capillary networks because they are metabolically active and require an abundant supply of oxygen and nutrients. Other types of tissue, such as connective tissue, have fewer capillaries. The lens and cornea of the eye, and the epidermis of the skin, do not have a capillary network [3].

Veins carry blood from the capillaries back to the heart. Veins have the same three layers as the arteries, but contain less smooth muscle and connective tissue. This makes the walls of veins thinner than those of arteries, and they operate at lower pressures than arteries. Because the walls of veins are more easily stretched by pressure than the walls of arteries, the veins typically contain more blood than the arteries at any given time.

In order to understand the movement of blood through the circulatory system, there is a need to differentiate between blood flow, pressure, rate or velocity, and resistance. Movement of blood through the vessels is termed blood flow. Blood flow rate is the volume of blood passing through the vessels per unit of time. A force is exerted by the heart to cause the blood to flow through the vessels. The measure of that force is the blood pressure. Blood, like other fluids, moves from an area of high pressure to one of low pressure. Thus blood flow rate is directly proportional to the pressure gradient (pressure difference between beginning and end of a vessel). Blood flow velocity is the distance traveled by blood per unit of time. Blood flow velocity is inversely proportional to the total cross-

sectional area of the blood vessel it is flowing through. The larger the area, the slower the flow velocity. Thus, blood moves slowest in the capillaries (which have the largest cross sectional area in the circulation) and fastest in the arteries (which have the smallest cross sectional area in the circulation). Resistance is the force that opposes the flow of blood, and it is primarily related to vessel diameter [4]. The smaller the vessel diameter, the greater the resistance, and the less the flow. By the time the blood is leaving the capillaries and moving into the veins, only a small amount of the pressure applied by the heart remains. Blood flow through the veins back to the heart is not pulsatile and is facilitated by skeletal muscle action, respiratory movements, and constriction of smooth muscle in the venous walls.

Cardiovascular Disease

Cardiovascular disease in general refers to processes that leads to narrowing or blockage of blood vessels, which eventually culminates in a heart attack, chest pain (angina) or stroke. Other conditions that affect the heart rhythm, heart valves, and heart muscles are also part of heart disease. Cardiovascular risk factors include a high body mass index (BMI), large waist circumference, high blood pressure and lipids, elevated levels of inflammatory markers/adhesion molecules, and diabetes [5]. Cigarette smoking is another important risk factor for cardiovascular disease.

Cardiovascular disease (CVD) is a cause of immense health and economic burden in the United States and even globally [6]. Heart and circulatory disease conditions include stroke, subclinical atherosclerosis, congenital heart disease, rhythm disorders, coronary artery disease (CHD), heart failure (HF), valvular disease, venous disease and peripheral

artery disease [7]. The World Health Organization estimates that CVD will cause the death of 23.6 million people by the year 2030 [8].

According to the American Heart Association (AHA) and the Centers for Disease Control and Prevention (CDC), heart disease is the number one cause of death in America, followed by cancer [6]. Approximately 17.6 million deaths were caused by cardiovascular disease globally in 2016, which is an increase of 14.5% from deaths in 2006. Coronary heart disease is the leading cause of death associated with CVD (43.2%) followed by stroke (16.9%), high blood pressure (9.8%), diseases of the arteries (3%), and other minor cardiovascular diseases combined (17.7%). Of the 840,678 deaths recorded in 2016 in America, 635,260 deaths were from coronary heart disease. About 610,000 people die every year of heart disease and about 735,000 people living in America have heart attacks every year. Altogether, cardiovascular disease costs the US about $200 billion every year [9].

Atherosclerosis

Atherosclerosis is a chronic inflammatory disease and is said to be the leading cause of coronary artery disease, peripheral artery disease and stroke [10] and the resultant deaths [11], resulting from loss of elastin, increasing accumulation of lipids, cholesterol and infiltration of additional cells. This pathological remodeling is as a result of repair responses of the arterial wall cells to the stress factors and injuries it experiences. Atherosclerosis affects medium and large arteries, and many cells types are involved in the development and progression of atherosclerosis, including endothelial cells (EC), smooth muscle cells (SMC), and inflammatory cells [12]. The SMC take on a synthetic

phenotype (as opposed to their normal contractile phenotype), and begin to migrate from the tunica media to the tunica intima (inward migration instead of the outward migration that happens in vessel growth). There is also increased synthesis of extracellular matrix fibers, calcification of the vessel wall, foam cell formation, and deposition of cholesterol-containing lipid. These processes are accompanied by inflammation of the SMC and increased migration of inflammatory cells and lymphocytes. Altogether, these events result in the formation of lesions in the intima called plaques, which consist of cholesterol or its esters covered by a fibrous cap. Plaque formation causes a mechanical obstruction in the bloodstream which alters blood flow patterns and can also cause formation of a thrombus (blood clot). As the lesion develops, the plaque can rupture, initiating platelet adhesion/activation and the formation of a thrombus, which occludes blood flow through the artery and causes organ dysfunction [13].

Causes/Risk Factors of Atherosclerosis

Innate/Genetic/Environmental factors

One might assume that atherosclerosis is a result of modern day dysregulation in lifestyle, but it is interesting to know that atherosclerosis dates back to at least 4000 years ago [14]. Studies of embalmed mummies have shown atherosclerotic lesions and calcifications in their arteries, suggesting that the causes of atherosclerosis also include innate, genetic, and environmental factors. Various forms of infection that prevailed in the premodern era may have contributed to promoting atherosclerosis as much as chronic, systemic inflammatory diseases do in today's human beings. Likewise, smoke inhalation from open flames can contribute to atherosclerosis just as well as cigarette smoking [15].

Genetic variations associated with increased susceptibility to atherosclerosis and cardiovascular disease also play a role [16-19].

Age

Evidence suggests that atherosclerosis starts of at an early stage in life and progresses as the individual ages. With repeated cycles of damage and local healing of the arterial wall occurring over a person's lifetime, age can be said to be the most common risk factor for atherosclerosis. However, research has shown that early forms of diet-type atherosclerosis may be reversible; that is, startup of a new lifestyle of a low-fat diet or taking of lipid-lowering drugs despite age, can reduce fatty deposits of plaque in the artery. This reduction is possible as a result of the breakdown of lipids in the plaques, infiltration of macrophages, and a differentiation of macrophages from the proinflammatory M1 to the anti-inflammatory M2 type [20].

Endothelial Dysfunction/Inflammation

Atherosclerosis begins with a response-to-injury-type of mechanism [21]. At first, the endothelium is intact, but when a micro-injury occurs, it leads to endothelial dysfunction characterized by increased permeability, increased secretion of adhesion molecules, and alterations in gene expression. Endothelial dysfunction has been shown to affect more than 100 genes in different signaling pathways, depending on the type of initial stimulus [22]. However, hypercholesterolemia is the major cause of EC dysfunction at the beginning of atherosclerosis. Initially, cholesterol crystals were thought to be passive in atherosclerosis, but their role as active triggers of atherogenic inflammatory response is now well established [23]. Chronic hypercholesterolemia or hyperlipidemia causes

accumulation of lipoproteins, which are then oxidized by locally produced reactive oxygen species to form oxidized low-density lipoproteins (LDL) and oxidized cholesterol esters which in turn stimulate the production of cytokines, chemokines and growth factors by EC and contribute to recruitment of monocytes [24]. In addition, disturbed shear stress patterns are a trigger for the development of atherosclerosis. These patterns naturally occur at branching sites of larger arteries. Therefore, atherosclerosis is more likely to occur at arterial branching sites [25].

SMC Proliferation and Migration

The secretion of growth factors, chemokines and cytokines from the macrophages and EC changes the SMC to a synthetic phenotype, which functions differently than its contractile phenotype in a healthy vessel. The SMC start to proliferate and migrate from the media to the intima. This leads to a formation of a mature atherosclerotic lesion (fibro-atheroma), in which there is continuous production and deposition of extracellular matrix (collagens and proteoglycans). Additionally, macrophages and SMC within the lesion phagocytose oxidized lipoproteins, including cholesterol crystals released by cell death, causing the formation of foam cells. This process causes a fresh release of cytokines that maintains the activation of macrophages and causes recruitment of other leukocytes including T lymphocytes. The end result is a chronic, continuous inflammatory situation [26, 27].

Plaque Formation and Rupture

It is worthy to note that as long as the necrotic core of cholesterol crystals and the calcification around it are enclosed by SMC and ECM proteins (the fibrous cap), then the

plaque remains in a stable state. Nevertheless, the continuous cycle of foam cell formation, production of matrix metalloproteinases that degrade the matrix proteins around the lesions, infiltration and recruitment of other immune cells, and death of SMC, can all lead to the rupture of the fibrous cap. If the cap ruptures, all these necrotic and inflammatory particles are then released into the bloodstream. This can lead to thrombosis and occlusion of other blood vessels, resulting in ischemia and associated cardiovascular events.

Thus, overall, atherosclerosis is established as a specialized inflammatory disease with some possibly immune mediated component [28-30].

Coronary Artery Disease (CAD)

CAD, as noted above, is the leading cause of morbidity and mortality amongst all the diseases of the cardiovascular system and is the leading cause of death in the United States. It results from pathological changes within the coronary artery walls that lead to plaque buildup and breakup, causing thrombosis and reducing blood flow. Clinically, patients with coronary artery disease often present with chronic chest pain or discomfort known as angina pectoris. Angina is the most common symptom of CAD because when the blood vessels do not get enough blood (myocardial ischemia), the first sign is chest discomfort or pain. In more advanced cases, patients may present with acute coronary syndrome (ACS) which can be manifested as acute angina, acute myocardial infarction (AMI; heart attack) in which a portion the heart muscle is deprived of blood and dies [31], or even sudden cardiac death.

There are three mechanisms by which arterial occlusion occurs in CAD. The first is vascular spasm of coronary arteries. This may even occur at the early stages of

atherosclerosis and happens when the damaged blood vessel constricts. The smooth muscle around the artery has a spasm (sudden contraction) which narrows the vessel and prevents oxygen and nutrients from being delivered to the heart muscle. Vasodilator medications may be able to treat this type of acute occlusion, and the disease process itself may still be reversible at this point, for example, if an unhealthy diet is replaced by a low-fat and -sodium diet. This type is brought on by physical examination. The second mechanism of arterial occlusion is due mainly to atherosclerotic plaque forming under the vessel lining, with a lipid core surrounded by an overgrowth and proliferation of smooth muscle and collagen deposition. This plaque bulges into the vessel lumen, causing partial to full occlusion and limiting blood flow. The plaque can also become calcified, "hardening" the artery. The third mechanism of arterial occlusion in CAD is thromboembolism, where breakdown of the plaque triggers platelet aggregation and clot formation (thrombosis) and/or a clot entering the circulation and lodging in a different vessel (embolism).

Currently, the best treatment for coronary heart disease is stent implantation. Various types of stents have been developed and used but the effect is not always permanent [32-35]. For over more than a decade, extensive studies have been performed to describe vascular responses to implanted stents of various type and to show what correlates to the pathobiology evidenced at the stent placement sites. [36]. Adverse reaction to stents is caused by many factors including stent characteristics, individual susceptibility determined by genetic predisposition, procedural factors, clinical factors, and inflammatory response. Thus, there is need for improved therapeutic interventions so as to achieve desired clinical outcomes [36]

Peripheral Artery Disease

The peripheral vascular system is that part of the circulatory system that transports blood and nutrients to the extremities (arms, legs, hands, and feet). Peripheral artery disease (PAD) causes narrowing of the arteries that supply the extremities, limiting blood flow to the skeletal muscles (especially the lower limbs). This reduced blood flow causes a range of clinical symptoms [37]. According to the National Health and Nutrition Examination survey (NHANES) in the United States, PAD affects more than 8.5 million adults, which accounts for ~12-20% of people above 60 years of age [6]. The number of deaths in America attributable to PAD in 2016 was 56,923 while globally, about 202 million people were living with PAD in 2010. That was an increase of 23.5% (164million in 2000 to 202million in 2010) [38].

Since atherosclerosis is a systemic disease, there happens to be a strong association between coronary artery disease, peripheral artery disease, and cerebrovascular disease. The clinical severity of one of these syndromes in a patient predicts the presence of the others. PAD is therefore a marker for systemic atherosclerotic disease, meaning that people with PAD are more likely to have atherosclerosis in other vascular beds (coronary, cerebral, and others) [39]. PAD is a strong predictor of death from vascular causes; the worse the severity of PAD in a patient, the more likelihood there is for death of the patient due to myocardial infarction or stroke. Patients who show symptoms of large vessel PAD have a 25% chance of death due to vascular disease within 12 months, and the mortality rate of patients with severe, chronic PAD is as high as 45% of all cases [40, 41]. Although PAD is thus a strong marker for vascular disease, it is unfortunately under-recognized and under-treated [42, 43]. Patients have no symptoms in almost half of all cases of PAD, and there

is no universal screening program in existence in most developed countries. Preventive measures to prevent the occurrence of PAD was advocated in the United States in 1999-2006 but was only adhered to by 24-35% adult patient [44]

Risk factors for PAD are the same as those for atherosclerosis in general, and include smoking, high blood pressure, hypercholesterolemia/hyperlipidemia, diabetes mellitus, obesity, and a family history of vascular disease. Out of these factors, smoking and diabetes are the strongest markers of the risk for mortality and morbidity from PAD [45]. In patients with a confirmed diagnosis of PAD, diabetes has been shown to increase amputation and mortality rates [46]. It also correlates with increased atherosclerosis development and accelerated rates of cardiac events, especially in patients with poor glucose control [47].

Diagnosis of PAD is made clinically by using the ankle brachial index (ABI) or by the use of imaging [ultrasound, computed tomography (CT), or magnetic resonance imaging (MRI)]. ABI is a ratio of blood pressure measured at the brachial artery (in the arm) to that measured at the dorsalis pedis (posterior tibial artery in the leg). Essentially, the index is calculated by taking the blood pressure at both the upper and lower limb and averaging it. Peripheral artery disease is then diagnosed if the ABI < 0.9. PAD is identified as mild in cases where the ABI is between 0.7-0.9, moderate in cases where ABI is between 0.5-0.7, and severe when the ABI is <0.5. ABI serves to indicate how severe the disease is, and also helps to monitor the progress of existing disease and any response to initiated treatments [48]. Vascular imaging techniques may be used for diagnosis of PAD in symptomatic individuals or in those whose ABI is abnormal for other reasons. It can

identify arterial obstruction in specific vascular sites and also help to demonstrate degree of stenosis, and can be used for assessing the effectiveness of treatment.

PAD is clinically classified into four groups based on symptoms: asymptomatic, intermittent claudication (IC), chronic limb ischemia (CLI) or acute limb ischemia (ALI). The asymptomatic group represents over 50% of patients with PAD and these are the ones that mostly go unrecognized and undertreated by their primary care physicians [49]. Though they look unaffected and still go about with daily functions and activities, they actually have a 40% risk for stroke, and a 20-60% risk for MI higher than in people without PAD [50]. The next group of patients, the intermittent claudication (IC) patients, is said to be the classic type of PAD patient. IC comes with itching pain in the calves, thighs, or buttocks, which the patient feels during activity but subsides at rest. It can also manifest as cramping in the lower legs, and may cause limping. Though it may significantly limit the quality of life, IC is generally stable. With time, only 1-3% of IC patients will require amputation [51, 52]. However, the presence of any of the risk factors mentioned above increases the risk of amputation. The third group of patients has chronic limb ischemia (CLI), which is defined as pain even when the patient is at rest, or by the presence of ulcers with or without tissue necrosis. These patients mostly have an ABI <0.5 and as such, may require amputation. Almost 25% of CLI patients will undergo amputation within one year of diagnosis. They have 1, 5, and 10 year mortality rates of 20-45%, 40-70%, and 80-95% respectively [53]. Finally, in the last group of patients with ALI, there is a sudden onset of limb ischemia which threatens limb viability. It is seen as increasing claudication and progresses quickly to pain even at rest. These patients experience what is known as the six P's of acute limb ischemia (pulselessness, pallor, poikilothermia, paraesthesia and

paralysis). ALI is caused by atherosclerotic plaque rupture and the resulting occlusive thrombus which is formed and blocks blood flow to the limb. In contrast to CLI, the obstruction happens so quickly that it does not allow for the formation of collateral vessels, hence limb viability is compromised. ALI requires prompt revascularization by means of thrombectomy or catheter-directed thrombolysis with a drug such as urokinase. Unfortunately, many times all attempts to save the limb still result in amputation in about 10-15% of cases, and there is a 12 month mortality period in such patients [54].

There are several currently accepted treatments for PAD. Some patients may be helped by lifestyle modification one such example is exercise therapy. Some other options for patients with more severe disease or those who cannot exercise are using drugs to dissolve clots, surgical removal of clots, angioplasty or stent placement to widen blocked vessels, and bypass surgery. In a bypass surgery the blood is diverted around the blocked area by grafting a vessel taken from elsewhere in the body. Despite the fact that these treatments are effective in many patients, they have various drawbacks and are not effective in all patients. That explains the need to develop new treatments for PAD [55].

Therapeutic Angiogenesis/Arteriogenesis for PAD

Finding a way to induce therapeutic angiogenesis/arteriogenesis has long been an important goal for PAD treatment. This process occurs in response to tissue ischemia and inadequate blood supply brought about by occlusion in the large vessels, and results in structural alteration in existing small collateral vessels to enable them to enlarge and function like a natural bypass. An increase in collateral growth (angiogenesis/arteriogenesis) would therefore bring about an effective solution for the

PAD burden [56]. Genetic and stem cell therapy to stimulate angiogenesis/arteriogenesis are potential approaches to PAD treatment. The use of growth factors in the treatment of PAD first began to be investigated about 20 years ago. Various angiogenic and arteriogenic growth factors stimulate blood vessel growth and can be administered as recombinant proteins intraarterially or intramuscularly to promote growth of collateral circulation [57]. Vascular endothelial growth factor (VEGF) and basic fibroblast growth factor showed some promise in early studies, but gave indefinite results in a much larger clinical trial testing [58, 59]. These results suggested the problem was more complicated than just a lack of growth factors. Collison et al put it this way: "impaired responsiveness to angiogenic stimuli…..rather than a problem with the availability of angiogenic growth factors" was the reason for the indefinite results. Other studies have investigated hematopoietic stem-cell transplant as a possible treatment, with research evidence showing that administration of these bone marrow derived progenitor cells promotes new blood vessel formation via a paracrine effect and leads to improvement in ABI by releasing pro-angiogenic growth factors and cytokines that causes blood vessel radial growth in patients with PAD.

Therapeutic Angiogenesis/Arteriogenesis for CAD

Similarly to PAD, CAD can be treated in a variety of ways, including by percutaneous coronary intervention (PCI; angioplasty and stenting), coronary artery bypass grafting (CABG), and by medical treatment to manage underlying disease processes such as diabetes and atherosclerosis. However, development of new and promising therapeutic processes that offer better options is needed. There has been a rise in the number of people for whom angioplasty and stenting is not fully effective. A prevalence of 25.8% of

incomplete revascularization has been documented, with impaired quality of life and increased mortality at three years for these patients as compared to patients with complete revascularization [60]. There has also been an increase in “no-option” patients (patients who cannot undergo PCI or CABG and who despite medical therapy, still suffer from the effects of coronary artery disease).

As discussed above, the body in itself can create vascular bypasses from naturally existing anastomoses. This is a noninvasive process and can prevent tissue ischemia by getting blood across to areas that are distal from the site of occlusion. Stimulation of this process of arteriogenesis may present a better therapeutic approach than current clinical interventions [61]. Improved coronary collateral circulation has been proven to increase survival in patients with CAD, therefore, there have been many studies conducted on interventions aimed at promoting a well-developed collateral circulation [62]. A better understanding of the mechanical factors and the signaling pathways that initiate and control arteriogenesis may identify new targets for treatments to promote the formation of these natural bypasses [63].

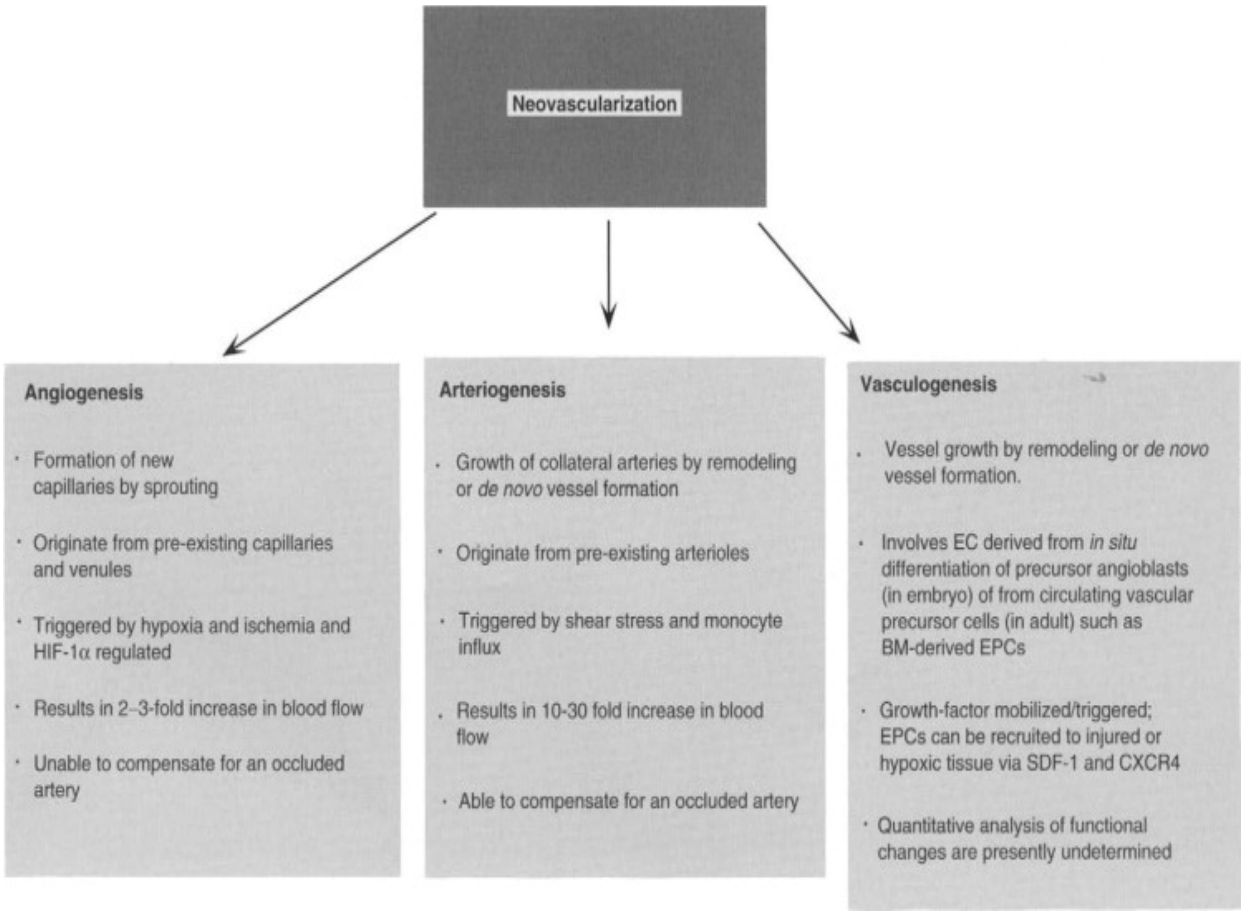

Figure 2. Illustration of key features in neovascularization. Modified from Jose Mann-Garcia 2007 Post-Genomic Cardiology

Coronary Collateral Circulation

Vasculogenesis is the process of development of the cardiovascular system during embryogenesis, where de novo formation of blood vessels occurs from endothelial progenitor cells (EPC) [64]. These EPCs sprout out to form dense vascular networks, with various anastomoses. This network is at its peak at birth, but declines as time goes on by physiological regression, a process called pruning. The remaining collaterals recede in caliber or size and have little blood flow moving through them under normal conditions. However, with changes in vascular pressure and resistance, they can again be recruited. This is often the case in the cause of CAD, where there is the development of a pressure

gradient across a stenotic lesion. This pressure gradient causes an increase in flow through these preformed arterial anastomoses/collateral arteries, and leads to a structural remodeling process termed arteriogenesis. Thus, it can be said that the development of fully functional coronary collaterals depends on the presence of CAD, and that collateral development is highest when there is a total coronary occlusion [62].

Without coronary atherosclerosis, coronary collaterals in patients are in the range of 10-200uM caliber, but in patients with CAD, there can be a four-fold increase and collaterals may be 100-800uM in diameter. This observation was confirmed in an experimental model, where occlusion of the femoral artery in rabbits increased the vascular diameter of pre-existing collaterals by four- to five-fold [65].

Interestingly, there are species-to-species variations in naturally existing collaterals. Humans have been found to have them, but there are variations in number from individual to individual. Guinea pigs have the most abundant naturally existing collaterals, with reports that those collaterals in a guinea pig are enough to compensate for blood flow during stenosis of a major coronary artery. Dogs and cats have also been shown to have well-developed coronary collaterals while rabbits, baboons, and rats have less [66, 67]. The precise basis for these differences between species is not known and is a very interesting question for comparative studies.

Collaterals are networks of arterial to arterial blood vessel connections in between vascular branches [77]. Ordinarily, they have very small radius which makes blood flow through them difficult due to high resistance. But in pathology (atherosclerosis) of a major

coronary artery, they undergo radial remodeling and act as alternative bypass to overcome the resultant induced ischemia.

Arteriogenesis, an outward remodeling of the collateral wall, progresses in three stages. The first stage, known as the initiation stage, starts with the release of chemokines, upregulation of adhesion molecules, and changes in the ECM. The second stage is called the growth phase and features the recruitment and infiltration of leukocytes (particularly monocytes), EC and SMC migration, and an increase in the radius of the lumen and length of the vessel, making it tortuous. The infiltrating monocytes produce a variety of growth factors that contribute to these processes in the growing blood vessel. Finally, in the third stage, maturation and stabilization of the vessel takes place, and the SMC return to their contractile phenotype as opposed to the synthetic phenotype they adopt during vessel growth [68]

Normally, in a non-growing collateral, vascular smooth muscle cells are arranged in a circular and regular structure. However, during the remodeling, the basement membrane disappears. The SMC then begin to migrate to the sub-endothelial layer after adopting a secretory phenotype characterized by prominent rough endoplasmic reticulum, golgi and mitochondria (this is different from their usual contractile phenotype with abundant α-smooth muscle actin and few organelles). They also loose desmin, an intermediate filament protein. This disintegrate the collagen basal matrix and enable smooth muscle cell proliferation, widening the diameter. When the remodeling is complete, SMC goes back into its contractile state [69, 70]. This event is very similar to the smooth muscle cell proliferation that occur in atherosclerosis but that causes an inward migration

of the smooth muscle cells and causes a decrease in arterial wall diameter, reducing pulsatile flow of the arteries. This happens chronically but arteriogenesis is actually a controlled process to restore blood flow and is self-limiting as soon as the process is complete. The collaterals disappear as soon as blood flow is restored to the distal parts of the occlusion [70, 71].

Arteriogenesis vs. Angiogenesis

There is a need to differentiate between angiogenesis and arteriogenesis. Both occur in the adult and cause vascular growth. Angiogenesis is the sprouting of capillaries formed from the existing vascular network. These capillaries elongate and sprout to give rise to a more dense capillary network. Angiogenesis is thought to be primarily hypoxia-induced and VEGF-driven. Hypoxia inducible factor-1α (HIF-1α) and vascular endothelial growth factor (VEGF) have been shown to be key contributors to angiogenesis. Angiogenesis is therefore mainly driven by metabolic demands of the tissue for oxygen [72-74]. Whereas capillary sprouting may decrease the diffusion distance for O_2 within a particular tissue, it cannot increase the overall amount of blood reaching tissues that are distal to occluded sites, because tissue blood flow is controlled at the level of the arterioles. Therefore, angiogenesis is not effective at increasing tissue perfusion [75].

Arteriogenesis, on the other hand, is a type of vascular growth that entails the remodeling of naturally preexisting arterioles, allowing for an overall increase in blood flow to the affected tissue. Unlike angiogenesis, arteriogenesis is regulated by mechanical stimuli and pro-inflammatory signaling pathways rather than hypoxia. It can be seen that hypoxia is not a major influence in arteriogenesis, because there is a long distance between

the occluded region where the collateral arteries are remodeling, and the hypoxic region which is the distal tissue that is being under perfused [76]. Atherosclerotic occlusion drives the adaptive response of arteriogenesis to compensate for lack of blood flow to distal regions of the tissue. Blood is diverted from the blocked vessel into collaterals, causing a change in blood flow and shear stress. The increased shear stress serves as the initial trigger for arteriogenesis to begin. The increase in fluid shear stress causes an increase in a variety of factors including nitric oxide, PLGF [77] and monocyte chemoattractant protein-1 (MCP-1), resulting in attraction and recruitment of monocytes [63, 68].

Interestingly, arteriogenesis shares many common mechanisms with atherosclerosis (a chronic inflammatory disease). Treatments to stimulate arteriogenesis are therefore potentially prone to cause harmful effects in what is termed a "Janus Phenomenon" [74, 78]. This could be one reason why many studies that focused on monocyte activation as a potential treatment, though initially promising, could not be translated into clinical settings [69, 79-82]. The benefit to risk ratio was unbalanced and these researches were discontinued. This is an important consideration for developing future therapies to stimulate arteriogenesis.

Exercise and Arteriogenesis

Arteriogenesis has been shown to be induced in humans as well as animals through exercise training. Exercise increases blood flow to the cardiac or skeletal muscle, altering the fluid shear stress (FSS) in the collateral vessels in the skeletal muscles, which activates the process of vascular remodeling and diameter growth [83-87]. It has also been shown to suppress inflammation and activities of proinflammatory immune cells and to improve

endothelial function [88-90]. Although exercise is clearly helpful, questions remain, such as what type and duration of exercise are effective to induce collaterization (especially in patients with limited physical abilities). In addition, the arteriogenic effect seems to be transient and disappears after the exercise program is terminated. Exercise is also time consuming, which serves as a discouragement to many patients [62]. Nevertheless, exercise has been recommended by the European Society of Vascular Surgery (ESVS) as a treatment guideline for patients with peripheral artery disease [91].

Signaling Pathways

Research shows that arteriogenesis is an active process in which the cells of the vascular wall multiply (as opposed to the vessel just dilating passively).

Mechanical forces such as altered shear stress in the collaterals are the primary stimulus for arteriogenesis [76]. Cell surface receptors on the EC of the arterioles sense this change in shear stress and transduce this signal into the cell to cause changes in its function through various signaling pathways. Through these pathways, transcription factors that control the expression of growth factors such as vascular endothelial growth factor (VEGF-A) and placental growth factor (PLGF) are activated. Shear stress has been shown to increase the levels of these growth factors [77]. Growth factors stimulated the endothelium and cause it to enter the cell cycle. Likewise, smooth muscle cells start to proliferate and produce metalloproteinases (MMPs) which breaks down the basement membrane and collagen, allowing SMC to migrate into the tissues [76]. Eventually, the cell mass of the vessel can increase to about 50 times its initial weight and its total diameter can increase to about 20 times its original diameter [92].

Activated EC also increase their expression of genes that serve as chemoattractant for circulating cells (especially monocyte chemoattractant protein-1, MCP-1), and upregulate the expression of adhesion molecules and surface receptors (such as selectins, VCAM-1, and ICAM-1) that bring about the rolling and tethering of leukocytes (especially monocytes) followed by their migration out into the adventitial space [93] Monocyte adherence to the vessel wall and their subsequent differentiation into macrophages is a very important part of the arteriogenic process. Monocytes produce numerous cytokines, growth factors and proteases that help further destroy the basement membrane to allow smooth muscle cell migration. They also phagocytize surrounding cells, making way for the growth of arterioles [94].

Chemokine receptor 2 on circulating monocytes binds to the MCP-1 on the surface of activated EC, which begins a signaling cascade of events leading to the production of many cytokines, including bFGF and TNFα. TNFα has been shown to cause increased collateral density and conductance and has several actions on EC and monocytes. The TNFα produced by monocytes upregulates the expression of adhesion molecules both on the EC and monocytes, and upregulates granulocyte macrophage colony stimulating factor (GM-CSF), which helps prolong the lifespan of the monocytes. Monocytes have been shown to line the remodeling collateral artery within 12 h after arteriogenesis is initiated by ligation of an upstream artery, but to disappear over time [65]. Monocytes have also been shown to respond to the chemoattractant effect of PLGF, an important arteriogenic factor that brings about more infiltration of monocytes into the vessel wall [95].

There are many growth factors that have been shown to stimulate angiogenesis and arteriogenesis. Of these, one of the best known is vascular endothelial growth factor (VEGF) which is critical in controlling endothelial function. Transforming growth factor (TGF-α, TGF-β), GM-CSF, MCP-1, FGF-2, and platelet–derived growth factor (PDGF) have also been reported to stimulate both angiogenesis and arteriogenesis.

Cell Types

Endothelial Cell

The endothelium is composed of endothelial cells (EC), which play an important role in maintaining normal vascular function. ECs are about 10-20μm in diameter, are flat, and cobble–like shaped with central nuclei. The entire vascular system is lined by a single layer of endothelial cells, with about ten trillion cells forming an almost 1kg organ (heart and blood vessels) in an adult. The endothelium acts as a semi-permeable barrier between the blood and tissues, hence it controls the passage of macromolecules [96]. Endothelial cells were once thought to be inert, but are now known to be active and wholly involved in homeostasis, immune, and inflammatory reactions. They produce and respond to different cytokines and adhesion molecules. It is now also clear that they can have both anti- and pro-inflammatory responses, depending on environmental cues, and that they are major immunoreactive cells.

When there is no disease present, the normal function of the endothelium is to maintain homeostasis, regulate vascular permeability, transport molecules (nutrients, oxygen, waste) between the blood and surrounding tissues, and maintain an atheroprotective environment in the blood stream through production of nitric oxide (NO) and other molecules. EC also release various vasoactive compounds (including NO, prostacyclin, and endothelin) that cause vasodilation or vasoconstriction of smooth muscles, thereby regulating the amount of blood flow into various organs in the body. These vasoactive substances released also prevent platelet aggregation and adhesion, hence creating a non-thrombotic environment and allowing the continuous and steady flow of blood [97-99]. However, endothelial dysfunction or activation can occur, and contributes to many diseases. An uncontrolled endothelial cell response gives rise to many disease states, such as atherogenesis, sepsis, hypertension and inflammatory syndromes. All these diseases are signs of endothelial injury, activation and dysfunction.

In response to pro-inflammatory and procoagulant scenarios, EC can develop an imbalance in oxidative signaling (oxidative stress). There is a decrease in nitric oxide and an increase in reactive oxygen species (ROS) as a result of increased degradation of nitric oxide and decreased eNOS expression [100, 101]. Cardiovascular risk factors such as hypercholesterolemia, diabetes mellitus, hypertension and smoking contribute to this effect. This is because persistent hyperlipidemia and hyperglycemia generate increased ROS, which leads to increased oxidative stress on the endothelial cells. Two common forms of ROS, superoxide and hydrogen peroxide, are produced by vascular cells through several oxidases (NADPH and xanthine oxidases) and by uncoupling of the mitochondrial respiratory process from oxidative phosphorylation. Reactive oxygen species activate EC

to cause increased permeability and synthesis of inflammatory cytokines and adhesion molecules, as discussed below.

In the healthy endothelium, junctional proteins and integrins help to maintain its integrity and play a major role in regulating its permeability, signal transduction, cell growth and survival. Intracellular junction proteins connected to the actin filaments of the EC cytoskeleton create strong cell-cell adhesion [96, 102, 103]. Integrins act as receptors for subendothelial extracellular matrix proteins such as fibronectin. They help to attach the endothelial cell monolayer to the extracellular matrix. This attachment of integrins to the extracellular matrix and intercellular junctions determines endothelial integrity, because lack of it results in increased vascular permeability [104]. Some other important proteins that help create the adhesions and tightness of the endothelial cell layer are occudins, claudins, adherins, and connexins. PECAM (platelet endothelial cell adhesion molecule) is also constitutively expressed on endothelial cells. However, under pathological conditions such as oxidative stress, EC become activated and these normal structures and activities become impaired. Increased vascular permeability can be seen as a result. Increased endothelial permeability causes lipoproteins to accumulate within the sub endothelial space [105, 106]. In this way, EC activation leads to the pathogenesis of many cardiovascular diseases.

Endothelial cells are exposed to blood flow and shear stress. It has been shown that EC are sensitive to shear stress variations, that is, changes in shear stress are then converted to intracellular signals that cause altered gene expression leading to structural and functional changes (vascular remodeling). Therefore, in the setting of atherosclerotic

vascular disease, EC also participate in the vascular remodeling process by downstream signaling that leads to the production and release of growth factors such as VEGF and PLGF [107], which induces arteriogenesis to maintain blood flow to distal sites following an upstream vascular occlusion [108].

Smooth muscle cells

Smooth muscle cells make up the second and thickest layer of the vascular wall, the tunica media. It is the region that gives strength to the vessel and allows it to change in diameter to regulate blood flow. Arteriogenesis involves the differentiation, growth and migration of vascular smooth muscle cells (SMC) and remodeling of the extracellular matrix after infiltration of leukocytes (monocytes) and release of growth factors such as MCP-1 and PLGF.

Formation of a thick new intima through a phenotypically changed and proliferating smooth muscle cell is a significant characteristic of a growing collateral vessel. During arteriogenesis, the vascular smooth muscle cells change from the contractile phenotype to the synthetic phenotype after the destruction of the basal membrane by proteases moves outwardly [109, 110]. At this step of arteriogenesis, there is a loss of SMC structural proteins, especially desmin [111]. With the switch from the contractile to the synthetic and proliferative phenotype, VSMC migrate through the internal elastic lamina into the lumen of the preexisting collateral vessel. Smooth muscle cells are thus primarily responsible for the outwardly remodeling pattern of collateral growth. In atherosclerosis, smooth muscle cell migration is an inward remodeling process, while in arteriogenesis, it is an outward remodeling process. As discussed above, there are many similarities between atherogenic

and arteriogenic signaling; therefore, the mechanism for this bidirectional switch has not fully been characterized. The smooth muscle cells regain their contractile phenotype as the collateral growth reaches completion [64].

An explanation of the relationship between the VSMC phenotype and arteriogenesis has only recently been shown by a study in which a ten-fold increase in smooth muscle contractile proteins (smooth muscle-MHC and smooth muscle α-actin) correlated with vascular smooth muscle cell proliferation and successful collateral growth in response to arterial occlusion in healthy animals and further showed no such correlation in metabolic animals with metabolic syndrome in which collateral growth was reduced.

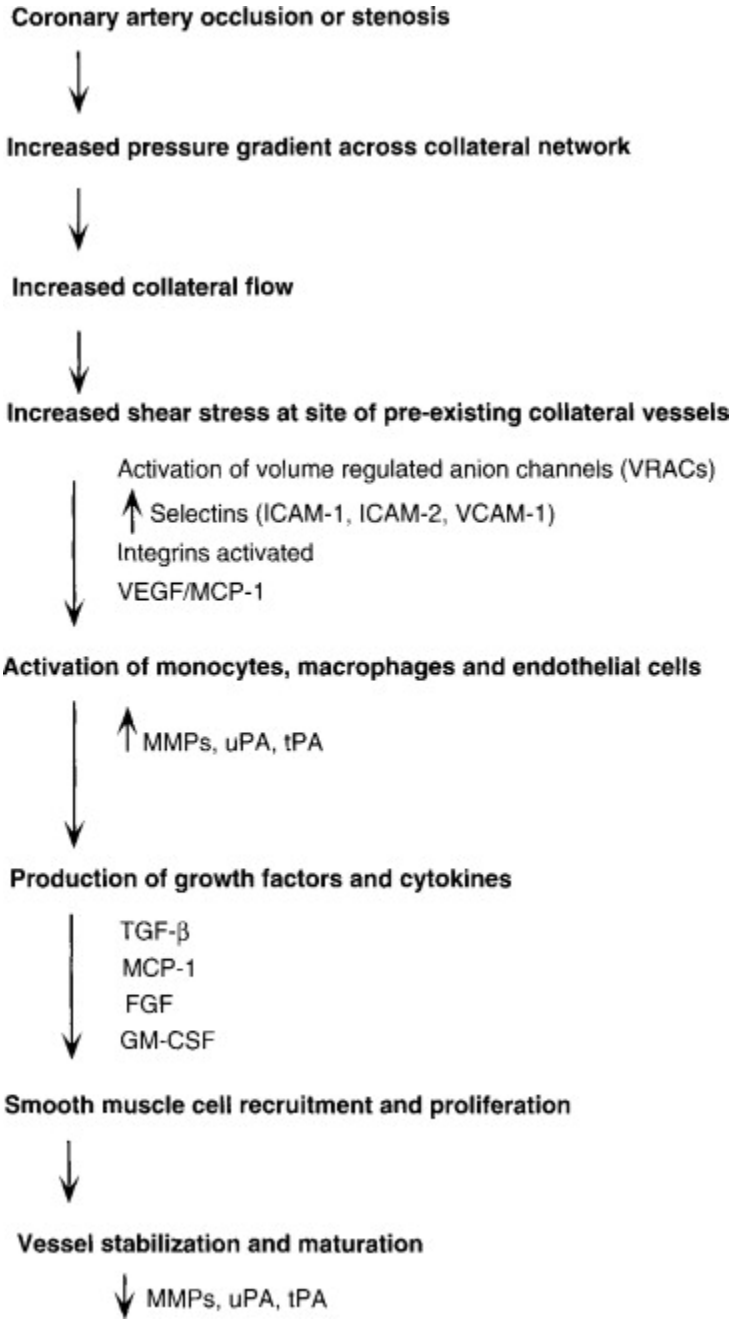

Figure 3. Flowchart depicting events in arteriogenesis, resulting in smooth muscle cell proliferation and collateral growth. Adapted from Post-Genomic Cardiology 2007.

Skeletal Muscle Cells

Skeletal muscles are specialized contractile muscles found in animals and are responsible for movement of the body. They are made up of muscle fibers and surrounded

with protective membranes. This makes them contract and move easily without much friction. Skeletal muscle cells are connected at their ends to the skeleton by tendons. These tendons are connected directly to the collagenous outer covering of the skeletal muscle, known as epimysium. Under the collagenous outer covering, skeletal muscles are grouped into bundles called fascicles, which are again covered by another protective covering made from collagen, the perimysium. This allows nerve and blood vessels to run through the muscles.

Skeletal muscles make up approximately 40% of total body mass, making them the largest tissue in the body. They play a major role in movement and metabolism. Skeletal muscle mass and function are directed in response to changes in nutrition, physical activity, and the environment and disease conditions. Blood from the heart is supplied to skeletal muscles by major arteries (peripheral arteries) and maintenance of their function is a key public health issue as reduction in function leads to unwanted clinical outcomes like poor glycemic control, impairment of bodily movement, reduced mobility, and mortality [112, 113].

Arteriosclerotic plaques can form in peripheral arteries, leading to reduced blood flow to the skeletal muscles (peripheral artery disease) [114]. Although revascularization by surgical intervention may be possible for some patients, patients with critical limb ischemia, do not have the option of revascularization due to extreme tissue damage and prolonged atherosclerotic disease. Skeletal muscles have been said to be the tissue most vulnerable to ischemic damage in the extremities [115]. Hence, there is a need to develop new tissue rescue strategies to preserve skeletal muscles from ischemia. In arteriogenesis, collateral arteries in the skeletal muscle remodel to increase their diameter. Skeletal muscle

produces growth factors such as VEGF, PLGF, and MCP-1 [116], although our lab has shown that the quantities of PLGF produced by skeletal muscle cells is less than that produced by endothelial cells. Skeletal muscle blood vessels are the main sites for arteriogenesis in peripheral artery disease, since the occlusion caused by atherosclerosis happens upstream in the major peripheral arteries. Therefore, studies on arteriogenesis using observations from skeletal muscle are very relevant in showing interactions between growth factors and their receptors and the corresponding radial collateral growth [117].

Diabetes

Diabetes mellitus is a chronic metabolic disease that affected about 171 million people in the world in the year 2000, and is predicted to affect about 366 million by the year 2030 [118]. In the year 2012, about 29.1 million people in America had diabetes, and another 8.1 million people were prediabetic (not aware they were at risk for diabetes), thus allowing more damage to be done to their health. Diabetes is a disease that has resulted in both human and economic loss. Diabetes is primarily characterized by hyperglycemia (persistent high blood glucose), leading to the cells of the body being exposed to excess glucose for a long period of time (both in the cytoplasm and extracellularly). It has been classified into three types: type 1, type 2 and type 3 (gestational diabetes).

Type 1

Type I diabetes is also known as juvenile or insulin-dependent diabetes. It is a situation where the beta cells of the pancreas that secrete insulin die as a result of an autoimmune disease process where the body attacks itself, thus making the cells no longer able to make insulin. Therefore, this means endogenous insulin is not present and the

person has to take synthetically made insulin to keep their glucose level under control. Insulin is the key that stimulates the release of glucose transporters that are then able to bring glucose from the blood into the cell.

Type 2

Type 2 diabetes mellitus is also known as non-insulin dependent or insulin resistant diabetes. The beta cells of the pancreas make insulin, but the issue is that the cells are not sensitive to the insulin; hence, there is no movement of glucose transporters to the cell surface to bring in glucose. Therefore, glucose builds up in the blood. This situation is called insulin resistance, since insulin is present but there is resistance to the insulin-insulin receptor interaction that otherwise activates intracellular signaling to cause translocation of the GLUT (glucose transporters) to the cell surface where they can act as carriers of glucose into the cell. Insulin resistance is a result of a feedback mechanism that results after a long period of high insulin production by the pancreatic cells into the bloodstream leads to a decreased sensitivity to insulin.

Gestational Diabetes

Gestational diabetes, the third type of diabetes, is pregnancy-induced. High blood sugar, obesity, unhealthy eating habits and hereditary factors are possible causes of gestational diabetes during pregnancy. Babies born during to mothers with gestational diabetes are sometimes premature, or may become obese and are at higher risk of developing diabetes themselves later in life.

Effect on Endothelial Function

Under normal physiological conditions, endothelial cells have stable interactions with the underlying smooth muscle cells, have scanty organelles, and contain a flat nucleus. They are in turn separated from the connective tissue underneath them by a thin extracellular matrix called the basal lamina. In addition, the EC are actively carrying out transcytosis, a process of transcellular transport across the endothelium.

The structure of the endothelial cells and their extracellular matrix is compromised in the presence of hyperglycemia. In hyperglycemia, there is an increase in the number of organelles such as the Golgi body and rough endoplasmic reticulum. The basal lamina begins to thicken, leading to impairment in metabolic transport between the blood and tissues. Diabetes also causes endothelial barrier injury, resulting in hyperpermeability and plasma leakage, an important feature of endothelial dysfunction [119].

In normal endothelial cells, specific transport systems move essential circulating blood macromolecules across endothelial cells to the subendothelial layer to meet the metabolic demand of the underlying tissue, while tight junctions (located in between endothelial cells) work as selective barriers to regulate the movement of molecules from circulation. The GLUT gene family transports glucose across the endothelial cells. Of this family, only GLUT-1 and GLUT-4 are expressed by endothelial cells [120]. The former is the most expressed, while the regulation of GLUT-4 expression is important in diabetes and hypoxemia.

Hyperglycemia is an important factor that leads to endothelial cell dysfunction. Chronic hyperglycemia in diabetes is a major factor in the development of both macro- and

micro-vascular disease. A study reported that treatment of human endothelial cells with 25mM glucose resulted in upregulation of several inflammatory genes, including IL-8 and ICAM-1 [121]

Although diabetes mellitus leads to alterations in nearly every organ and cell type in the body, one of the most dramatic effects is on the endothelium, where diabetes leads to endothelial dysfunction and cardiovascular disease. Five main hypothesis have been put forward by different researchers to explain how diabetes causes endothelial cell dysfunction and vascular complications. These include 1) increased polyol pathway flux; 2) increased advanced glycation end-product (AGE) formation; 3) activation of protein kinase C isoforms; 4) increased hexosamine pathway flux; and 5) increased formation of reactive oxygen species and other products of abundant oxidative stress. For the purposes of this discussion, we will focus on AGE.

Advanced glycation end products (AGE), are formed as a result of modification of proteins and lipids and are found in pathological settings including hyperglycemia. When they bind to enzymes, they sometimes affect the physiological activity or even totally inactivate it. The importance of AGE formation especially in vivo started to be recognized in the 1970s with studies on HbA1c, a naturally occurring small human hemoglobin type that is increased in diabetic patients. HbA1c is a post translational product formed when glucose reacts with the N-terminal valine amino group of the b-chain of hemoglobin. It is used as a marker for assessing metabolic indices in diabetic patients and reflects the average blood glucose concentration for a 60-120 day period [122]. AGE found in the diabetic tissues have been found to cause the destabilization of the extracellular matrix. AGE formation is associated with alteration of homeostasis in DM. Intracellular molecules

modified by AGE leads to impairment in growth factor functions [123]. In endothelial cells exposed to high amount of glucose, intracellular AGE has been seen to form within a week. bFGF is one of AGE modified growth factors in EC. Thus, bFGF loses its ability to fully stimulate EC due to its glycation and cannot induce angiogenesis [124]. The receptor for AGE, RAGE, have been shown to be expressed at low levels in the adult in different cell types including EC, macrophages, lymphocytes, VSMC, podocytes and neurons [125] But a significant increase in RAGE expression is caused by diabetes mellitus [125, 126] with increased AGE ligands in the blood and tissues. This report shows the relationship between RAGE expression and its ligand AGE in the diabetic blood, as well as that of RAGE expression in circulating cells and resultant increase in ligand levels in that circulation [127-129]. In EC, SMC and monocytes, ligation of RAGE causes activation of ras21, MAPK signaling pathway and GTPases, which in turn, results in transcriptional activation by NFkB, and other factors [130]. Increased expression of adhesion molecules on EC and monocytes such as VCAM, ICAM and E-selectin follows. Apart from transcriptional activation of various adhesion molecules, RAGE ligation also leads high expression of proinflammatory cytokines IL-1, IL-6, tumor necrosis factor-α [131]. Some of these are known to induce RAGE transcription. Therefore, there is a resultant increase in cellular adhesiveness in DM and hyperglycemia.

Compared with those of similar age groups, diabetic patients often present with more widespread atherosclerotic disease, more severe atherosclerotic lesions and larger number of occlusions. This is because diabetes is linked to endothelial dysfunction hence; it increases the progression of atherosclerotic disease. Endothelial dysfunction is easily

observed and detected in young patients who presents with early signs of insulin resistance with or without other types pf cardiovascular risk factors.

Endothelial dysfunction is a complex process that leads to an imbalance in the production and degradation of biological mediators of normal body function. Increase in oxidative stress is one such imbalance. Normally, reactive oxygen are produced to help to accomplish some functions in the body and thereafter broken down by certain oxidants into nontoxic substances. In diabetes, there is increased production and decreased breakdown of these reactive oxygen species hence leading to damage and alterations in cellular activities. NADPH oxidase is an enzyme that donates an electron to oxygen, leading to the formation of superoxide (ROS), which in turn is converted to hydrogen peroxide (H^2O^2), another ROS, in the presence of superoxide dismutase. Catalase then finishes the degradation by breaking down hydrogen peroxide to oxygen and water but in an immune cells, for example neutrophil that contain phagosome that destroys and engulfs foreign materials, hydrogen peroxide is converted to hypochlorous acid and water in the presence of myeloperoxidase and chlorine in the neutrophil to enhance phagocytosis. Pharmacological inhibition of these enzymes resulted in overproduction of ROS.

Effect on Arteriogenesis

Diabetes mellitus has been shown to be a negative predictor of collateral growth [132] [133]Variations shown by different individuals in the extent of arteriogenesis suggest that the presence of an additional disorder may cause a suppression of the innate ability to develop new vascular growth in response to occlusion. Diabetic patients with coronary disease were studied with non-diabetic patients having similar disease. The former were

shown to have reduced arteriogenesis [134] . This may be the cause for high mortality rates in diabetes-associated coronary and peripheral artery disease. The mechanism by which this occur is still under investigation and is not fully characterized.

The severity of coronary artery and peripheral artery diseases in diabetic patients have been linked to an impaired or altered process of arteriogenesis [135, 136]. Assessment of this impaired mechanism in diabetic patients have been limited to the descriptive imaging of collaterals as shown with the use of angiography or magnetic resonance imaging type of angiography, collateral blood flow and resistance invasive assessment, based on either temporary or chronic coronary artery occlusion and collateral flow measurements and pressure [137, 138]. These studies casted some lights on the intrinsic mechanisms causing impairment in vascular growth in diabetes.

Further studies on effect of diabetes on collateral growth (arteriogenesis) were further illustrated from studies on functional abilities of circulating cells that are essentially involved in arteriogenesis. These includes monocytes, lymphocytes and endothelial circulating cells. It was shown that monocyte response is impaired in an ex vivo analysis [136, 139] Monocytes from diabetic patients cannot move towards VEGF-A, a relevant arteriogenic growth factor. This show a functional defect in the downstream signaling as the kinase function of the receptor, VEGFR-1, was not affected. This reduction in monocytes migration was also seen with other types of growth factors involved in arteriogenesis process [140, 141]

Most documented report on impairment of collateral artery growth resulting from diabetes mellitus are from animal studies. Other reports are also from cell culture in vitro

studies of the different pathophysiological reasons for diabetes-arteriogenic derailment. These studies show that growth factors and their receptors are affected in terms of production and expression; extracellular matrix and other cells that contributes to collateral growth are also affected in same manner. Therefore, the resultant effect of diabetes on arteriogenesis is as a result of defects in arteriogenic growth factor signaling (reduced perception, promotion and interpretation of arteriogenic signal or stimuli [136]

Early evidence that revealed that diabetics may have an inadequate or reduced arteriogenesis was a study of autopsied hearts of diabetes with a history of myocardial infarction. Their vessels reveal on examination intimal layers with endothelial proliferations and focal bulges that narrowed the walls. There was also increased arterial wall thickness due to fibrosis and mucopolysaccharide accumulation that altered the elastic fibers. In patients without DM, arterial wall thickness was observed with increase in vessel diameter but these were both decreased in the diabetics [142]

Another mechanism by which diabetes have been shown to adversely affect arteriogenesis is by elevated production of antiangiogenic factors. Angiostatin, can go against the expression of arteriogenic growth factors and cause reduced blood vessel growth. It is a product of the breakdown of plasminogen by matrix metalloproteinases (MMP-2 and MMP-9). These two are activated when there is oxidative stress or reduced nitric oxide production in vitro. It is also produced by diabetic blood vessels in vivo. Reports have shown that there is increase in angiostatin levels in the diabetic vasculature [143, 144].

As shown earlier, current interpretations for impaired preexisting collateral growth indicates that this is not only based on existing vascular cells found in the vessels but also on circulating cells. Diabetes and hyperglycemia causes endothelial cell dysfunction[145] and so counteracts the rescue mechanism for cardiovascular ischemia. Monocyte function is adversely affected by diabetes. A very important characteristic of monocyte is their ability to migrate towards a created gradient of growth factors or cytokines whenever that is present (called chemotaxis). In a study where freshly isolated monocytes from diabetic subjects were isolated, there was total reduction of monocytes to VEGF-A compared to a strong migration towards an inflammatory mediator tripeptide fMLP. CD34+ cells from the diabetic did not also migrate to growth factor VEGF but displayed cytoskeletal rigidity due to reduced nitric oxide signaling [146]. Monocytes from type 1 diabetic subjects also did not trans-migrate towards proinflammatory CCL2 and CCL3 [147]. Circulating cells from diabetic patients are in an activated status [148], which is replicated by exposure of these cells to hyperglycemia in cell cultures. Significant increase in transcription factors are observed, but displayed a tumor necrosis factor (TNFα) induced adhesion of the circulating cells to EC. Upregulation of cell surface adhesion molecules were also observed, which increases monocyte adhesion but there is generally a reduction in trans-endothelial migration towards growth factor (reduced chemotaxis). Hyperglycemia and diabetes causes the activation of several signaling pathway, hence increase in markers of activation and adhesion molecules. The resultant effect of this is that it makes vascular cells in diabetic to be preactivated and not to respond as they should to migratory signals [148, 149]

There is also evidence of reduced blood vessel growth in diabetics from studies that showed glycation of growth factors, glycation of extracellular matrix proteins that reduces proteolysis and hence affect smooth muscle proliferation and growth in expanding the vascular diameter. bFGF, a growth factor, was glycated following exposure to hyperglycemia reduced its high heparin binding and mitogenic activity. It also lowered its migration towards the EC. This also continued even when it was injected into normal glycemic mice. The glycated bFGF displayed less angiogenic effect than the non-glycated bFGF [124] Advanced glycation end products (AGE) have also been implicated in diabetic reduced arteriogenesis. As shown in a mouse hind limb model, due to AGE deposition, angiographic imaging showed a reduced blood perfusion in the ischemic leg. This was revered by amino guanidine, a substance that inhibit AGE formation , which decreased the amount of AGE been formed and restored impairment in arteriogenesis AGE has been shown to prevent matrix degradation due to its accumulation and amino guanidine restored the process [150].

Studies on bone marrow mononuclear cells (BM-MNC), also revealed the effect of diabetes on collateral growth. There was a decrease in proangiogenic response, which was shown by a reduced capacity of the diabetic BM-MNC to differentiate into endothelial progenitor cells and also by reduced angiogenic function in cultured cells. Placental growth factor (PLGF), belonging to the VEGF family, was able to reverse this impaired function of the diabetic BM-MNC and promote arteriogenesis in the diabetic ischemic model [151].

Placental Growth Factor (PLGF)

Placental growth factor (PLGF) was discovered in 1991 by an Italian scientist, Maria Graziella Persico. She cloned and purified the protein and characterized its structure by crystallography. She also was able to identify VEGF receptor-1 (VEGFR-1) as its receptor, and thus classified it as one of the members of the vascular endothelial growth factor (VEGF) family. She worked along with Dr. Peter Carmeliet in Leuven, Belgium, to show evidence that PLGF is actually quiescent in postnatal growth, but very necessary in vascular embryonic development. PLGF is expressed abundantly in the placenta but is increased elsewhere in several pathological states [152]. Besides the placenta, PLGF is also expressed in vascular endothelial cells, smooth muscle cells, the heart, skeletal muscle, and activated inflammatory cells [116, 153]. In comparison to VEGF-A, PLGF is only expressed in certain tumor cell types [154]. Human PLGF is composed of four isoforms, which are derived after the primary transcript undergoes alternative splicing. PLGF 1-4 differ in size, in how they are secreted, and in binding attributes. PLGF 1-3 bind to VEGFR-1, which is mainly expressed on the EC cell surface, while PLGF 2 binds VEGFR-1 and also neuropilin-1 and -2 (which are also expressed on EC); this is heparin-dependent binding. PLGF-4, the last isoform, also has a heparin binding domain [155]. In the mouse genome, only one isoform of PLGF is seen, which has a 65% similarity to the human PLGF-2 isoform [156]

PLGF is an N-glycosylated secretory protein and exists mainly as a homodimer but can also form heterodimer with VEGF. Its structure contains six cysteine residues within each monomer that form three disulfide bridges, creating a three-dimensional shape called

a cystine-knot. Cysteine residues of each monomer also form two inter-linked disulfide bonds to make up the homodimer. So each homodimer express two cysteine-knot at opposite ends of the PLGF molecule. Even though the human isoform of PLGF has only a 42% amino acid sequence resemblance to VEGF-A, its three-dimensional shape is comparable to that of the latter [157]. The PLGF dimer contains two α-helices and seven β-sheets per each monomer. If one of the two glycosylated ends of the PLGF molecule is mutated, then the binding ability can become reduced which shows that unlike VEGF-A, glycosylation is very important in PLGF receptor binding ability [155].

The angiogenic/arteriogenic properties of the VEGF family of growth factor are mediated by their binding and activation of two tyrosine kinase receptors, VEGFR-1 and VEGFR-2. These two receptors have seven immunoglobulin-like domains that are expressed extracellularly. They also have a transmembrane and intracellular domain, the latter having tyrosine kinase activity. When a ligand binds to the extracellular domain, receptor dimerization takes place, followed by phosphorylation. Though PIGF shares a three-dimensional conformational structure that resembles that of VEGF-A, it has the ability to bind solely to VEGFR-1 receptor [158] with more affinity than VEGF-A or VEGF-B. The immunoglobulin-like domain 2 of VEGFR1 is the site of primary binding of PLGF, VEGF-A and B [159, 160], but PLGF and VEGF-A can also bind to domain 3 on the VEGFR-1 receptor. Binding to domain 3 is more important for PLGF than VEGF-A, since it has been shown that deletion of domain 3 only causes a 50% decrease in VEGF receptor binding, but a 500% decrease in PLGF binding [161].

Though PLGF binds primarily to VEGFR-1, it can indirectly activate VEGFR-2, by binding to VEGFR-1 and throwing off VEGF-A, making it available to bind VEGFR-2

[162]. Another indirect way PLGF can activate VEGFR-2 is if both PLGF and VEGF-A coexist in the same cell, they may form a heterodimer [163].This heterodimer form can bind and activate VEGFR-1 and then stimulate a VEGFR-1/VEGFR-2 dimerization, once the two receptors are upregulated on the surface of the cell [164]. Furthermore, one report shows that when PLGF is the first to bind to the VEGFR-1 receptor, a transphosphorylation of the VEGFR-2 receptor can occur to cause its activation as well [165]. VEGFR-1 (Flt-1) is needed in the first stages of embryonic vascularization and gene knockout results in death in the embryo [166]. Even though endothelial proliferation appeared normal in the knockouts, dysregulated vascular formation was still observed when the receptor was deleted.

In angiogenesis, when both PLGF and VEGF co-exist, it has been shown that PLGF potentiates the effect of VEGF in vitro and in vivo. When bovine adrenal cortex-derived capillary endothelial cells or human umbilical vein endothelial cells were exposed to both VEGF and PLGF, endothelial cell proliferation was 50-fold higher than with VEGF-A alone. This effect of PLGF to increase proliferation was only observed with VEGF-A and not with other growth factors, for example, bFGF. In vitro, heterodimer formation of VEGF/PLGF potentiated the mitogenic endothelial cell response, with more VEGFR-2 binding and tyrosine kinase activation observed. Also, there was greater EC migration with heterodimers, compared with PLGF homodimers [158].

PLGF does not directly promote angiogenesis (capillary proliferation) because it does not stimulate a mitogenic response in EC, but PLGF can increase the mitogenic response of capillary EC to VEGF. The combination of PLGF and VEGF has been shown to stimulate the growth of new capillaries 36 h earlier than treatment with FGF-2 alone,

illustrating that together they induce a faster growth of cells to form capillary endothelium [167]. Lack of PLGF has been shown to disrupt EC responses to VEGF but not to bFGF, which makes PLGF a kind of control switch for VEGF action [162]. In another experiment, PLGF was overexpressed in the skin by using a keratin-14 promoter. This caused an increase in the number of growing blood vessels. This was more evidence of PLGF's strong arteriogenic property [168].

PLGF induces collateral growth more effectively than other growth factors such as VEGF and PDGF because it causes a stimulation of both EC and SMC at the same time, while VEGF modulates just the EC and platelet derived growth factor (PDGF) modulates just the SMC [169].

PLGF acts to potentiate the effect of VEGF by recruiting monocytes/macrophages [170]. It does this by causing an increase in the mRNA levels of interleukin-1β, interleukin-8, MCP-1, and VEGF in peripheral blood mononuclear cells [171]. PLGF is involved in tumor angiogenesis by causing increased infiltration of circulating hematopoietic progenitor cells and also of macrophages to proliferating tumor vasculature [162]. In another experiment, PLGF directly increased the bone marrow recovery at the early stages of ligation by inducing an increased chemoattactiveness of VEGFR-1 bone marrow progenitor cells. In another mouse model, human PLGF was shown to act in synergy with human granulocyte colony-stimulating factor (GM-CSF) to increase the number of circulating hematopoietic blood progenitor cells [172]. In a diabetic wound closure experiment, it was shown that PLGF is needed for attracting monocytes and macrophages to the site of injury. PLGF was produced by infiltrating keratinocytes and by the endothelial cells of the collaterals. An absence of PLGF resulted in lack of wound healing. PLGF was

also shown to cause increased adhesion of monocytes/macrophages to sites of collateral growth during an ischemic hindlimb experiment

As discussed before, monocyte infiltration into the site of collateral growth is essential in arteriogenesis [173-175]. The importance of PLGF in arteriogenesis is evidenced by the discovery that PLGF has chemoattractant properties towards monocytes. Monocyte express only one type of VEGF receptor: VEGFR-1, which is the particular receptor of choice for PLGF to bind to [173]. Therefore, it is clear that there is an important biological role for PLGF and the VEGFR-1 receptor in human monocyte infiltration and activation. VEGFR-1/PLGF binding also stimulates smooth muscle cell growth, another process important in arteriogenesis, and causes an increase in the release of the arteriogenic cytokines TNFα and MCP-1 through recruitment of the monocytes/macrophages [176]. Therefore, we can say that PLGF is an arteriogenic growth factor that acts primarily on monocytes while VEGF is angiogenic and acts primarily on endothelial cells [141, 176, 177].

In tumor studies, PLGF was seen in hyper vascular renal cell carcinomas but not in the normal healthy kidney. VEGF expression was high in human primary thyroid tumors, but PLGF was low [178, 179]. High PLGF expression appears to be limited to certain types of tumors, such as breast and gastric tumors. Low PLGF in other tumors has been suggested to be due to methylation of the PLGF promoter region [180]

In vivo, the decreased arteriogenic response to femoral artery ligation in PLGF knockout mice was recovered by administering exogenous PLGF. This rescue was associated with a PLGF-induced increase in monocyte number at the site of collateral

growth. Conversely, when pharmacological action was used to deplete the monocyte population before femoral ligation was carried out, PLGF mediated collateral growth was hampered. In another experiment in PLGF knockout mice, arteriogenesis was rescued by infusion of exogenous PLGF protein, but infusion of exogenous protein did not restore collateral growth if monocytes were depleted first [181]. Similarly, another study showed that impaired arteriogenesis in response to femoral artery ligation was due to deficient monocyte/macrophages in circulation. There was reduced blood flow 7 d after the ligation in monocyte/macrophage deficient mice in comparison to control mice that had normal levels of circulating monocytes. Immunohistology showed reduced monocyte infiltration at the site of collaterals [182].

In an experiment in PLGF knockout mice, there was no visible defects in blood vessel growth at baseline. But when hindlimb ischemia or myocardial infarction was induced, evidence of a reduced arteriogenic response was observed. Increased collateral growth response to the ischemic insult was only observed in the wild type mice that had PLGF present, with tortuous and enlarged vessels providing evidence of an arteriogenic response to ligation. PLGF levels in the wild type had increased by about 45% after the ligation, resulting in infiltration and migration of monocytes. Interestingly, fibronectin (a glycoprotein of the extracellular matrix that provides a scaffold for migrating SMC) had extravasated or leaked in the wild type at a higher rate compared with the PLGF knockout where it only extravasated at 25%. This observation confirms that the presence of PLGF is essential for vascular permeability and plasma extravasation, an important process in arteriogenesis, to occur [162, 183]

Increased expression of PLGF was also observed after an acute myocardial infarction that showed improved patient therapy as a result of increase in left ventricular ejection [184]. Therefore, a pharmacological intervention to improve PLGF expression or signaling will be a breakthrough strategy that will compensate for atherosclerotic stenosis by improving collateral growth and development [185].

In arteriogenesis, PLGF has been shown to more strongly induce the growth in size and increase in number of collateral arteries following femoral artery ligation compared to VEGF. This was evidenced by a 3-fold improvement in collateral growth with PLGF [176]. Also, knockout of the PLGF gene in a mouse model resulted in an impaired arteriogenic response in the knockout animals when compared to wild type in the situations of ischemia, wound healing and cancer, showing that PLGF is an important determinant of adaptive collateral vessel formation. This scenario was reversed by transplantation of bone marrow from PLGF positive mice, increasing circulating bone marrow progenitor cells induced by presence of PLGF [174].

Shear stress has been shown to be the main activator of arteriogenesis and therefore, our group hypothesized that shear would be the main regulator of PLGF expression in vascular cells. Co-culture experiments using human coronary artery endothelial and smooth muscle cells showed an elevation in PLGF mRNA and protein expression when the cells were subjected to high shear (mimicking the effect of a 60% occlusion in an upstream artery on shear stress in collaterals) [77].

Similarly, PLGF has been shown to be upregulated by mechanotransduction, as seen in an experiment where bronchial epithelial cells were exposed to stretch. PLGF and

nitric oxide were increased in a manner dependent on the amount of stretch and the duration [186]. In another study, when blood was withdrawn from human patients with chronic total coronary occlusion and CAD, more PLGF was seen at the distal coronary vessels. This was correlated to shear stress but not to duration of the occlusion [187].

PLGF mediated signaling has not been extensively studied. PLGF signaling in placenta and in HUVEC showed a difference in downstream components of the signaling mechanism although the upstream activation was the same [188]. In the former, VEGFR-1 activation by PLGF was seen to induce stress activated kinase, JNK and p38 protein kinase, with a small induction of ERK1/2. However, in the latter, PLGF strongly induced ERK1/2 but had low effect on the JNK and p38 signaling pathways. This confirms that there is a difference in the downstream MAPK signaling even with the same upstream signaling (PLGF activation of VEGFR-1). This effect may be due to cell type-dictated influence [189]

PLGF regulation has been shown to be affected by diabetes, though detailed characterization is yet to be described. Streptozotocin induced diabetic mice had abnormal PLGF expression in wounds. When PLGF was overexpressed by gene transfer, wound healing was restored in these diabetic mice. The improvement was a result of increased vascularization and monocyte/macrophage recruitment [190].

The infusion of growth factors has been considered as an alternative therapy to percutaneous revascularization. Vascular endothelial growth factor (VEGF) and fibroblast growth factor treatment have been shown to temporarily improve myocardial blood flow and function in experimental models, but clinical trials of their use were not successful. A

recent study examined whether PLGF might be able to induce revascularization without some of the negative side effects observed for VEGF. In this study, mice were fed a high cholesterol diet for 4 wks. and chronic myocardial ischemia was induced by transient ligation at 8 wk. Either recombinant PLGF-2 or vehicle was then randomly infused into the mice. The results showed an increased PLGF level in the plasma, without any negative systemic inflammatory or hemodynamic effects. Furthermore, there was no negative effects on atherosclerotic plaque growth, composition, or vulnerability as had previously been seen following VEGF infusion [191] and cell adhesion molecules were not upregulated by PLGF compared to vehicle. PLGF treatment significantly improved myocardial function and increased capillary and collateral density [192]. The researchers in this particular experiment used PLGF-2 because compared to PLGF-1; it has a heparin binding motif that confers an interaction with coreceptors neuropilin 1 and -2 on it, which makes for enhanced signaling and increased chemotaxis and sprouting of endothelial cells [193].

A similar study conducted in pigs with chronic myocardial ischemia found that administration of recombinant PLGF-2 improved myocardial blood flow and function. In addition, there were no adverse effects observed. The lack of adverse effects is particularly important because other studies had shown an adverse effect of exogenous PLGF on early formation of atherosclerotic plaque and on carotid artery injury of rabbits 9 d after local adenoviral delivery of PLGF [194, 195].

Advanced Glycation End Products (AGE)

As discussed earlier, advanced glycation end product (AGE) formation is one of the mechanisms by which diabetes hampers arteriogenesis. One aspect of the metabolic syndrome in diabetes is hyperglycemia, which is characterized by an excess level of glucose in the blood plasma for a long time. AGE, a heterogenous class of compounds formed from modified proteins and lipids and nucleic acids, are formed in the hyperglycemic state. Formerly, AGE was thought to only be formed outside the cell through a non-enzymatic reaction between glucose and the proteins that exist extracellularly, but now we know that AGE is also formed within the cells at a much greater rate [196]. The whole process starts with the Maillard reaction, in which the carbonyl group of glucose reacts with the amino group of another molecule (protein, lipids, nucleic acid) to form a Schiff base. This is formed within a few hours. This Schiff base can further undergo a rearrangement inside the molecule itself to form what is known as Amadori products, which are more stable. It takes several days to form an Amadori product. The Amadori product can further form irreversible products [197] or it may break down into various compounds. If it does not break down, it can begin to cross-link other proteins, eventually leading to the formation of AGE. AGE formation on an enzyme may cause its complete inactivation (lack of function) since protein shape or structure correlates with their functions. Similarly, extracellular matrix proteins, when glycated, have impaired function and may not be easily broken down by proteolysis (an important process in vessel proliferation and growth). Carboxymethyllysine (CML) has been identified as the most abundant AGE in vivo due to its high degree of accumulation in tissues [198, 199].

High amounts of AGE in skeletal muscle and blood have been found to cause reduced muscle mass, reduced grip strength and movement in the elderly and in patients with diabetes mellitus [200-203]. This shows that there is a negative correlation between AGE and skeletal muscle function. This relationship was found to occur as a result of dietary consumption of foods rich in AGE. Foods cooked at high temperatures, high fat foods such as butter and sesame oil, and tobacco have all been found to contain AGE products which can be absorbed into the body through consumption [204, 205]. AGE are not well absorbed, so this had made the ill effect of dietary consumption go unnoticed for a while, but recently data collected from a randomized trial implicated dietary AGE as a cause of chronic diseases such as diabetes, CVD and chronic kidney diseases, which they promote by causing increased inflammation and oxidative stress [206]. When a reduced dietary intake of AGE-enriched foods was established, insulin resistance, endothelial dysfunction and oxidative stress was improved [207].

In an experiment on the long term effect of consumption of an AGE-rich diet on skeletal muscle function, mice were fed either a high AGE diet or a low AGE diet for 16 wk. Mice fed the high AGE diet had less muscle mass, reduced skeletal muscle function, and the accumulation of a CML type of AGE product in the skeletal muscle [208].

AGE is associated with homeostatic imbalance in diabetes [209] where it causes extracellular cross-linking and alterations in the interactions between extracellular matrix and cells. On collagen, AGE cross-linking causes an expansion of the molecule, impairing its function as an intact barrier. It also changes the structure of the cell binding domain of type IV collagen, decreasing endothelial cell adhesion [210]. AGE also leads to glycation of growth factors [123] and affects enzymatic signaling pathways (for example, production

of nitric oxide). AGE has been shown to decrease vessel wall elasticity in diabetic rats, and to cause a rise in fluid leaking across the vessel wall [211]. In all the above examples, the alterations caused by AGE were not due to specific interactions with cellular proteins. However, AGE has also been shown to interact with some cell-surface proteins that normally function in the removal or neutralization of AGE (for example, macrophage scavenger receptors) [212]. There are also receptors that can lead to modified gene expression when they interact with AGE [213, 214]. An important example of this is the receptor for AGE (RAGE), which has been much studied and has been shown to act as a signal transduction receptor [215].

The receptor for AGE, RAGE, was discovered as a cell surface receptor for AGE in cultured vascular cells from studies on diabetic complications [215] and RAGE was also found to be present on blood cells. Through this receptor AGE can interact with endothelial cells, peripheral blood-derived monocytes/macrophages and vascular smooth muscle cells. The effects of AGE can be reversed by treating cells with antibodies against the receptor or by the use of soluble RAGE, which is a decoy form of the RAGE receptor that possesses an extracellular domain but lacks the intracellular domain to cause downstream signaling [213, 214, 216-218]. Studies have shown that RAGE is expressed at low levels in different cell types including endothelial cells, macrophages, lymphocytes and vascular smooth muscle cells [125, 219], but diabetes causes an increase in RAGE expression [125, 126]. This is in line with increased AGE levels in the blood [220] and in the tissues as well [221, 222]. This shows an overlap of RAGE/AGE interaction in diabetic blood vessels, as well as RAGE presence on circulating cells with increased AGE levels in the blood [127-129] and underscores the importance of RAGE as a propagator of cellular dysfunction.

The RAGE receptor has a molecular weight of 35 kDa, and it is made up of three extracellular domains, with the V-type acting as the principal domain for ligand binding [223]. It also has a transmembrane and an intracellular domain that transmit signals intracellularly for a RAGE-mediated response to occur [224]. Upregulation of RAGE on the cell surface is regulated by nuclear factor kappa B (NF-kB), interferon-γ response element and IL-6 DNA factor, which all have sites on the RAGE promoter [225].

In EC, SMC and monocytes, RAGE-AGE binding can cause cellular activation leading to ras21 and MAPK signaling cascade activation [130, 226]. There is also an increase in oxidative stress in endothelial cells as a result of RAGE/AGE signaling due to an increase in NADPH oxidase, which then leads to the activation of many other signaling pathways [227]. With MAPK and oxidative stress signaling, the NF-kB transcription factor is activated, which further leads to an increase in the upregulation of adhesion molecules on the EC and monocyte. These include ICAM-1, VCAM-1 and E-selectin. This increase in adhesion molecule expression increases the adhesion of monocytes to EC and ECM [131, 147]. In addition, there is high proinflammatory cytokine production (TNF-α, IL-1 and 6, MCP-1) which further causes more RAGE upregulation on the cell surface [131, 225, 228]. MCP-1 was the first cytokine to be discovered because it is highly abundant and plays the role of attracting monocytes/macrophages to sites of vascular growth/inflammation. It aids the migration of monocytes/macrophages into the vessel wall. Different cell types such as EC, SMC, monocytes, epithelial cells and fibroblasts also produce it. TNF-α also acts to further promote the chemoattractant action of MCP-1 [229]

Continuous signaling through RAGE/AGE produces an increased activation of MAPKs and GTPases, key factors for the needed migratory response of

angiogenic/arteriogenic cells during new blood vessel growth. Therefore, along with the increase in monocyte cellular adhesiveness in diabetes and hyperglycemia, monocytes lose their natural ability to migrate towards the normal gradient of angiogenic/arteriogenic growth factor stimuli created, for example, by VEGF [139] and PLGF [230].

Negative coronary artery remodeling in diabetes has been associated with an increase in glycated blood protein (albumin) and a decrease in the soluble RAGE receptor in diabetic patients [231].

Endothelial cells in diabetic patients have been shown to be constantly exposed to circulating AGE. Nitric oxide, a substance that promotes vasodilation, has been shown to be reduced in the vessel wall of patients with type 2 diabetes due to elevated production of superoxide caused by an interaction of RAGE/AGE and TNF-α signaling. In this experiment, RAGE was upregulated in diabetic coronary arterioles and as a result augmented the expression of TNF-α. To prove this, sRAGE and NF-kB inhibitor, was used to attenuate the increase in TNFα and RAGE [232].

Diabetes-associated accumulation of AGE is thought to induce overproduction of TNF-α from macrophages. This effect has been implicated in reduced vascular growth [233].

Monocytes

The blood, the medium of nutrient transport to all parts of the body, has three specialized cellular elements (erythrocytes, leukocytes and platelets), all suspended in the liquid part, also called plasma. Monocytes are one of the five types of leukocytes (white blood cells) found in the blood and represent about 2-10% of leukocytes. The others are

neutrophils, eosinophils, basophils and lymphocytes. Monocytes and lymphocytes are known as mononuclear agranulocytes because they have one nucleus but lack membrane-enclosed granules in their cytoplasm. The monocyte is the larger of the two, with a kidney shaped nucleus, while lymphocytes have a spherical shaped nucleus and are the smallest of all five leukocyte types. The other three are termed polymorphonuclear granulocytes. They have many-lobed nuclei and contain granules [4].

Monocytes are migratory because they can leave the blood under certain pathological conditions and during inflammation and move into surrounding tissues. They can divide and when stimulated, can differentiate into macrophages (macro=large; phages=eater). The reaction that occurs between the blood and the surrounding tissues in response to injury is called inflammation. It results in redness of the site, pallor, swelling and hotness (heat). The macrophages work as phagocytes, ingesting foreign materials, bacteria, and also work in tissue repair [234]. They are involved in both innate immunity and also influence the process of adaptive immunity. They have multiple roles in immune function, including: acting as tissue resident macrophages in normal conditions (ready to phagocytize any incoming foreign object), migrating to sites of resolution or injury within approximately 8-12 hours in response to signals of inflammation, and differentiating into macrophages or dendritic cells in order to cause an immune response. Half of the monocytes produced are stored in the spleen [235]. They can then transform into macrophages after penetrating the appropriate tissues. They can also transform into foam cells in the endothelium especially after ingesting lipids.

There are three classes of monocytes in human blood which are classified based on the type of receptor they upregulate on their cell surface [236]. The first is known as

classical monocytes. They have a high expression of CD14 cell surface receptor ($CD14^{++}$ $CD16^{-}$). The second is known as the non-classical monocyte. It expresses a low level of the CD14 receptor but has an additional co-expression of the CD16 cell surface receptor ($CD14^{+}$ $CD16^{++}$). The third class is the intermediate monocyte with high level of CD14 and low level of CD16 receptor expression ($CD14^{++}$ $CD16^{+}$) [237]. The level of CD14 cell surface receptor on a macrophage can be used to differentiate between the different classes of monocyte, especially between the non-classical and intermediate. Studies show that the intermediate monocytes is a unique class of monocytes because of their high expression of surface receptors which are involved in reparative processes, especially VEGFR-1 and 2 and because they are produced abundantly by the bone marrow [238]. They also produce an increased amount of proinflammatory cytokines like TNF-α and IL-12 after stimulation with microbial agents.

In mice, monocytes have two classes. Inflammatory monocytes ($Cx3CR1^{low}$, $CCR2^{+}$, $Ly6C^{high}$). This class compares with the human classical $CD14^{++}$ $CD16^{-}$ class of monocytes. Second in mice is the resident monocytes ($Cx3CR1^{high}$, $CCR2^{neg}$, $Ly6C^{low}$) that compares to the human non-classical $CD14^{low}$ $CD16^{+}$ monocytes. Resident macrophages in mice roam around the endothelial cell wall both in physiological and inflammatory state [239-241]. The classical and non-classical monocytes in man crawl and do not roam along the endothelial wall [242].

Monocytes are the largest of the leukocytes [243] and are produced in the bone marrow and circulate in the blood for 1-3 days before moving into tissues in different parts of the body where they differentiate into macrophage and dendritic cells. Monocytes can be made to differentiate into dendritic cells in vitro by adding the GM-CSF cytokine and

IL-4 [244]. Therefore, overall monocytes are involved in phagocytosis, antigen presentation to T cells, and cytokine production.

Substances produced by other cell types can influence the chemotactic behavior and other functions of monocytes. These include chemokines such as monocyte chemotactic protein-1 (MCP-1 or CCL2) and monocyte chemotactic protein-3 (MCP-3 or CCL7) and some arachidonic acid metabolites, like N-formyl methionine leucyl-phenylalanine which is made by bacteria and can activate the formyl peptide receptor 1 on the monocytes [245]. Some microbial products can even directly activate monocytes. This gives rise first to production of pro-inflammatory cytokines and later, anti-inflammatory cytokine production. Cytokines produced by monocytes include TNF-α, IL-1 and IL-12.

It is important to note that the monocyte count, expressed as a percentage of monocytes relative to other leukocytes, can be used to express a prevailing situation or disease. Monocytosis is when there is the presence of an excess amount of monocytes. It gives an indication of certain disease states such as chronic inflammation, atherosclerosis, necrosis, and immune-mediated disease [246]. For instance, a high count of CD14+CD16++ monocytes may indicate sepsis [247] while a high count of CD14++ CD16+ intermediate monocytes is a predictor of cardiovascular disease in high risk populations [248, 249].

Monocytopenia occurs when there is a decrease or deficiency of monocytes. This is usually the case after treatment with glucocorticoids (immune-suppressive agents) [250].

Adherence of monocytes to the vessel wall and differentiation into macrophages is a very important event in collateral growth. Monocytes produce a host of cytokines, growth

factors and proteases (to break down ECM) needed in collateral growth [65, 251]. Monocyte infiltration into growing collaterals has been observed as early as day 3 after occlusion in an experiment of hindlimb ischemia that revealed abundantly growing collaterals [251]. Others also have reported that an increase in migration of blood monocytes increases collateral growth, and that a reduction in the number of infiltrating monocytes reduces arteriogenesis. In that study, when an exogenous infusion of monocytes was given, arteriogenesis was improved, demonstrating the importance of migrating monocytes in arteriogenesis [252].

PLGF is a key growth factor produced during the early stages of arteriogenesis. It acts as a chemoattractant for monocytes and interacts with the VEGFR-1 receptor on the monocytes [177, 253]. Similarly, activated EC produce MCP-1 in response to shear stress which activates the MCP-1 receptor, chemokine receptor 2 (CCR-2). In response to these signals, monocytes infiltrate the vessel wall and produce additional factors that stimulate arteriogenesis. For example, monocytes produce TNF-α and bFGF at sites of collateral growth [254]. TNF-α was shown to contribute to arteriogenesis in a study where LPS (a known inducer of TNF-α) was administered. TNF-α further promotes the monocyte response by causing an upregulation of adhesion molecules on both endothelial cells and monocytes. TNF-α and bFGF are mitogenic for EC and SMC and bFGF also potentiates the effect of VEGF [63]. The binding of CCR- 2 to MCP-1 also stimulates the monocytes to produce MMPs that break down the extracellular matrix as they progress into the wall to create space for expansion of the vessel.

In an experiment to illustrate the importance of monocyte migration, New Zealand white diabetic rabbits were subjected to femoral artery ligation. They developed collateral

arteries with a narrow diameter, which had reduced blood flow compared to collaterals in control rabbits. When monocytes from these animals were checked for VEGF-A and MCP-1 mediated chemotaxis, the response of the diabetic monocytes was significantly reduced compared to the non-diabetic monocytes. This was a very specific reduction, as the monocytes only failed to respond to VEGF-A and MCP-1 but responded to other chemoattractant such as FormylMetLeuPhe (fMLP) [255].

In another experiment, cell transplantation was used to enhance homing of macrophages to an area of collateral growth, yielding a strong arteriogenic response. This result suggests that targeting the innate and adaptive immune response might be an effective way to clinically boost collateralization. Similarly, another study systemically transplanted human macrophages into a mouse model of hindlimb ischemia. Collateral growth was strongly increased, as shown by more collateral arteries within the adductor muscles and better clinical scores. There was also a significant reduction in TNF-α, IFN-γ, IL-4, IL-5, IL-6, and IL-10 8 h after the systemic monocyte transplantation. These authors speculated that treatment with one growth factor produced by macrophages cannot be as effective at improving arteriogenesis, but that monocyte transplantation would allow the monocytes to locally secrete all of the necessary growth factors and cytokines in the correct amount and at the appropriate time, and to communicate with other cells involved in collateral growth, thereby successfully promoting a localized process of arteriogenesis [256]

Inflammation

Inflammation is a local response of a tissue to damage or infection that has a strong vascular component. In inflammation, there is an increase in the blood supply to the affected area. The endothelial cells shrink, causing an increase in capillary fluid filtration and allowing cells from the blood to extrasavate into the tissues. Leukocytes, monocytes, and lymphocytes crawl out of the bloodstream to participate in the inflammatory response and resolve the injury. The cytokines produced by these infiltrating cells and by the endothelial cells can penetrate the tissues as well.

Cytokines are small proteins that act in an autocrine (acting on cells that produce them) or paracrine (acting on neighboring cells) manner. They produce their action at a short distance around where they are secreted rather than at a distance (no endocrine action). They are very potent, and induce their action even at picomolar or femtomolar levels. In it all, their production is usually transient and tightly regulated. Examples include interferons, interleukins, colony stimulating factors and chemokines (which have chemoattractant properties and direct the movement of cells during migration from bloodstream into inflammatory tissues [94] Generally, cytokines are classified into two categories. First are the pro-inflammatory cytokines which include interleukin-1 (IL-1), tumor necrosis factor (TNFα), interferon gamma (IFN-γ), IL-6, IL-12, IL-18, and granulocyte-macrophage colony stimulating factor (GM-CSF), a chemokine [257]. The second group are the anti-inflammatory cytokines, which include IL-4, IL-10, IL-13, IFN-α, and transforming growth factor-β (TGF-β) [258]. The interplay between these two groups determines what type of inflammation would occur at a given site of action. An imbalance of one group over the other drives certain pathological states.

Adaptive collateral vessel growth (arteriogenesis) is an inflammatory process that involves the production and release of pro-inflammatory cytokines, infiltration of monocytes into the tissues and production of growth factors that trigger the growth and expansion of small existing collaterals [259]. Shear stress is the triggering force that initiates arteriogenesis, and is closely followed by the migration of inflammatory cells. Inflammatory cell infiltration is critical for arteriogenesis, since a reduction in the number of infiltrating cells, a blockage of their adhesion [260] to endothelial cells, or prevention of their migration attenuates arteriogenesis [261]. Infiltrating bone marrow derived macrophages have been shown to be a major determinant of growth factor production. In a mouse hindlimb ischemia model, bone marrow transplantation from PLGF knockout mice to wild type mice led to a significant reduction in blood vessel growth [262]. In collateral growth, monocytes/macrophages release inflammatory cytokines such as TNF-α, IL-1 and IFN-γ. These cytokines cause endothelial adhesion molecules like ICAM-1 and VCAM-1 to be upregulated at sites of inflammation. Signaling through pathogen associated molecular pattern receptors (PAMPs) and Toll-Like Receptors (TLRs, especially TLR-2 and TLR-4) expressed by macrophages has also been implicated in arteriogenesis in some studies [69, 263-265].

Advanced glycation end products (AGE) and inflammation

As discussed in earlier sections, AGE (products formed from irreversible non–enzymatic glycation of proteins, lipids and nucleic acid), are toxic substances found in the plasma which are implicated in diabetes and cardiovascular diseases. AGE are also associated with inflammation and oxidative stress [266, 267]. AGE have been proven to be antigenic and to induce an immune response in the body [268, 269]. AGE can interact with

two types of receptors: macrophage receptors and the receptor for AGE (RAGE) [270]. Macrophage scavenger receptors (mentioned above) are responsible for AGE capture, removal, and breakdown. Recently, studies have shown that AGE can also signal through receptors found on the surface of macrophages. Examples include the TLRs and CD14 [271]. RAGE is expressed on endothelial cells, on the surface of macrophages, and in various tissues including heart and skeletal muscle. Activation of RAGE stimulates a specific signaling pathway in response to AGE binding. In macrophages, AGE has been shown to cause elevated production of IL-1β and TNF-β via RAGE signaling [272].

Many studies have linked AGE to the endothelial dysfunction observed in diabetes, by causing overproduction of proinflammatory cytokines in macrophages, leading to macrophage malfunction and death [273, 274]. Glycated albumin, one type of AGE, stimulates excessive production of IL-8, IL-6 and TNF-α [275]. In fact, regression analysis shows that the glycated albumin concentration is correlated with the level of TNF-α and the glucose concentration in the blood of diabetic patients [276]. Interestingly, some researchers have suggested that AGE affects TNF-α production and TNF-α in turn, affects AGE levels [275, 276]. Therefore, given the strong relationship between AGE, inflammatory signaling, and diabetic complications, concerted efforts have been made to develop agents that would break down AGE or reduce the damage caused by AGE. Amongst these initiatives are the use of AGE inhibitors and breakers (aminoguanidine, alagebrium, statins, antioxidants, natural phenolic substances) and also the use of anti-inflammatory molecules [277-280]. Anti-inflammatory drugs and AGE inhibitors that act on activated macrophages have been used to suppress AGE induced overexpression of cytokines [281, 282]. Studies have shown that inhibitors of AGE have anti-inflammatory

actions, and can deactivate macrophages and reduce excessive production of cytokines that cause tissue damage. Interestingly, these studies also show that macrophages themselves can secrete AGE and that blocking the production of AGE in macrophages can reduce AGE levels and excessive cytokine production [283]. In summary, AGE is involved in the production of pro-inflammatory mediators, which in turn is implicated in the pathogenesis of diabetes-associated cardiovascular disease.

From this literature review, it has been established that arteriogenesis is reduced in coronary and peripheral arteries by diabetes, but that the mechanism by which this occurs is not well defined. Therefore, the key questions that our lab seeks to answer are: 1) how does diabetes inhibit arteriogenesis? and 2) what are the signaling pathways involved? Since PLGF is the key arteriogenic growth factor involved in monocyte recruitment and collateral growth, our group is currently working to determine how diabetes affect its expression as a step towards answering these key questions.

Previous results from our lab indicated that PLGF is highly expressed by endothelial cells, and is also found in skeletal muscle and cardiac myocytes. Critically, we found that the PLGF protein level in skeletal and cardiac muscle was reduced in mice fed a Western diet. This reduction was apparent both at the basal (unstimulated) level, and in the response to femoral artery ligation (a physiological stimulus for arteriogenesis). Therefore, it seems likely that reduced PLGF expression could play a key role in the inhibition of arteriogenesis, which has been observed in diabetes.

To investigate the mechanism for this reduction, our group previously conducted in vitro treatment studies of endothelial and skeletal muscle cells in which we attempted

to isolate the effect of various metabolic factors involved in the diabetic phenotype by exposing cells to cholesterol, glucose, insulin, and oxidative stress. Since PLGF levels were not affected by any of these factors, we were led to consider what additional factors found in vivo could be tested. In our prior in vivo study, advanced glycation end product (AGE) levels were elevated in hindlimb skeletal muscle. As described in this review, there is an established connection between AGE and vascular dysfunction in diabetes. Therefore, the overall goal of the present study was to test the hypothesis that AGE inhibits PLGF expression in endothelial and skeletal muscle cells.

Previous results in our lab also revealed that PLGF levels in human coronary endothelial cells were decreased by treatment with recombinant tumor necrosis factor alpha (TNFα). AGE is known to induce TNFα expression, as discussed in this review; therefore, we further hypothesized that AGE would exert its effects on PLGF through TNFα.

In the present study, we tested this hypothesis by examining both the direct and indirect effects of AGE on PLGF expression in HCAEC and SKMC. We also investigated the potential role of macrophages in this signaling pathway, as well as the influence of endothelial cell activation state on responsiveness to AGE. These studies have generated new insights into a potential mechanism contributing to the diabetes-induced impairment of arteriogenesis and may lead to future therapies aimed at enhancing collateral growth in diabetics.

CHAPTER II

MATERIALS AND METHOD

Human Primary Cells

Human coronary endothelial cells (HCAEC) and human skeletal muscle cells (HSKMC) were purchased from Lonza. Cells were grown in either endothelial cell growth medium (EGM-2MV) or skeletal muscle cell growth medium (SKGM), respectively, supplemented with growth factors (Lonza). Cells were grown to confluence at 37^0C in a humidified incubator containing 5% CO_2. Culture medium was changed the day after cells were subcultured and refreshed every other day thereafter. For experiments, equal numbers of cells were seeded into each well of 6- or 12-well plates. HCAECs were used at passage 4 and HSKMC were used at passage 5.

Established Cell Lines

Mouse hemangioendothelioma endothelial cells (EOMA) was purchased from American Type Culture Collection (ATCC). Cell were grown in Dulbecco's Modified Eagle's Medium (DMEM/High Glucose, Invitrogen), containing L-glutamine, and supplemented with 5% fetal bovine serum and 1% penicillin/streptomycin. Mouse skeletal muscle cells (C2C12) and RAW 264.7 murine macrophages were provided by

Drs. Stephen Clarke and Ranjan (OSU) respectively and were grown in DMEM supplemented with 5% and 10% fetal bovine serum and 1% penicillin/streptomycin respectively. Cells were grown to confluence at 37^0C in a humidified incubator containing 5% CO_2. Culture medium was changed the day after cells were subcultured and refreshed every other day (for the C2C12) and every three days (for the RAW 264.7 cells) thereafter. For experiments, RAW 264.7 cells were used at passage 10 and MSKMCs were used at passage 8. EOMA cells were at passage 7.

Advanced Glycation End Products (AGE) Preparation

AGE was prepared through the non-enzymatic glycation of bovine serum albumin (BSA) in the presence of D-(+) glucose or DL-glyceraldehyde. Briefly, a 50mg/ml or 25mg/mL solution of BSA (Sigma-Aldrich, St. Louis, MO) for glucose or glyceraldehyde derived AGE respectively, was incubated in the presence of 45mg/ml D-glucose or 20 mM DL-glyceraldehyde (Sigma-Aldrich) in phosphate buffered saline (PBS, pH 7.4). The solution temperature was maintained at 37˚C for 8weeks or 1 wk. respectively, to fully glycate the albumin and to prepare irreversibly formed AGE. As a control, 50mg/ml or 25 mg/mL BSA (for glucose or glyceraldehyde controls respectively) was also incubated in PBS in the absence of DL-glyceraldehyde for 8weeks or 1 wk. at 37˚C. After the incubation period, both the control, glucose and glyceraldehyde-glycated samples were dialyzed in fresh PBS at 4˚C every 2 h for a total of 4 changes. The glucose or glyceraldehyde derived AGE was centrifuged at 3,000 rpm for 30 min in Amicon concentrator tubes (Millipore, Billerica, MA) to concentrate the AGE solution. Total protein concentration in the control and AGE preparations was quantified using the BCA assay (Pierce, Rockford, IL). BSA and AGE samples were determined to be endotoxin

free by testing with the LAL chromogenic Endotoxin Quantitation Kit (Life Technologies Corporation, Grand Island, NY). Endotoxin values were less than 1.0EU/mL. The AGE level in the BSA and AGE solutions was measured by ELISA (OxiSelect Advanced Glycation End Product Competitive ELISA kit, Cell Biolabs).

Treatment of Cells

AGE treatment. Confluent HCAECs, HSKMCs, MSKMCs, and RAW 264.7 cells were treated with either BSA or AGE at 1000-1500 μg/mL for 24 h. Untreated cells served as a control group.

Growth factor treatment. In some experiments, HCAECs and HSKMCs were treated with recombinant TNFα protein (R&D Systems) at concentrations ranging from 0 ng/mL to 100ng/mL for 24 h. In other studies, cells were treated with recombinant IL-1 protein at concentrations ranging from of 0 pg/mL-100,000pg/mL.

RAW-conditioned media treatment. For experiments on RAW-conditioned media, RAW cells were treated as described above. Media was then collected and centrifuged at 1500x g for 10 min to pellet any cells. The RAW media was then added to confluent EOMA cells and incubated at 37ºC for 24 h.

Endothelial cell activation. For studies of endothelial cell activation, HCAECs were pre-incubated with BSA or AGE at 1000μg/ml at the moment of subcultuing of cells into the 6 or 12- well plates and when confluent, was treated with a combination of inflammatory mediators: IL-1 (ThermoFisher) at 1 ng/mL, LPS (InvivoGen) at 2 μg/mL, and IFN-γ (PeproTech) at 20 ng/mL and further incubated for 24 h.

Macrophage activation. For studies on macrophage cell activation, confluent RAW 264.7 cells were treated with two concentration of LPS (InvivoGen) at 50ng/ml and 500ng/ml.

All treatments were added directly to the cell medium. Samples of culture medium were collected 24 or 48 h post-treatment into fresh tubes containing protease inhibitors for measurement of PLGF and TNFα protein levels.

Enzyme-Linked Immunosorbent Assay (ELISA)

Mouse and human PLGF and TNFα protein concentrations were quantified in the media samples by ELISA (Mouse and Human DuoSet ELISAs, R&D Systems) following the manufacturer's protocols. All samples were assayed in duplicate. The assay plates were read using a BioTek Synergy HT plate reader (BioTek Instruments) at 450 nm. All data were normalized to total protein concentration in the samples, using the bicinchoninic acid assay (BCA, Pierce), except in experiments where BSA and AGE were added (since the additional protein being added confounded the analysis of total protein).

RT-PCR

The reverse transcription-polymerase chain reaction (RT-PCR) technique was employed to quantify the mRNA expression of mouse and human PLGF, TNFα, VCAM-1, ICAM-1, MCP-1, RAGE, and β-actin in HCAEC, HSKMC, RAW 264.7, and EOMA. Total RNA was collected using the RNeasy mini kit (Qiagen) following the manufacturer provided protocol. The quantity and purity of total RNA was analyzed by the A260/280

ratio using a Take3 micro volume plate on the BioTek Synergy HT Multi-mode Plate Reader (BioTek Instruments). RNA samples were either directly used for PCR or stored at -80°C until use. RNA was reverse transcribed to cDNA using the QuantiTect reverse transcription kit (Qiagen) according to the manufacturer's instructions. Target genes were amplified from cDNA in an ABI 7500 Fast instrument (Life Technologies, Grand Island, NY) using PerfeCTa SYBR Green FastMix, Low ROX (Quanta Biosciences) with the appropriate forward and reverse primers. Forward and reverse primers were used as shown in the table below. The relative PLGF, TNFα, MCP-1, RAGE, VCAM-1, and ICAM-1 mRNA expression (RQ value) was quantified using the comparative ΔΔCt method. Target gene expression was normalized to β-actin gene expression.

Mouse Multiplex Cytokine Assay

Cytokines secreted by the RAW 264.7 cells into the media were analyzed using a multiplex Pro Mouse Cytokine 6-Plex panel and cytokine reagent kit (Bio-Rad), following the manufacturer's protocol. A Luminex-100 instrument with Bio-Plex Manager 6.1 software (Bio-Rad Laboratories) was used.

Statistical Analysis

All data are presented as mean ± SEM. The GraphPad Prism statistical package was used for data analysis. One way analysis of variance (ANOVA) followed by post-hoc test was used in all data with p value of <0.05 considered significant

List of Primers

Mouse	PLGF	Forward	CTGCTGGGAACAACTCAACAGA
		Reverse	GCGACCCCACACTTCGTT
Mouse	TNFα	Forward	GACGTGGAACTGGCAGAAGA
		Reverse	GAGGCTGAGACATAGGCACC
Mouse	VCAM-1	Forward	TGAAGATGGTCGCGGTCTTG
		Reverse	AGCTTGAGAGACTGCAAACAG
Mouse	ICAM-1	Forward	TCCGCTGTGCTTTGAGAACT
		Reverse	TCCGGAAACGAATACACGGT
Mouse	MCP-1	Forward	GGCTCAGCCAGATGCAGTTAA
		Reverse	CCTACTCATTGGGATCATCTTGCT
Mouse	RAGE	Forward	CAAGGAGGAACCACCCATCC
		Reverse	CAACCAACAGCTGAATGCCC
Mouse	B-actin	Forward	AGTTCGCCATGGATGACGAT
		Reverse	TGCCGGAGCCGTTGTC
Human	PLGF	Forward	CCTACGTGGAGCTGACGTTCT
		Reverse	TCCTTTCCGGCTTCA TCTTCT
Human	TNFα	Forward	GAATCGGATCAGGGAGGATG
		Reverse	AAGTTGGGGACACACAAGCA
Human	VCAM-1	Forward	ATGTCAATGTTGCCCCCAGA
		Reverse	TGCTCCACAGGATTTTCGGA
Human	ICAM-1	Forward	CCATCTACAGCTTTCCGGCG

		Reverse	TCAGCGTCACCTTGGCTCTA
Human	MCP-1	Forward	TGCTCATAGCAGCCACCTTC
		Reverse	GGGCATTGATTGCATCTGGC
Human	RAGE	Forward	ATTGGTGGTGGAGCCAGAAG
		Reverse	CAGGTCAGGGTTACGGTTCC
Human	B-actin	Forward	TGCCGACAGGATGCAGAAG
		Reverse	CTCAGGAGGAGCAATGATCTTGAT

CHAPTER III

RESULTS

AGE had no direct effect on PLGF expression

To test the effect of AGE on PLGF expression, confluent cell cultures of HCAEC, HSKMC and MSKMC were treated with BSA or AGE at 1000μg/ml for 24 hours. Treatment with AGE or BSA had no significant effect on PLGF protein levels in media in HCEAC and HSKMC (Fig 4). This lack of direct effect of AGE was also observed in PLGF mRNA (Fig. 5). However, there was a slight but significant direct effect of AGE on PLGF in MSKMC (Fig. 6).

Recombinant TNFα reduced PLGF protein levels in HCAEC but had no effect on HSKMC and MSKMC

We next sought to see if AGE could possibly indirectly affect PLGF protein levels in HCAEC, HSKMC and MSKMC via a mediator such as TNFα. As a first step in testing this hypothesis, HCAEC, HSKMC and MSKMC were treated with recombinant TNFα protein. There was a significant reduction in PLGF protein levels at TNFα concentrations between 0.1ng/ml-50ng/ml (Fig. 7 P=0.0001). TNFα had no significant effect on HSKMC and MSKMC (Fig. 8).

Recombinant IL-1 Treatment had no significant effect on PLGF protein levels in HCAEC

IL-1 is another mediator we tested on HCAEC, to show its effect on PLGF. IL-1 had no significant effect on HCAEC (Fig.9)

Direct Effect of AGE on TNFα stimulation in EC and SKMCs

We next tested whether direct treatment with AGE could increase TNFα levels in HCAEC or HSKMC. Confluent HCAEC and HSKMC were treated directly with AGE or BSA at 1000-1500μg/ml. TNFα protein levels were measured. TNFα was undetectable in the cells across all treatments. Therefore, we concluded that AGE had no direct effect on TNFα production in EC (data not shown).

AGE increased TNFα protein levels in murine macrophages

To further explore our hypothesis that AGE could be having indirect effects on EC, we next characterized the effect of AGE on TNFα expression in macrophages. RAW 264.7 murine macrophages were treated with AGE or BSA at 1000 or 1500μg/ml. TNFα was significantly increased in AGE treated RAW cells as compared to BSA-treated or untreated control cells (Fig. 10 $P<0.0001$).

We also quantified the PLGF protein level in the RAW 264.7 macrophages to see whether macrophages themselves make PLGF protein when activated by AGE. PLGF was undetectable across all treatments in the macrophage cells (data not shown).

AGE-treated macrophage media increased TNFα production in endothelial cells while AGE and BSA-treated macrophage media both reduced PLGF in EC

Because we have seen that PLGF levels was reduced by treatment with recombinant TNFα and that AGE stimulated the production of TNFα in macrophages, we sought to determine the effect of conditioned media from macrophages (media from the BSA or AGE treated RAW 264.7 cells), on PLGF expression in EC. To do this, we cultured EOMA cells (murine ECs) and incubated them with BSA and AGE treated RAW cell conditioned media that had been centrifuged (to ensure that just the media was added to EOMA cells).

TNFα protein was significantly increased in EOMA cells incubated with AGE-treated RAW media, but not in cells incubated with BSA-treated and untreated (control) RAW media (Fig. 11 $P< 0.0001$). However, TNFα mRNA was not significantly different across the three treatments (Fig. 12).

Excitingly, PLGF was significantly reduced in EC incubated with AGE-treated RAW media; unexpectedly, PLGF was also significantly reduced in EC exposed to BSA-treated RAW media (Fig. 13 $P<0.001$).

Endothelial cells pre-incubated with AGE and activated with inflammatory mediators increased TNFα expression

EOMA ECs were pre-incubated with BSA or AGE at 1000μg/ml and when cells were confluent, they were activated and treated with a combination of three inflammatory mediators (IL-1, LPS and IFN-γ; ILI). TNFα expression was increased in the AGE

treated cells, in comparison with the BSA or untreated control cells (Fig. 14 $P<0.001$). There was no significant difference in TNFα mRNA (Fig. 12).

TNFα was the most abundantly produced cytokine by AGE activated RAW macrophages, followed by IL-10 and IFN-γ

Macrophage media treated with BSA or AGE was further analyzed using a 6-panel multiplex mouse cytokine assay, to check for other cytokines that may be produced by the RAW cells in response to AGE or BSA. TNFα was the most significantly increased of all the cytokines quantified ($P=0.007$). There was also a trend towards an increase in IL-10 and IFN-γ with AGE treatment (Fig. 15 $P=0.06$).

Stimulation of murine EC (EOMA) with LPS further decreased PLGF.

Confluent EOMA cells were directly treated with LPS at 0, 50, and 500ng/ml to see the effect of LPS on PLGF levels. LPS is a known stimulator of TNFα, and since LPS was amongst the previous three inflammatory cytokines that caused overexpression of TNFα, it was used to treat EOMA cells directly without incubation with AGE. The results showed a significant decrease in PLGF. This is consistent with a TNFα-mediated effect on PLGF regulation (Fig. 14B)

Direct AGE treatment of HCAEC had no significant effect of ICAM-1, VCAM-1, TNFα, MCP-1 and RAGE mRNA.

Following AGE treatment, there was no significant change in the mRNA levels of adhesion molecules that would normally be upregulated when there is EC activation. Intracellular adhesion molecule (ICAM-1), vascular cell adhesion molecule (VCAM-1) macrophage chemoattractant molecule (MCP-1), and tumor necrosis factor (TNF-α)

mRNA levels were not significantly affected by direct treatment of AGE in HCAEC. RAGE mRNA, on the other hand, tended towards an increase which may become significant with increase in sample size. RAGE is the receptor for AGE which is found on many cell types (Fig 16)

TNFα mRNA was not significantly affected in RAW-AGE treated media and RAGE is downregulated in activated RAW media

RAW 264.7 cells were stimulated with AGE or BSA at 1000μg/ml for 24 hours. TNFα mRNA was not significantly increased in the RAW cells, despite the increased expression of TNFα protein. Interestingly, mRNA for RAGE, the receptor for AGE, is downregulated in the RAW cells (Fig 17). This finding suggests that AGE may not be signaling through RAGE on macrophages, since there are other receptors also present on the macrophage cell surface like TLRs and macrophage scavenger receptors.

MCP-1, RAGE and TNFα were insignificant in EOMA cell incubated with AGE-treated RAW media

After incubation of EOMA cells with AGE or BSA treated RAW media, the mRNA levels of MCP-1, RAGE, TNFα and PLGF were measured. There was no significant change observed in the mRNAs measured in the AGE-RAW media incubated EOMA cells when compared with the BSA-treated RAW media incubated EOMA. TNFα mRNA tended towards an increase, but was insignificant when compared between the two treatments (Fig. 18)

From these results, we see that AGE had no direct effect on PLGF regulation, but that AGE treated macrophage media caused a very significant change in the effect of PLGF, possibly via increased TNFα.

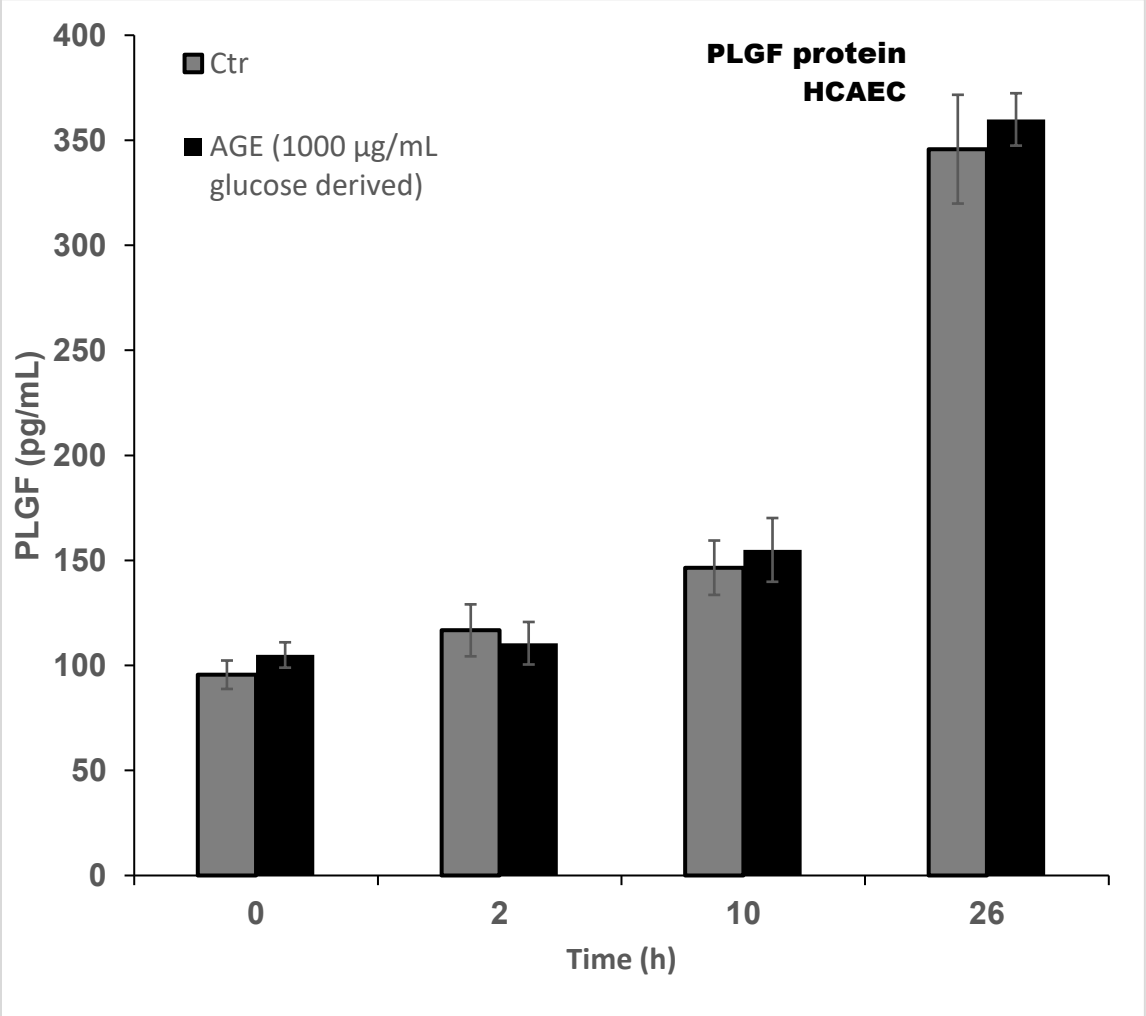

Figure 4. AGE had no direct effect on HCAEC. PLGF protein level was measured at different time points using ELISA. There is no statistical difference between the BSA-control treated and the AGE-treated cells. N=6, P=NS.

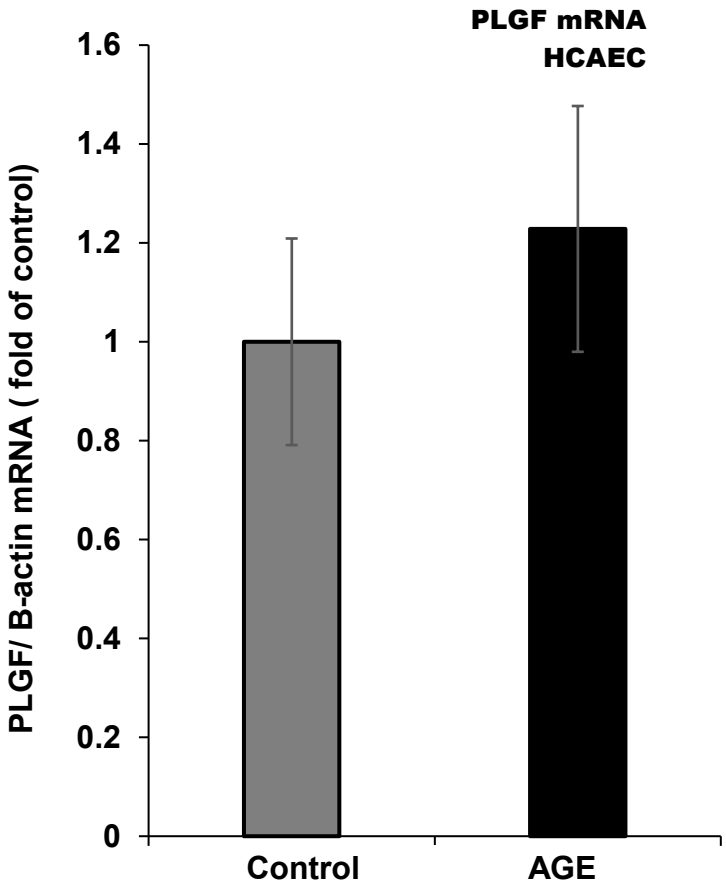

Figure 5 AGE had no direct effect on PLGF mRNA in HCAEC. PLGF mRNA was not significantly different between AGE and BSA treated cells. N=6, P=NS

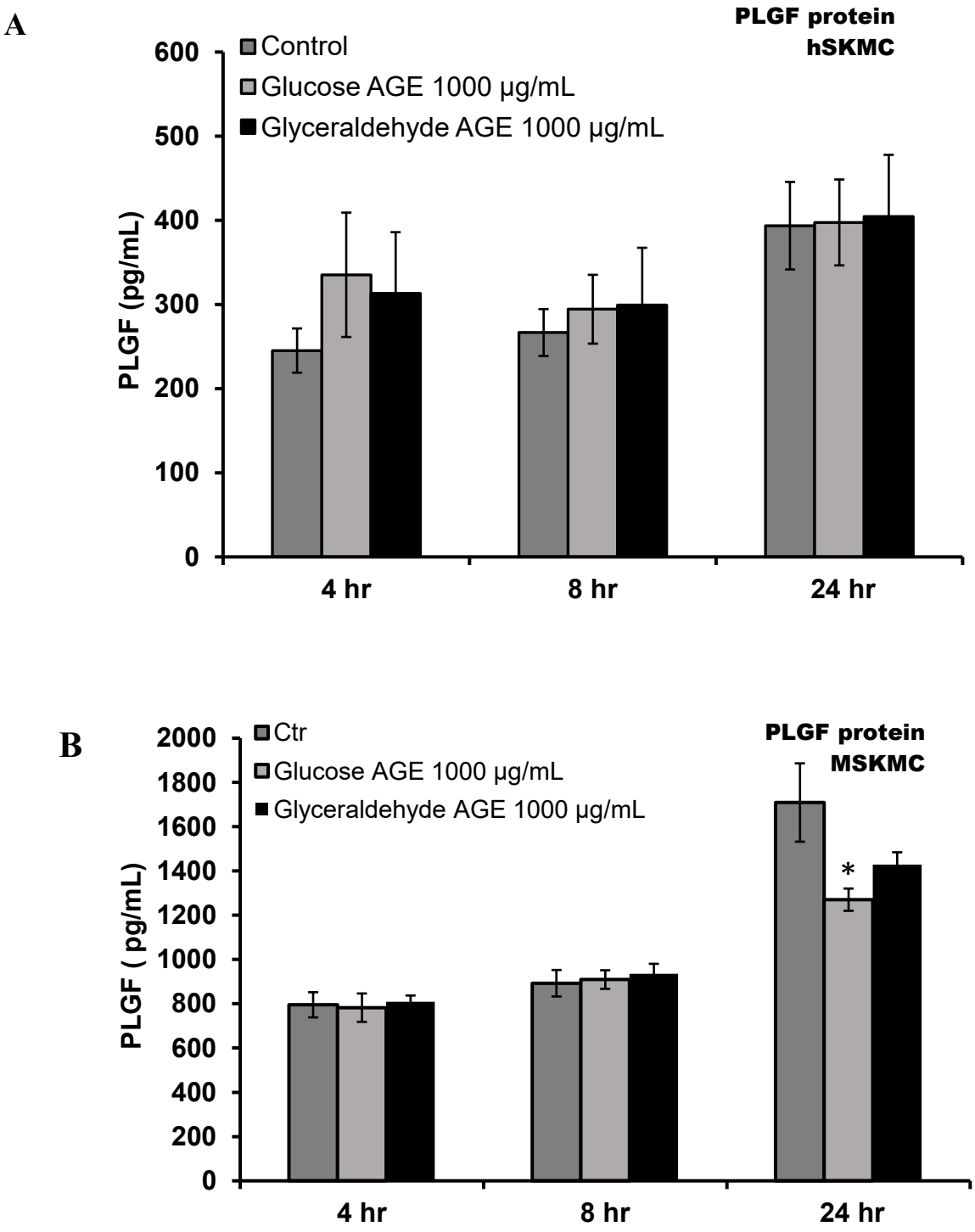

Figure 6. AGE had no direct effect on HSKMC but had an effect on MSKMC. A) PLGF protein levels was not significant between the AGE-treated and the BSA-treated cells in HSKMC (N=6, P=NS) but in B) there was a significant decrease in PLGF in MSKMC glucose-AGE-treated cells in comparison to the BSA treated cells. N=6, P≤0.05.

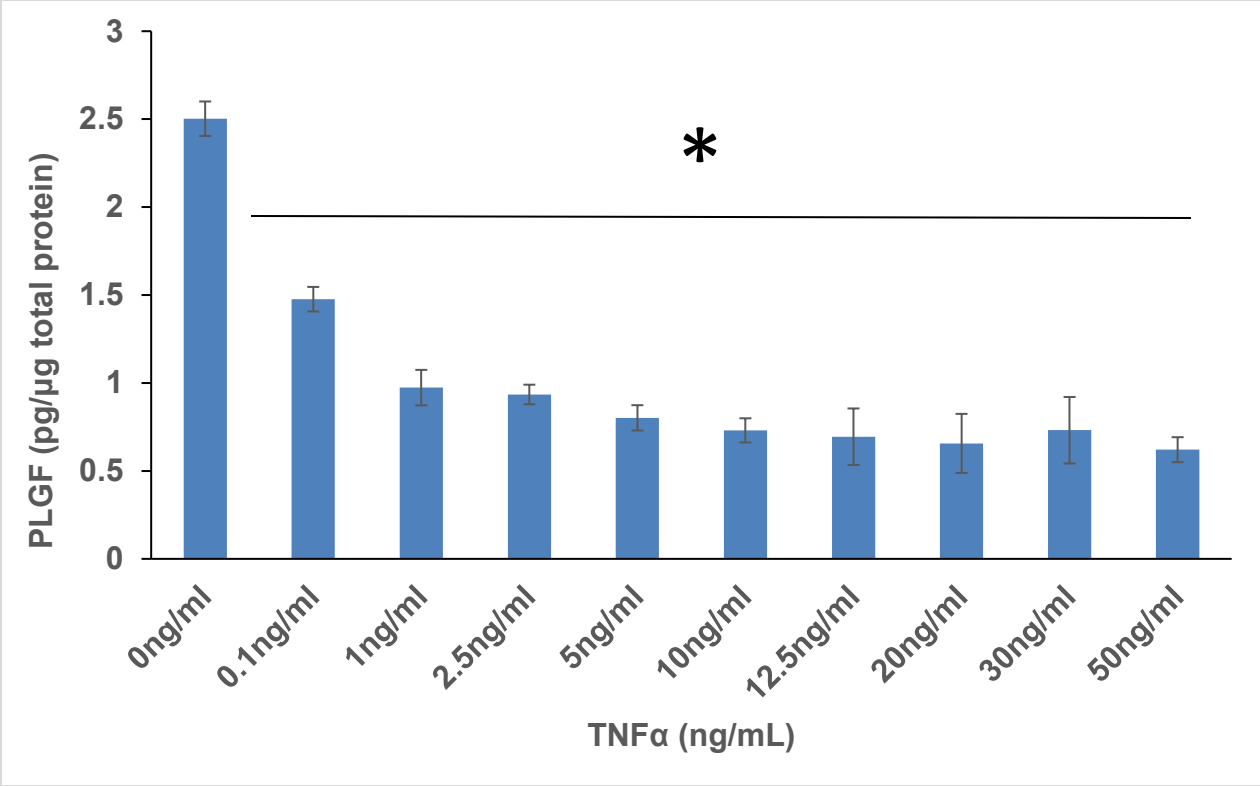

Figure 7. Recombinant TNFα decreased PLGF levels in HCAEC. Direct treatment of confluent HCAEC with recombinant TNFα between 0ng/ml-50ng/ml concentrations significantly reduced PLGF protein levels in HCAEC. N=4, P=0.0001

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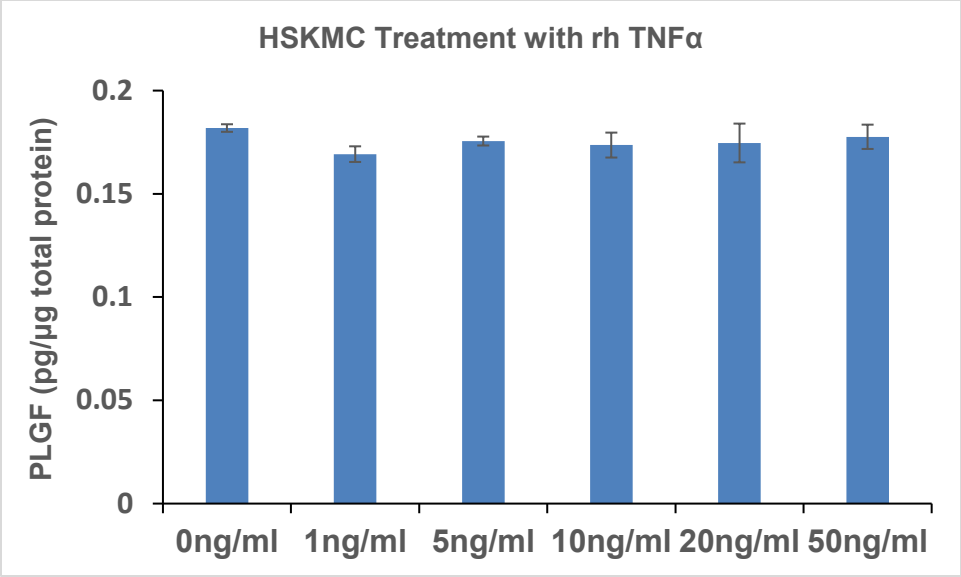

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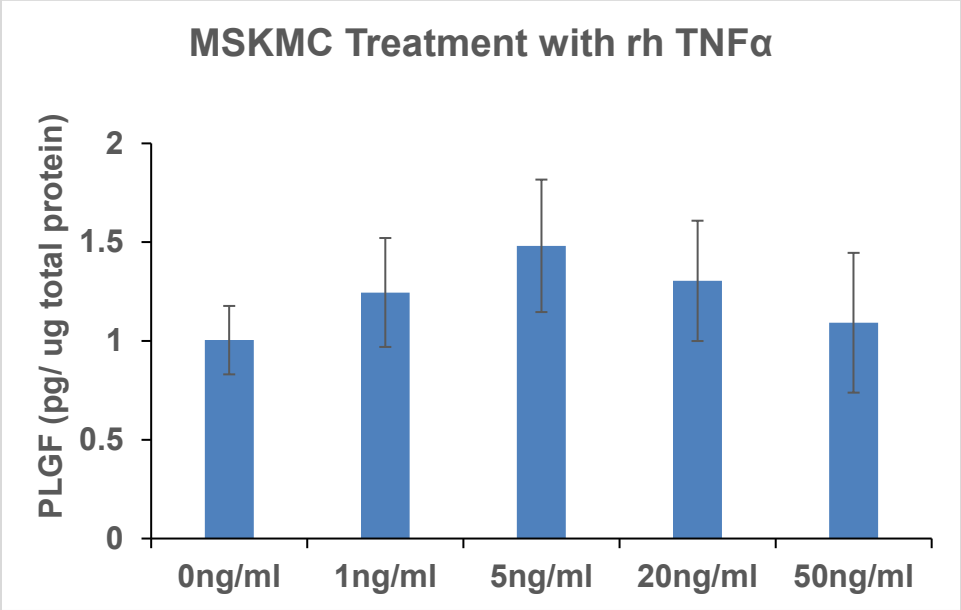

Figure 8. Recombinant TNFα treatment in HSKMC and MSKMC. A) Recombinant TNFα had no effect on PLGF levels in HSKMC B) and MSKMC. N=4, P=NS

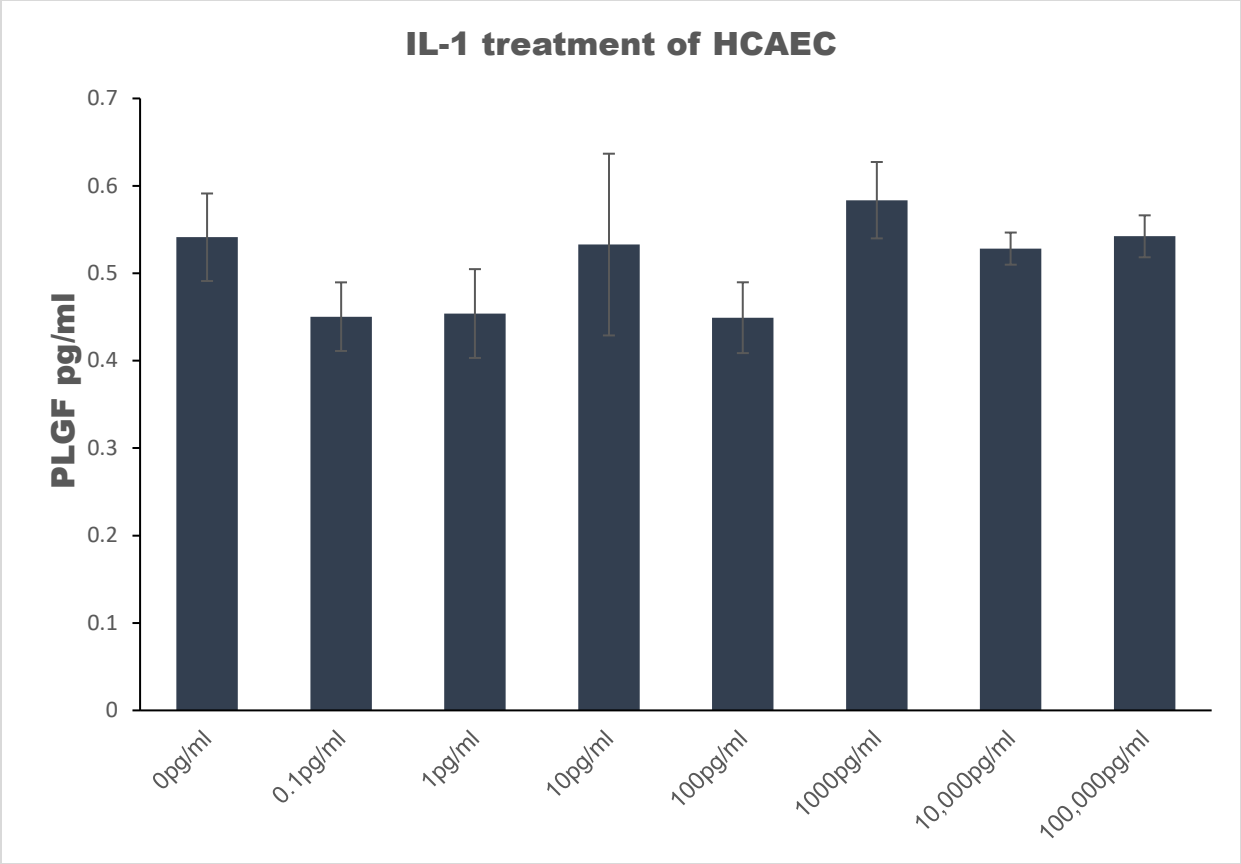

Figure 9. IL-1 had no significant effect on PLGF in HCAEC. Treatment of confluent HCAEC cells with other inflammatory markers such as IL-1 showed no consistent effect on regulation of PLGF protein levels in HCAEC. N=6, P=NS

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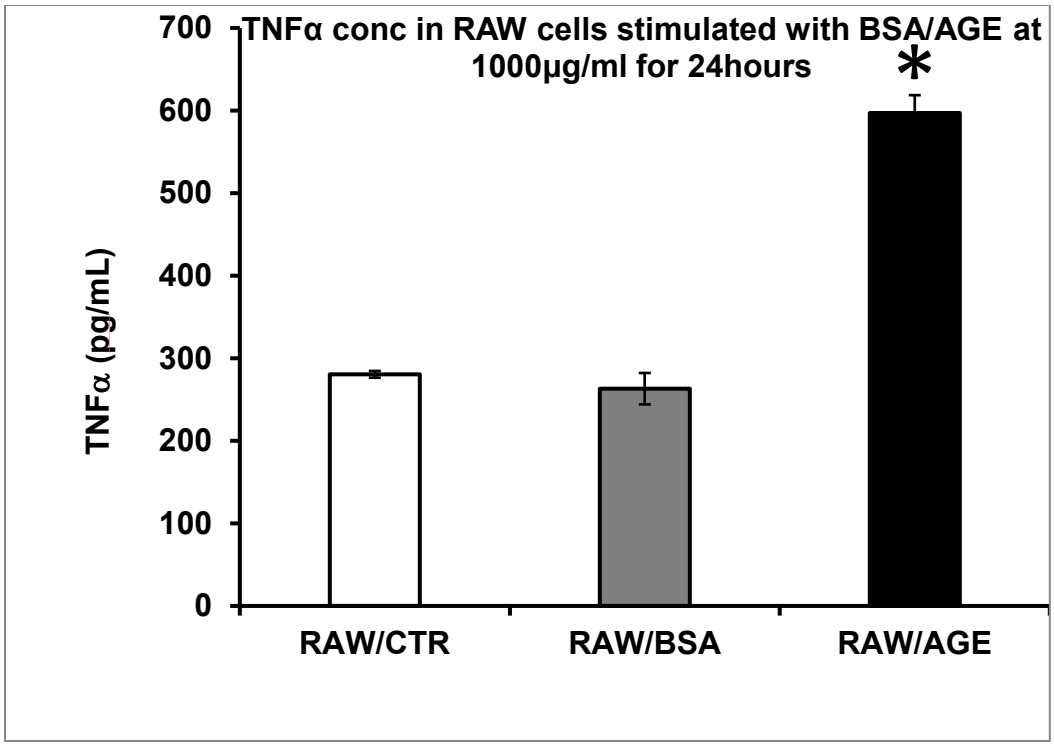

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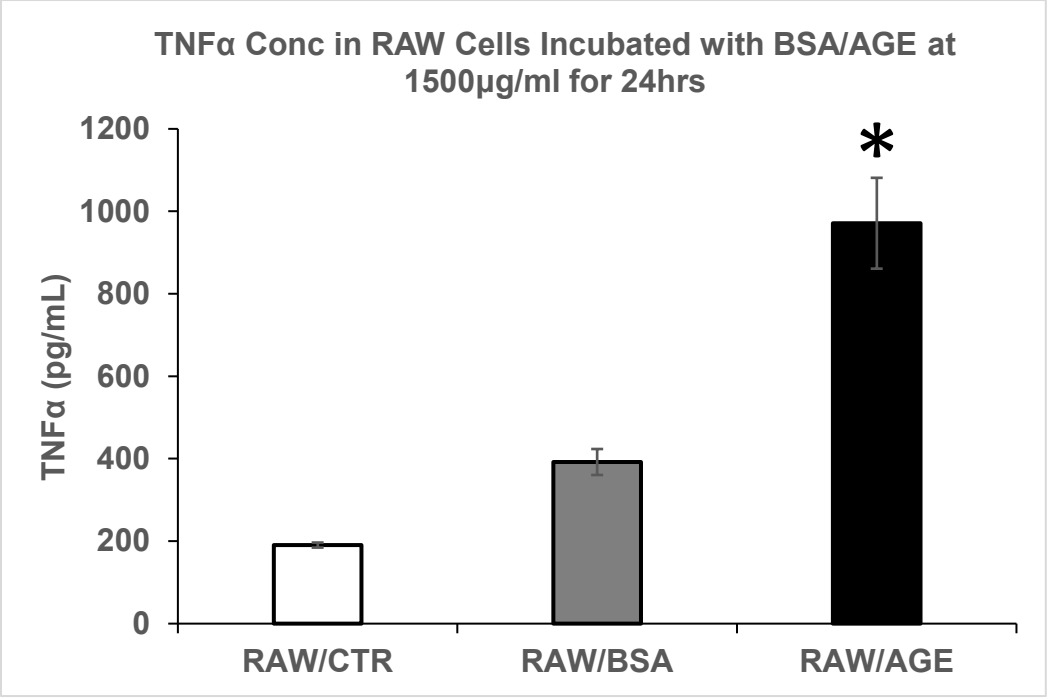

Figure 10. AGE increased expression of TNFα in macrophages. AGE-treated cells showed overexpression of TNFα production in RAW 264.7 murine macrophage cells in comparison to BSA or untreated control cells. A) At 1000µg/ml and at B) 1500µg/ml of BSA or AGE concentration for 24 hours. N=4, P=0.0001. PLGF protein levels were undetectable in RAW 264.7 cells (data not shown)

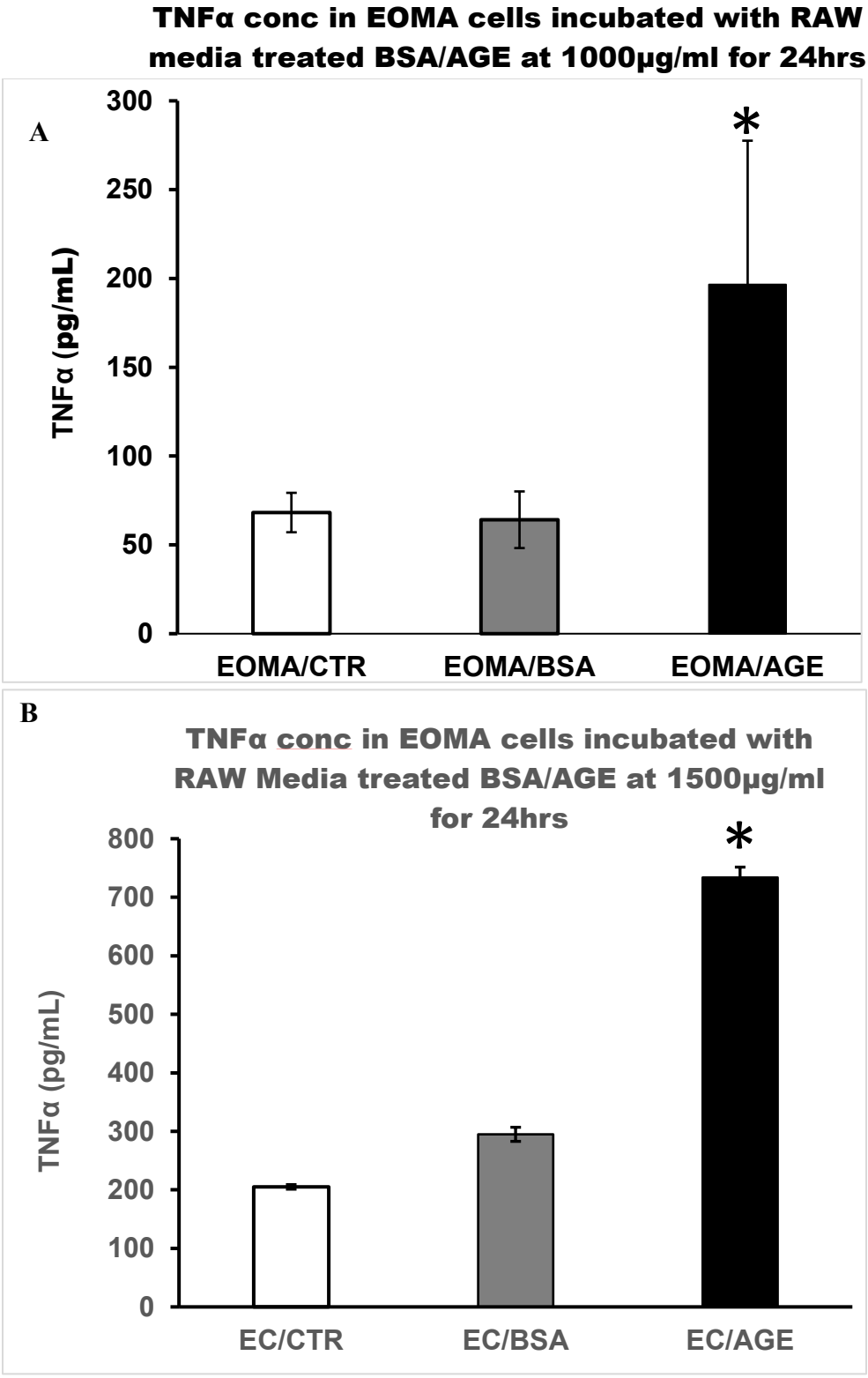

Figure 11. RAW media treated with AGE significantly increased TNFα protein levels in EOMA cell media than in BSA-treated or untreated control-RAW media incubated EOMA cells. RAW media stimulated with BSA or AGE was collected, centrifuged, added to growing EOMA cells in 12-well plates and incubated for 24 hours. A) TNFα was increased significantly in the EOMA

wells incubated with 1000μg/ml and B) 1500μg/ml AGE-treated RAW media than in the BSA or untreated control RAW media incubated EOMA cells N=4, P=0.0001

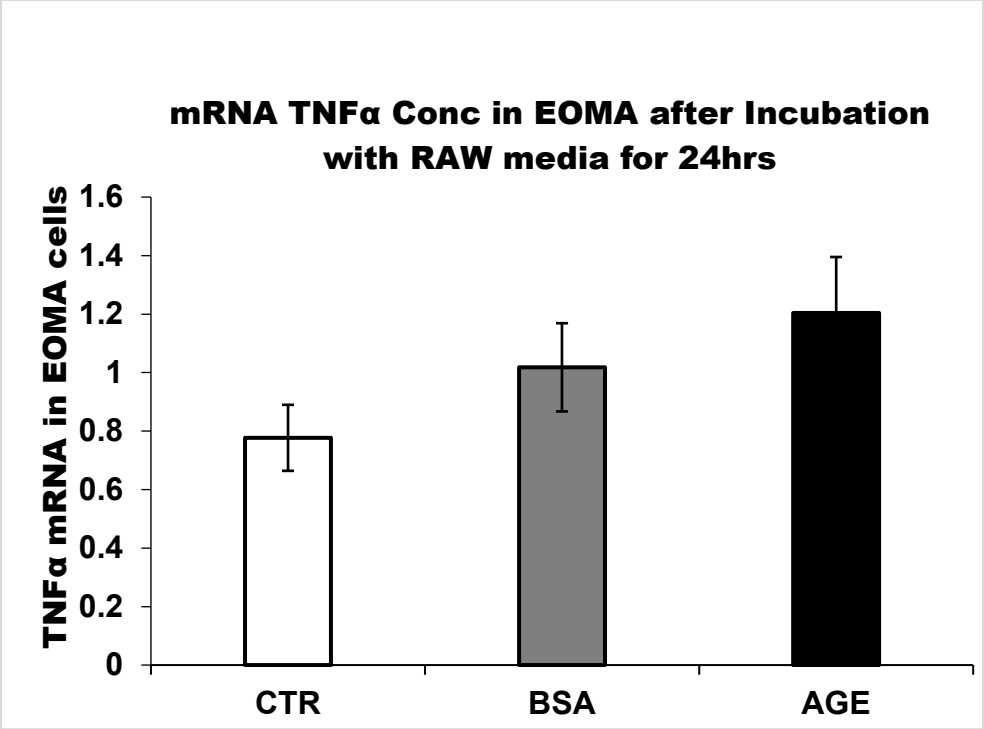

Figure 12. TNFα mRNA was not significantly different between EOMA cells incubated with RAW-AGE treated media and RAW-BSA or RAW-untreated incubated EOMA cells. N=4, P=NS

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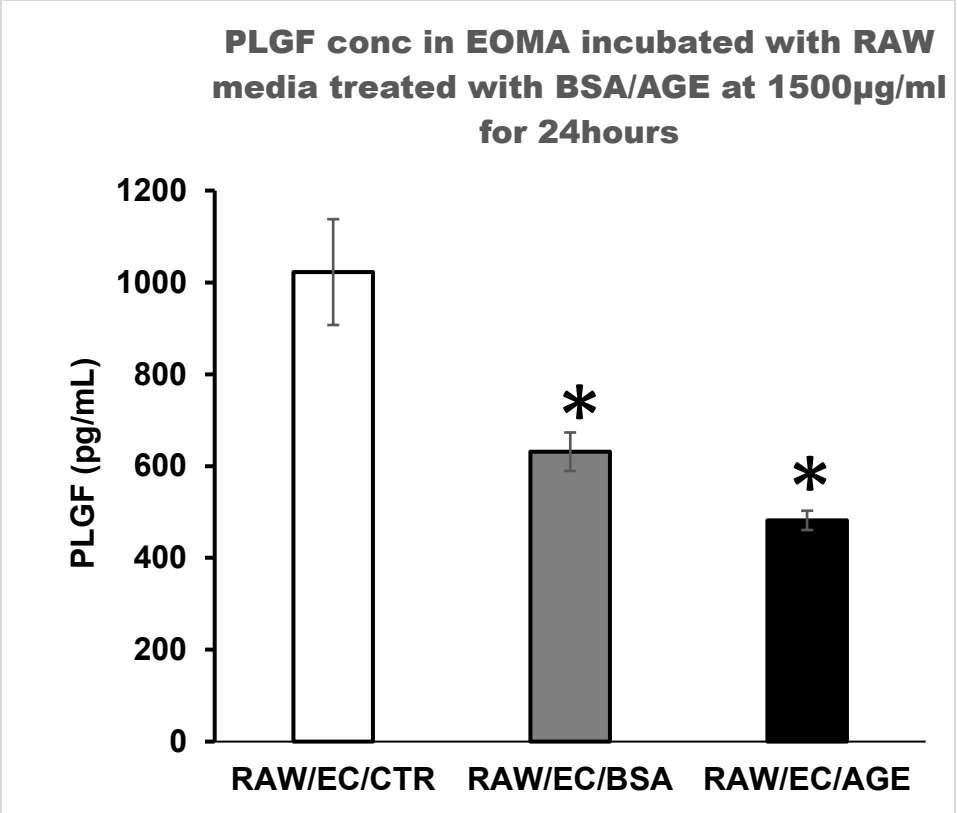

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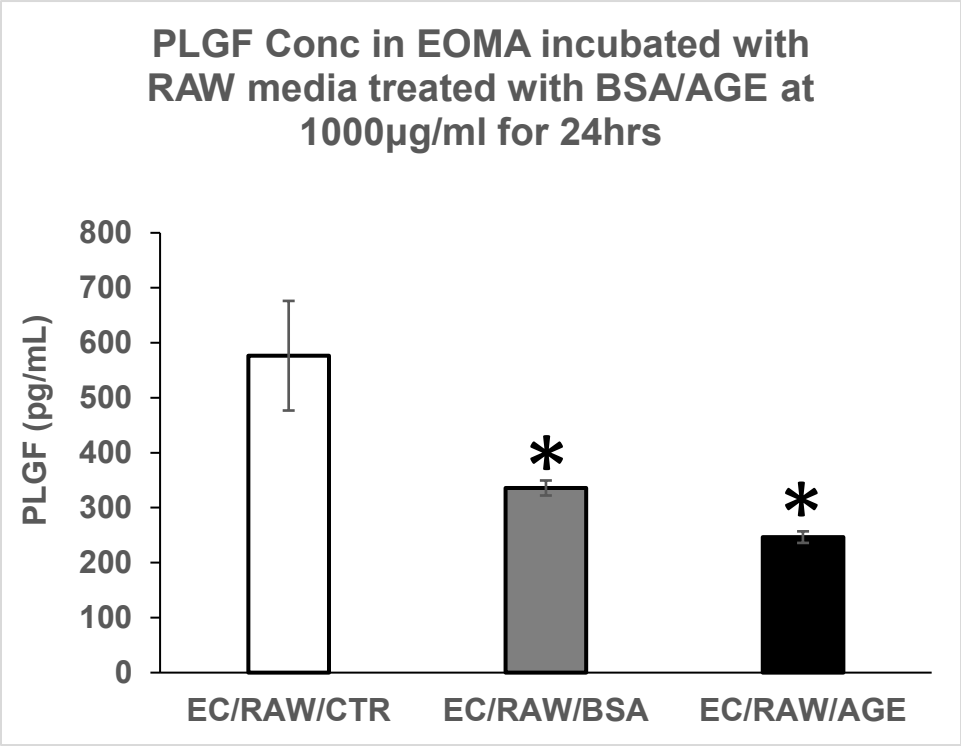

Fig 13. RAW media treated with BSA or AGE decreased PLGF levels in EOMA EC. A) at 1500µg/ml B) 1000µg/ml BSA or AGE concentration, PLGF protein levels was significantly

reduced in the AGE-treated RAW media incubated EOMA EC and in the BSA- control, RAW media incubated EOMA cells.

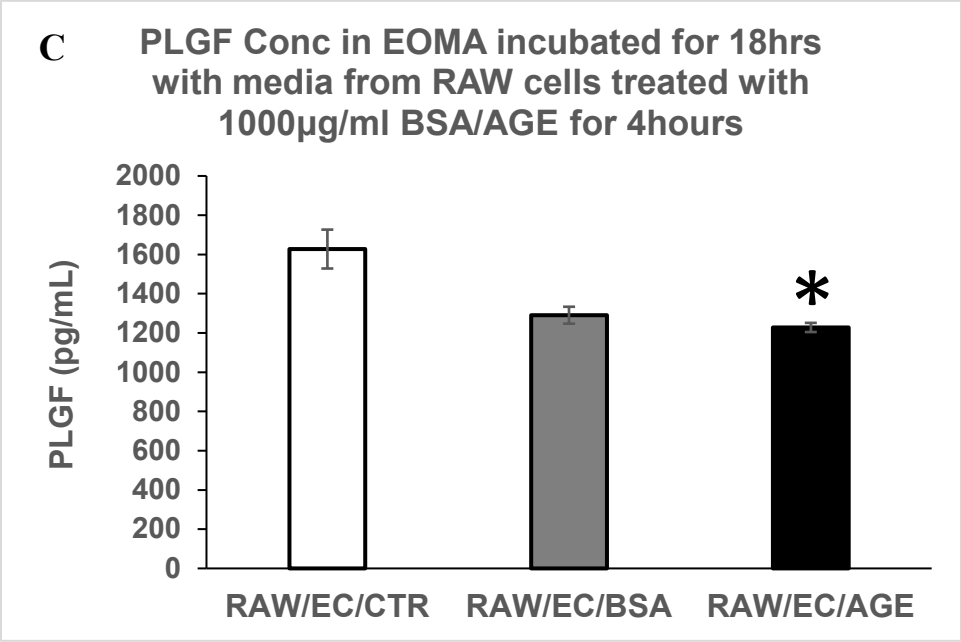

Fig 13 C. RAW media treated with BSA or AGE decreased PLGF levels in EOMA EC. C) in RAW media treated with 1000µg/ml BSA or AGE for 4 hours and then added to EOMA EC and incubated for 18hours, PLGF was also reduced in the AGE-treated RAW media incubated EOMA EC than in the BSA or untreated control-treated RAW media incubated EOMA cells. N=4, P=0.0035

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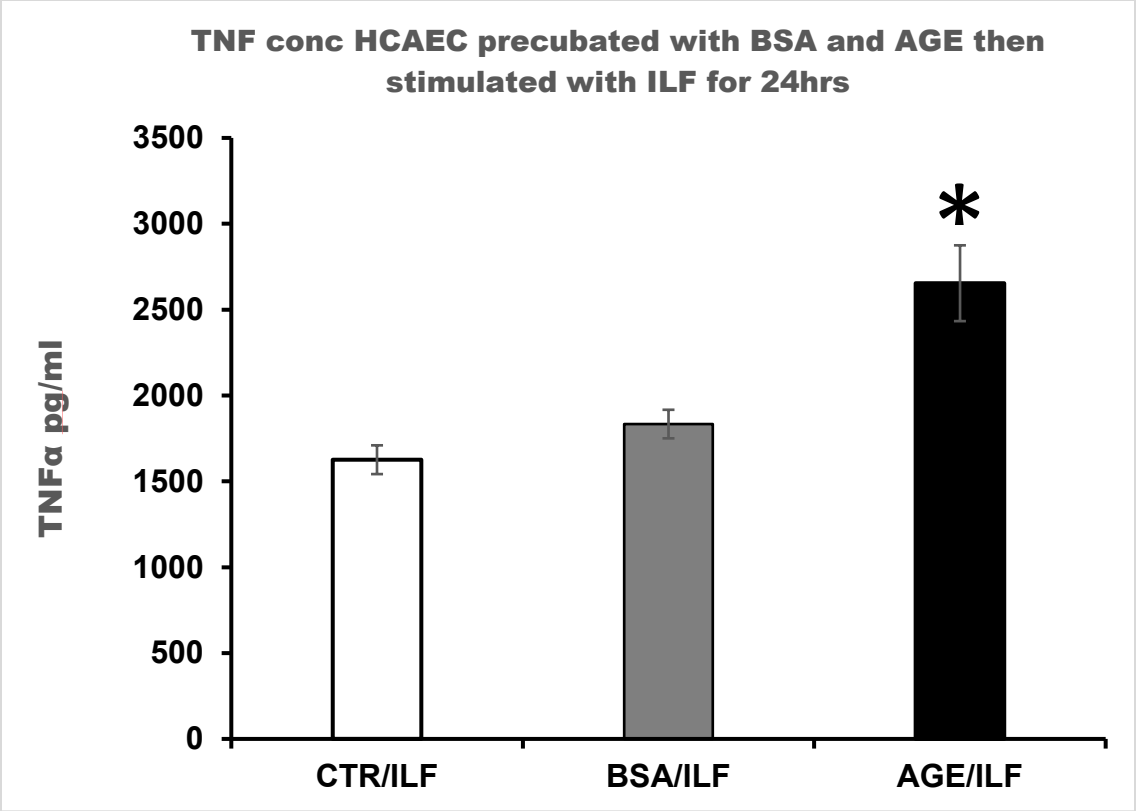

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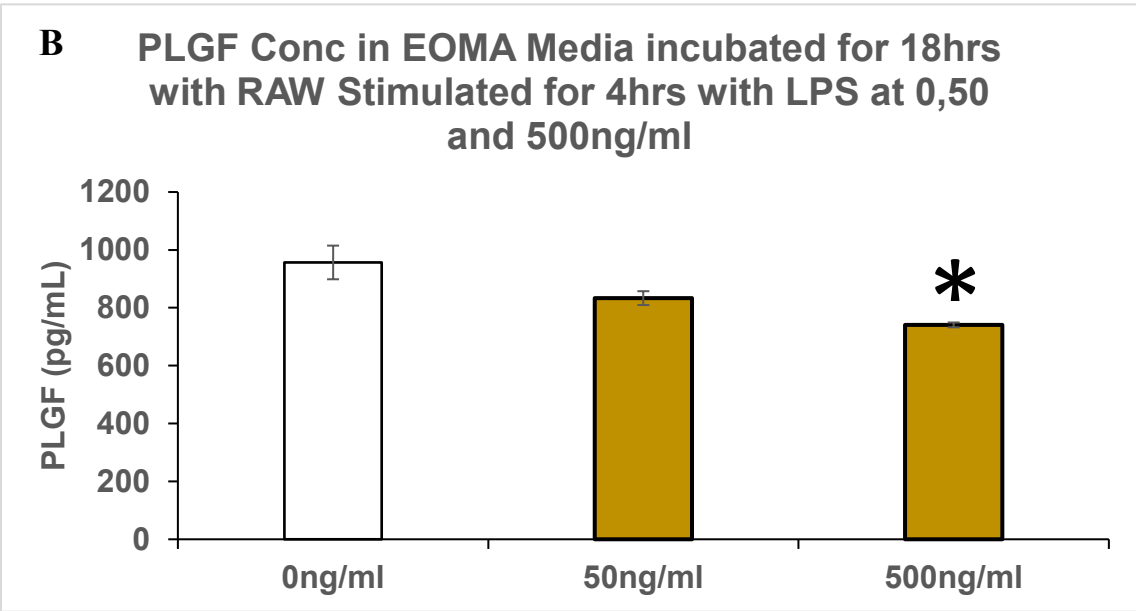

Fig 14. TNFα is overexpressed in HCAEC pre-incubated with AGE and activated by treatment with inflammatory markers. HCAEC were pre-incubated with AGE or BSA at 1000μg/ml for 24hours and then activated with a combination of inflammatory markers, IL-1 1ng/ml, LPS 2μg/ml and IFN-γ 20ng/ml (ILF). A) AGE pre-incubation caused an increased expression of TNFα in the HCAEC than BSA or untreated control pre-incubated ECs. N=4, P=0.001. B) LPS also reduced PLGF levels when used to treat RAW 264.7 cells for 4 hours and incubated on EOMA cells for 18 hours N=6, P=0.0078. LPS is a known inducer of TNFα.

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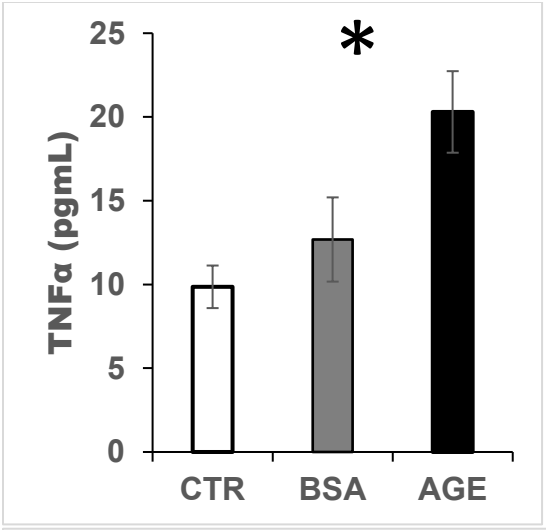

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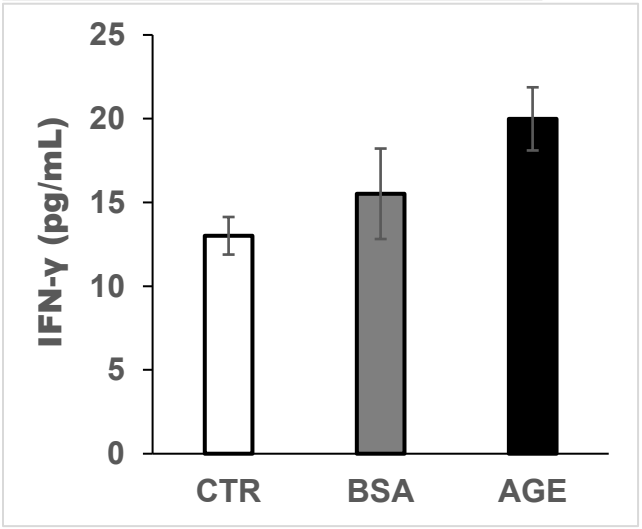

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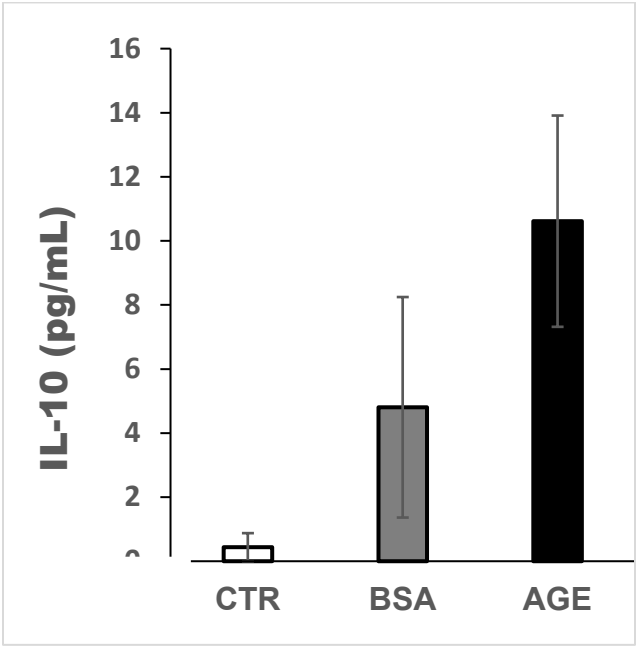
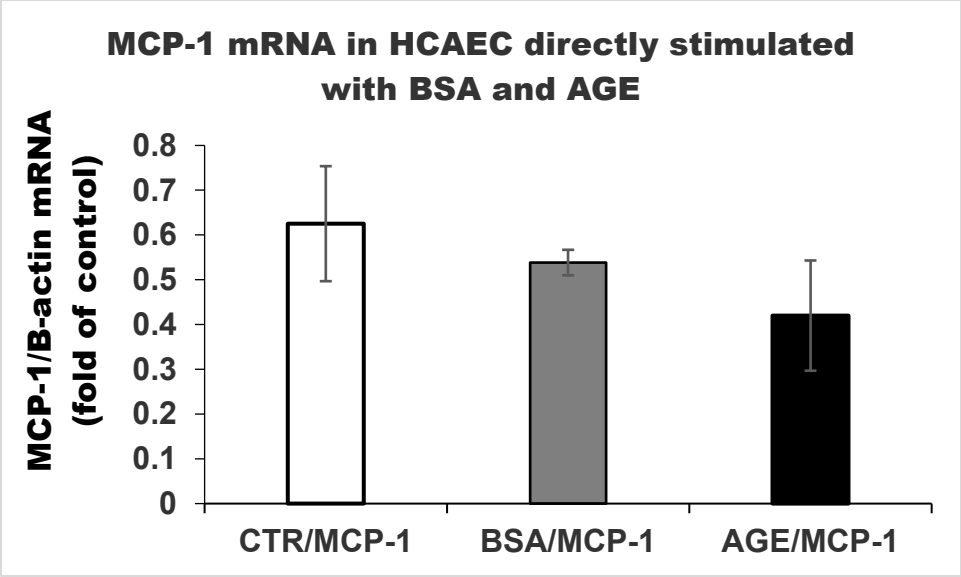


Fig 15. TNFα is the cytokine most secreted by RAW 264.7 murine macrophages. Further testing to confirm other cytokines secreted by RAW macrophages into the media, that may be having an effect on PLGF regulation was carried out using a multiplex mouse cytokine assay. A) TNFα was most significantly increased in the media of AGE treated RAW cells compared to BSA treated or untreated-control RAW cell media N=6, P=0.007. There was a trend towards increase in B) IL-10, N=6, P=0.006 and C) IFN-γ N=6, P=0.006.

A



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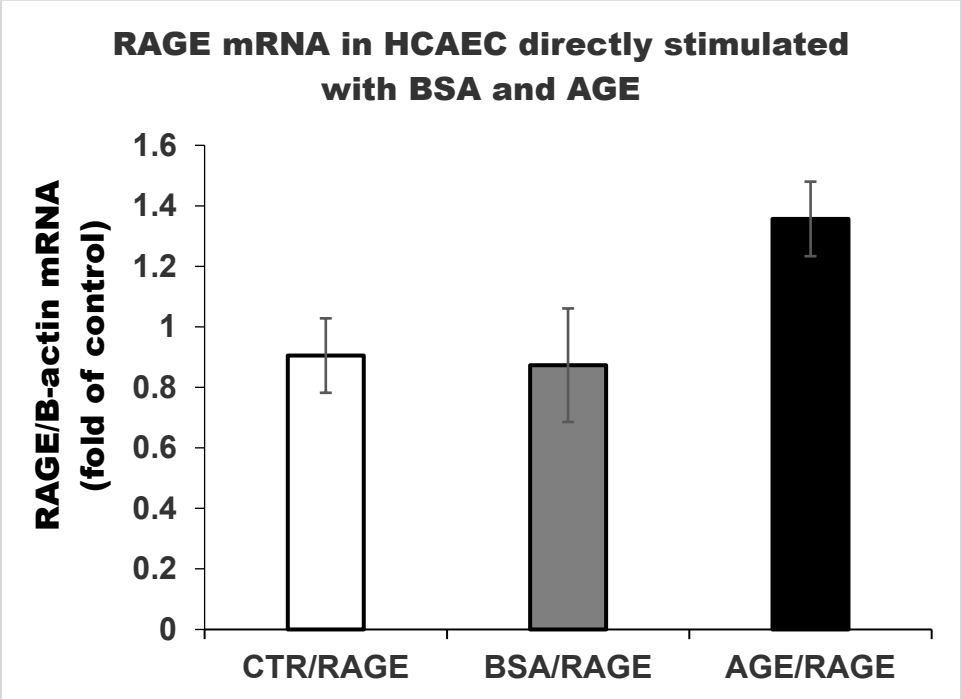

Fig 16. AGE did not directly activate endothelial cells. mRNA gene expression of adhesion molecules, cell surface receptors, that are normally upregulated when EC is activated, were measured. A) MCP-1 gene expression between AGE and BSA treatment was not significant; N=4, P=NS B) RAGE mRNA was also not significantly affected by AGE treatment in HCAEC but showed a trend towards significance. N=4, P=0.08.

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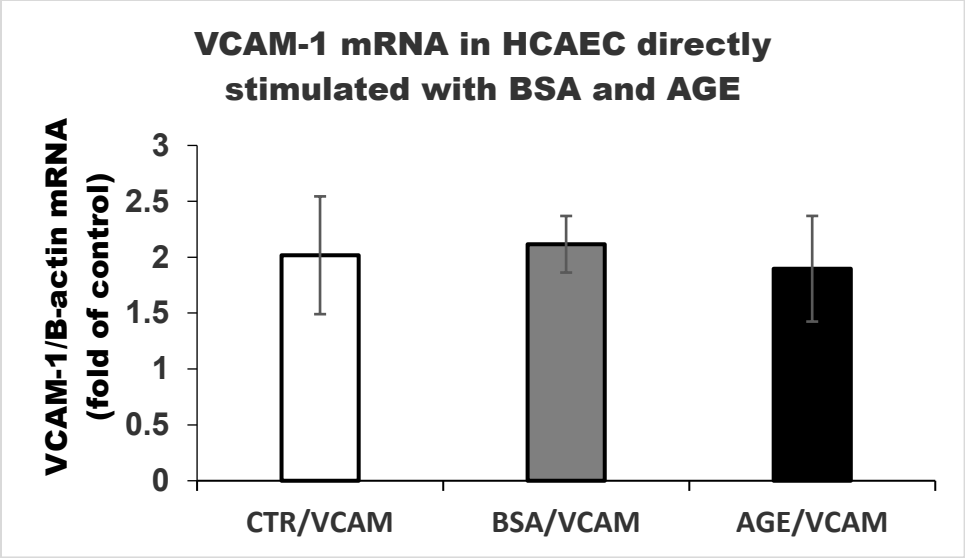
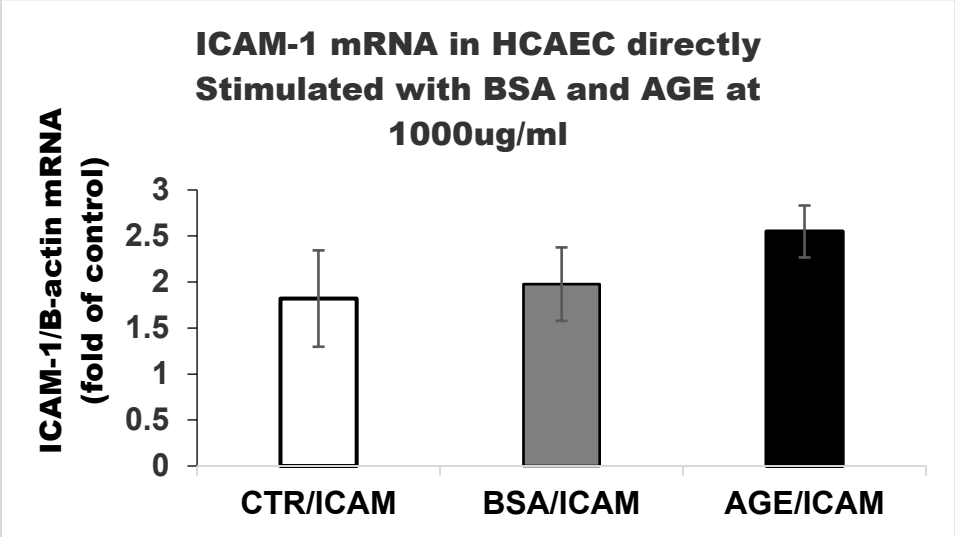


Fig 16 C) VCAM-1, D) ICAM-1 and E) TNFα mRNA were insignificant between treatments N=4, P=NS

D



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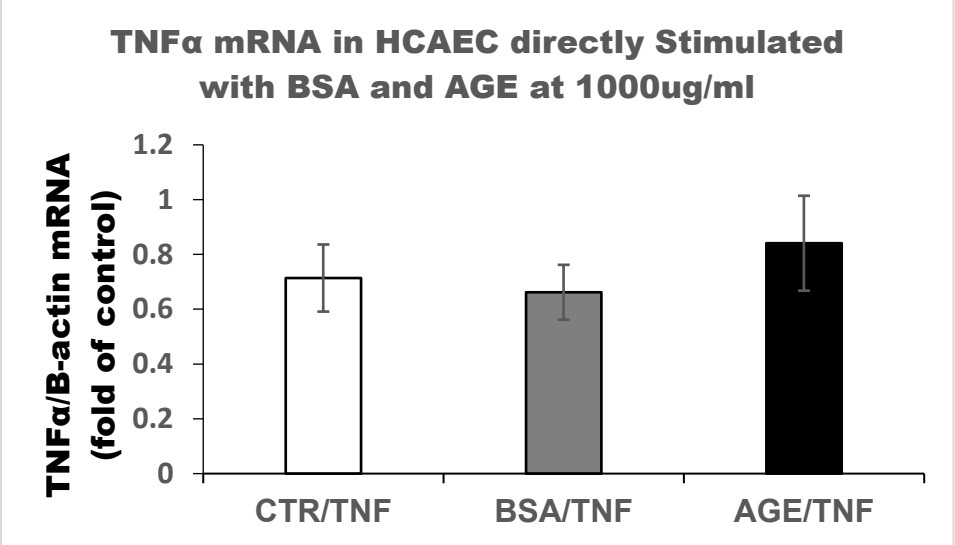

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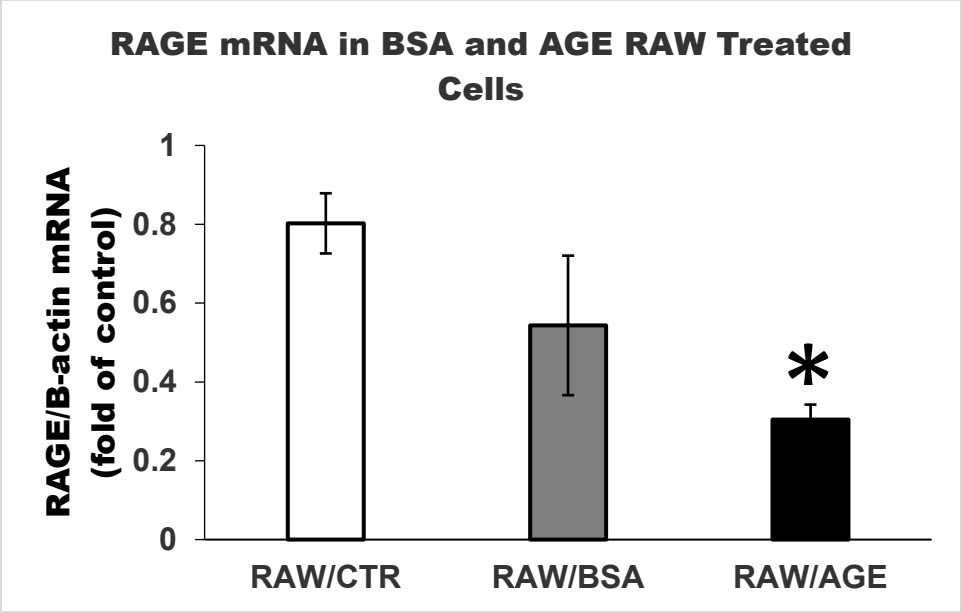

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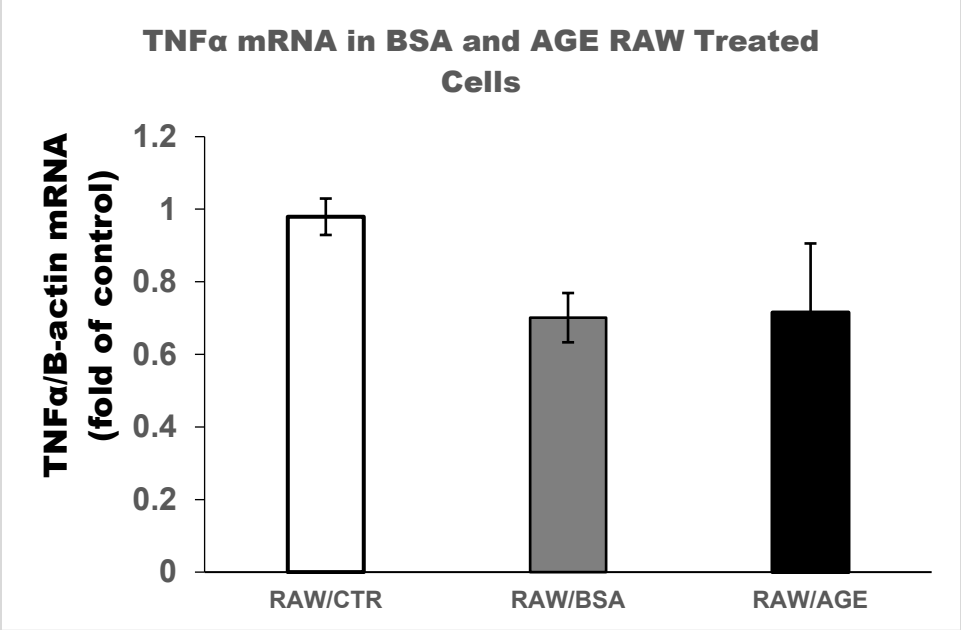
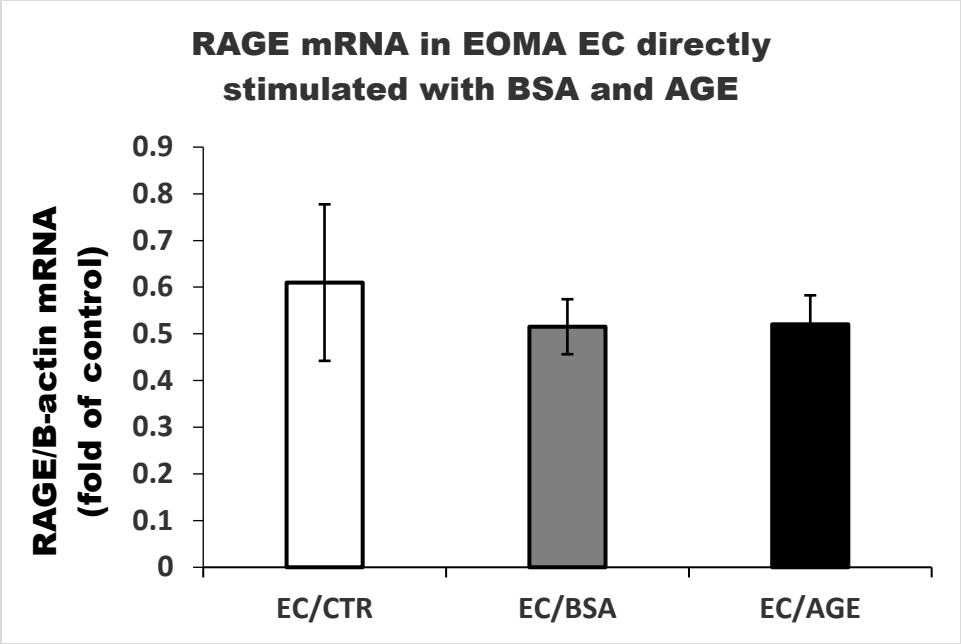


Fig 17. AGE may not be signaling through the RAGE receptor in RAW 264.7 murine macrophages. A) RAGE, the receptor for AGE is downregulated in AGE stimulated RAW cells compared with BSA and untreated control treated wells N=4, $P<0.05$. B) TNFα mRNA is not significant across all treatments in the RAW murine macrophage. N=4, P=NS

A



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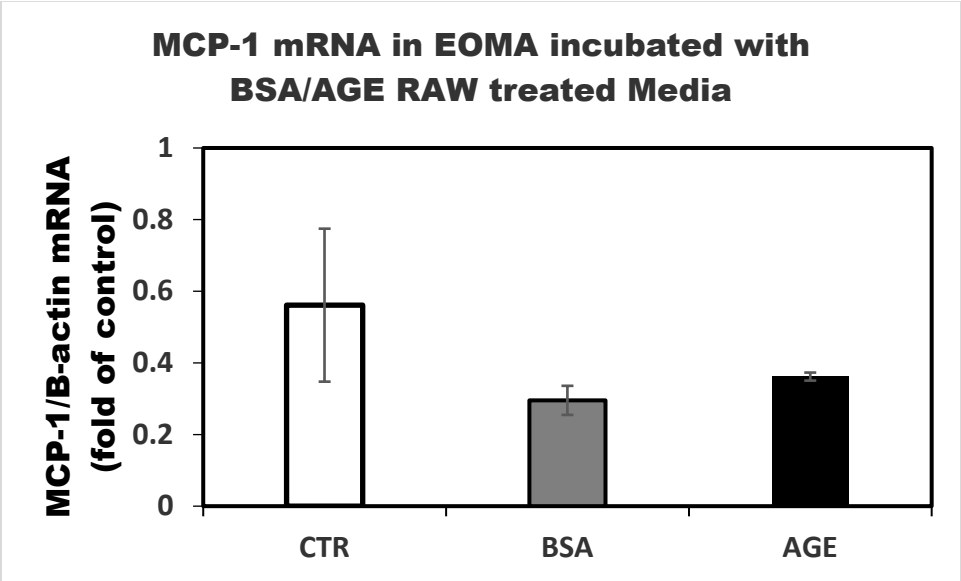
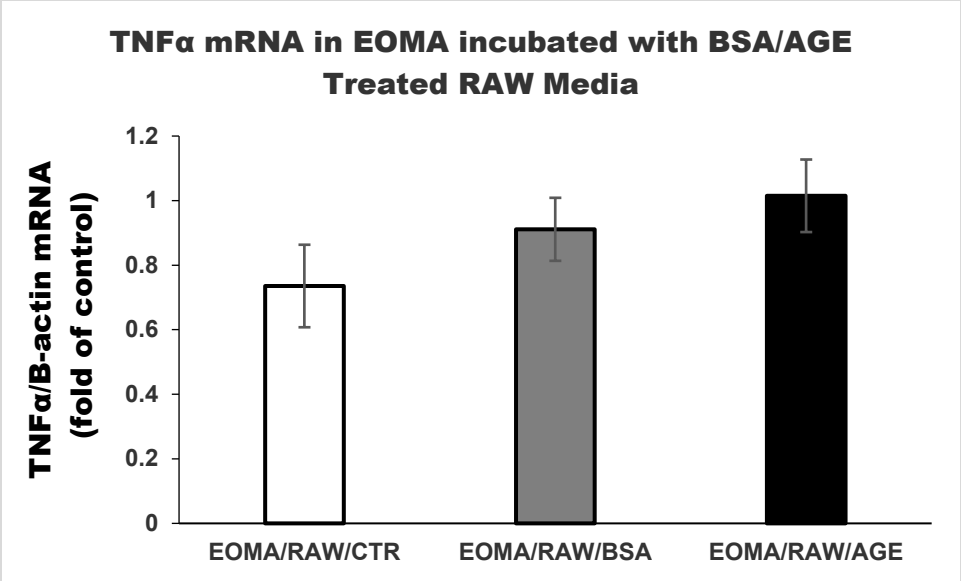


Fig 18. AGE indirect effect on EC does not affect gene translation. A) RAGE mRNA B) MCP-1 mRNA gene expression is not significantly different between EOMA cells incubated with RAW-AGE treated or RAW-BSA treated media. N=4, P=NS

C



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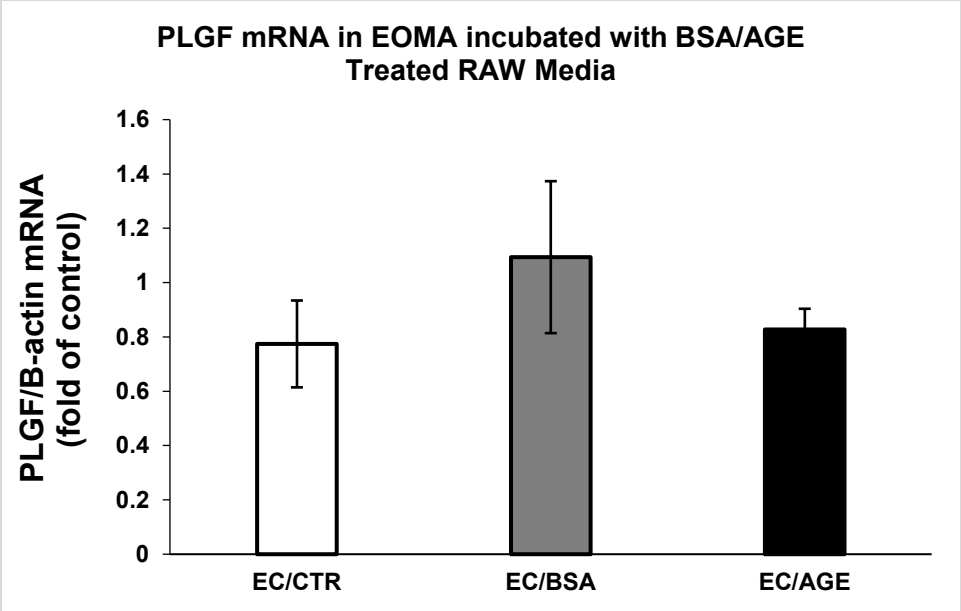

Fig 16 C) AGE stimulation of EC does not affect gene translation. C) TNFα and D) PLGF mRNA are not significantly different in AGE treated RAW media incubated EOMA cells when compared with BSA or untreated control treated RAW media incubated EOMA cells. N=4, P=NS

CHAPTER IV

DISCUSSION

There is extensive documentation in the literature from in vitro cell culture experiments, animal studies, and human studies that establishes that arteriogenesis is reduced by diabetes. However, the mechanism by which this reduction occurs is not well defined.

Our lab has been investigating the hypothesis that diabetes may inhibit arteriogenesis by interfering with the regulation of PLGF expression. In previous studies, our group showed that mice fed a Western diet for 6 months had a reduced level of PLGF expression in skeletal muscle. This effect was seen both as a reduction of baseline PLGF levels, and as an impaired ability to upregulate PLGF after the physiological stimulus of femoral artery ligation [1]. Further in vitro studies were carried out in an attempt to identify the metabolic factor(s) causing this reduction. However, treatment of mouse and human skeletal muscle cells with cholesterol, oxidative stress, glucose, and insulin failed to produce significant and consistent effects on PLGF expression. Since the mice had been exposed to long-term hyperglycemia, a possible role for advanced glycation end products (AGE) was next considered. AGE was quantified in mouse quadriceps femoris and gastrocnemius-plantaris-soleus muscles and was found to be significantly

increased [117], suggesting that formation of AGE could be a major contributing factor to the decrease in PLGF caused by the Western diet in our study.

In the present study, we set out to build on these findings by 1) determining whether AGE could reduce PLGF expression in EC and SKMC and 2) if so, identifying the signaling mechanism by which AGE exerts this effect. We initially hypothesized that AGE would act directly on EC and SKMC to reduce PLGF by acting through its receptor, RAGE to generate TNFα. Therefore, we first tested the direct effect of AGE treatment on PLGF expression in EC and SKMC.

Effect of AGE Treatment on PLGF Expression in EC and SKMC

Contrary to our expectations, direct treatment of HCAEC, HSKMC and MSKMC with 1000μg/ml and 1500μg/ml of AGE or BSA control for 24 h had no significant effect on PLGF mRNA or protein expression in HCAEC and HSKMC. However, there was a significant direct effect of AGE to reduce PLGF expression in MSKMC, compared to BSA. These findings are consistent with a previous report that AGE treatment does not affect TNFα secretion in quiescent endothelial cells [284]

Effect of AGE Treatment on TNFα Expression in EC and SKMC

In addition to assessing the effect of AGE/BSA on PLGF expression in EC and SKMC, we also tested whether AGE or BSA could induce production of TNFα by these cell types. Interestingly, even after treatment of HCAEC and HSKMC with 1000 μg/ml of BSA or AGE for 24 h, TNFα was undetectable across all treatments and cell types (data not shown). Along with our results showing a lack of effect of AGE on PLGF expression in EC and SKMC, these data suggest that there is a general lack of

responsiveness of these cell types (at least when quiescent) to AGE treatment. At least one study has reported that AGE can induce secretion of TNFα in EC [284]. However, in agreement with our findings, another study found that HCAEC which were treated with the RAGE ligand S100A12 were unresponsive to direct treatment, despite the presence of the RAGE receptor [285]. These differences could be due to different EC types/lines being studied and/or may reflect differential responses in quiescent vs. activated EC.

Based on our results showing a lack of direct effect of AGE on PLGF and TNFα in EC and HSKMC, we revised our research question to ask whether AGE could be having an indirect effect on PLGF in the in vivo Western diet studies. Previous studies by our lab have shown that PLGF is highly expressed by EC, and to a lesser extent by SKMC, and that the Western diet reduced PLGF in skeletal muscle (which is primarily composed of SKMC and EC) [117]. Therefore, we focused on measuring PLGF in these cell types. However, many other cell types interact with EC and SKMC in vivo. Therefore, we considered the possibility that AGE could be having an indirect effect on PLGF expression in EC and/or SKMC in vivo, by activating signaling pathways in other cell types which then in turn release mediators that signal to EC and SKMC. Other previous results from our lab showing that recombinant TNFα and IL-1 reduced PLGF expression in HCAEC were consistent with this possibility (unpublished). Therefore, we developed a revised hypothesis: AGE induces TNFα production by macrophages, and this macrophage-derived TNFα then acts on EC and/or SKMC to reduce PLGF.

Effect of AGE Treatment on TNFα and PLGF Expression in RAW 264.7 Cells

AGE is said to act as an antigenic substance that is recognized by macrophages and induces inflammatory cytokine production [286], similarly to the response of differentiated monocytes/macrophages in inducing inflammatory signaling to resolve an injury or clear foreign material from the body [254]. To test our revised hypothesis that macrophages (not EC or SKMC) respond directly to AGE by releasing TNFα, RAW 264.7 murine macrophages were treated with BSA or AGE at 1000 μg/ml or 1500 μg/ml for 24 h. AGE treatment stimulated TNFα overexpression, compared to BSA-treated or untreated cells (Fig 10). Thus, we conclude that RAW 264.7 macrophages are activated by AGE, causing them to secrete inflammatory cytokines.

Our results are consistent with reports from other cell types. There is an established relationship between inflammation and AGE, where glycated albumin is associated with stimulating monocytic cells to produce high amounts of pro-inflammatory cytokines. For example, AGE has been shown to induce inflammation that leads to diabetic retinopathy by inducing retinal microglial cells to over secrete TNFα. These authors also showed that production of TNFα by microglial cells in a glycated albumin environment increases with time of exposure [287]. In our study, we also measured TNFα secretion by RAW 264.7 macrophages treated with AGE for 24 h and 48 h and found that TNFα increases as time increases (data not shown). In our study, we also saw that the effect of AGE was concentration dependent, with 1500 μg/ml AGE causing more TNFα production than 1000 μg/ml AGE. Similarly, others have shown that more AGE formation results in a greater effect on macrophage stimulation and more TNFα production [275]. The relationship between AGE and TNFα is also well established in

human patients. In a study using a multivariable stepwise linear regression analysis, AGE levels were correlated with TNFα and glucose levels in the blood of Type 2 diabetic patients. Therefore, TNFα and AGE have now been proposed as clinical measurements to predict the presence and severity of vascular complications in patients with type 2 diabetes [288].

In addition to inflammatory cytokines, macrophages have been reported to produce numerous growth factors [65]. Therefore, we tested the protein level of PLGF in the RAW 264.7 cells. PLGF protein was undetectable in all treatment groups, suggesting that macrophages do not make significant amounts of PLGF compared to EC and SKMC [289]. This finding, along with our results from EC above, demonstrates that our proposed AGE-TNFα-PLGF signaling pathway cannot occur entirely within either EC, SKMC, or macrophages, but must involve intercellular communication.

Effect of Recombinant TNFα and IL-1 Treatment on PLGF Expression in EC and SKMC

To determine whether PLGF expression in EC and SKMC is responsive to inflammatory cytokines, HCAEC, HSKMC and MSKMC were treated with recombinant TNFα and IL-1. In HCAEC, TNFα significantly reduced PLGF across all concentrations tested (0-50 ng/ml, Fig 7). Interestingly, given the marked effect of TNFα on HCAEC, there was no effect of TNFα on PLGF expression in HSKMC and MSKMC (Fig 8). Treatment of HCAEC with IL-1 had no effect on PLGF expression (Fig 9). These results suggest that EC may be more important than SKMC as a cell type in which PLGF expression is modulated by inflammatory mediators. A possible further conclusion is that altered expression of PLGF in EC, not SKMC, may have primarily been responsible for

the reduced PLGF levels we observed in skeletal muscles of Western diet fed mice. These findings also suggest that TNFα, but not IL-1, is a potential mediator of this effect. Additional studies are necessary to determine whether these conclusions are correct.

TNFα has a multifaceted role in arteriogenesis. Growth factors like PLGF and MCP-1 are produced by EC in response to shear stress and act as chemoattractant to direct the migration of macrophages to the site of remodeling, where they produce TNFα. TNFα stimulates smooth muscle cell proliferation and migration to enlarge the vessel, but can also lead to alteration in shear stress perception [290] and monocyte function [291]. Furthermore, overproduction of cytokines leads to poor vessel growth, e.g. associated with diabetes [292]. Therefore, although a certain level of TNFα is needed in the process of arteriogenesis, overstimulation of TNFα production leads to reduced blood vessel growth [293].

Effect of RAW 264.7 Conditioned Media on PLGF Expression in EOMA cells

We next treated EOMA mouse endothelial cells with media aspirated from AGE-treated RAW cells. After 24 h of incubation with EOMA cells, media from AGE-treated RAW cells still contained a significantly higher level of TNFα than media from BSA or untreated-control RAW cells (Fig 11). In the present study, it is not possible to determine whether all of the TNFα measured at the conclusion of the incubation with EOMA cells was derived from the RAW 264.7 cells, or whether treatment of EOMA with the RAW 264.7 media was able to induce some additional TNFα production by EOMA cells. RAW 264.7 media was centrifuged before use to remove any cells; therefore, any TNFα present

at the conclusion of the experiment was either present in the media when it was first added to EOMA, or was produced by EOMA. To gain some potential insight into whether EOMA cells were producing TNFα themselves, we assessed the level of TNFα mRNA in EOMA cells following treatment with RAW 264.7 media. TNFα mRNA was not significantly increased in the AGE-treated RAW media incubated EOMA cells compared to BSA or untreated control RAW-treated media EOMA cells (Fig 12). This observation suggests that most, if not all, of the TNFα present in the media at the conclusion of the incubation with EOMA cells was derived from RAW 264.7 cells, in support of our intercellular signaling hypothesis.

This was similar to the results obtained from a study where HCAEC were incubated with monocyte conditioned medium, obtained from LPS or S100A12-stimulated primary human monocytes. There was high expression of inflammatory cytokines, for example, TNFα, IL-8 and IL-6, illustrating that monocytes as well as monocyte-derived cytokines could interact with vascular endothelial cells [285].

We then assessed PLGF levels in the EOMA cells treated with RAW 264.7 conditioned media, and obtained the very striking result that PLGF protein levels were significantly reduced in EOMA cells exposed to both the AGE-treated and BSA-treated RAW media for 24 h (Fig 13A-B). This was a fairly rapid response, as we also found that stimulation of RAW cells for as little as 4 h was sufficient to produce a reduction in PLGF when the media was transferred to EOMA cells for 18 h (Fig 13C). As discussed above, AGE-stimulated RAW media contained high levels of TNFα, which we would expect to be the most likely cause of the reduced PLGF (consistent with our results from treatment of HCAEC with recombinant TNFα discussed above). However, the

unexpected observation that media from BSA-treated RAW 264.7 cells also had an effect to reduce PLGF levels is not consistent with our hypothesis, since TNFα was not significantly increased in media from BSA-treated RAW 264.7 cells. Since direct treatment of EC with BSA did not affect PLGF in our earlier experiments, we conclude that BSA treatment of RAW 264.7 cells induced some type of response resulting in the secretion of additional mediator(s) that affected PLGF in the EOMA cells. It is possible that our BSA solution induced an inflammatory response in the RAW 264.7 cells, since the BSA is bovine-derived whereas RAW 264.7 cells are murine in origin. There appears to be a complex and incompletely understood relationship between macrophages and albumin. For instance, although the liver is known to be the major source of albumin production in the body, there are recent reports of non-hepatic production of albumin, including by activated macrophages [294, 295]. Interestingly, another study has shown that albumin can elicit an inflammatory response under certain circumstances [296].

Effect of Endothelial Cell Activation on Responsiveness to AGE

A study comparing the response of quiescent endothelial cells and activated endothelial cells to AGE found that whereas quiescent endothelial cells were unresponsive, activated endothelial cells responded to AGE by production of TNFα [284]. Therefore, we wondered whether the lack of response of HCAEC to AGE in the present study could be due to the relatively quiescent state of the cells. To test this possibility, HCAEC were incubated with BSA or AGE at 1000μg/ml and grown to 80% confluence, then activated with a combination of inflammatory mediators (IL-1, LPS and

IFN-γ) for 24 h. Interestingly, in the activated HCAEC, AGE treatment resulted in an increase in TNFα production in HCAEC, compared to the BSA and untreated control cells (Fig 11A). This finding is consistent with the above-mentioned study and underscores that AGE may have a greater effect on TNFα production in an inflammatory environment such as that found in type II diabetes. In a separate experiment, HCAEC were also stimulated directly with LPS, a known inducer of TNFα [63]. LPS is found in the outer wall of gram-negative bacteria and induces production of TNF in macrophages. PLGF was significantly decreased in response to the higher concentration of LPS (500ng/ml; Fig 14B), consistent with an effect of TNFα to reduce PLGF.

Mouse Multiplex Cytokine Assay

Since we obtained the unexpected finding that PLGF was reduced in EOMA cells by BSA-treated RAW media even though TNFα was not induced, we sought to determine what other cytokines were contained in the BSA-treated RAW media that could be having an effect on PLGF. To gain insight into this question, we carried out a multiplex 6-panel mouse cytokine assay to measure IFN-γ, IL-1, IL-6, IL-10, IL-17 and TNFα in both BSA- and AGE-treated RAW cell media. Our results showed that TNFα and IFN-γ were the most abundantly secreted cytokines in the RAW media after both treatments (Fig 12A). The results also confirmed that media from AGE-treated RAW cells had a higher level of TNFα than media from BSA and untreated control RAW cells, consistent with our earlier results using ELISA. Interestingly, IL-10 and IFN-γ also showed a trend towards increased expression in AGE-treated RAW cell media, with p=0.06 (Fig 15B-C). It is possible that this trend could become significant with an increased sample size.

However, our multiplex assay did not identify an obvious potential mediator for the effect of BSA-treated RAW cell media on PLGF expression in EOMA cells.

Arteriogenesis is a complex process which involves first an upregulation of inflammatory mediators and then a downregulation. This could explain the presence of IL-10, which is an anti-inflammatory cytokine produced by macrophages in order to end an on-going inflammatory process. In an interesting experiment in a mouse model of hindlimb ischemia, TNF-α, IFN-γ and IL-10 were initially increased in the presence of ischemia. However, after human macrophages were transplanted systemically, TNF-α, IFN-γ, IL-4, IL-5, IL-6, and IL-10 were reduced but collateral growth was strongly increased [256]. Similarly, we observed TNF-α, IFN-γ and IL-10 to be increased (or tending towards increase) in our study.

Macrophages are known to exist as M1 (proinflammatory) and M2 (anti-inflammatory) subtypes. Interestingly, both subtypes were found to be present in ischemic limbs of wild type and a high fat diet fed mice model. IFN-γ and TNFα are markers for the M1 proinflammatory subtype, whereas arginase-1 and IL-10 are markers for the M2 anti-inflammatory subtype. Markers for both subtypes were increased in the ischemic hindlimb. It is thought that this reflects the body's attempt to balance out the rise in proinflammatory stimulation by increasing the differentiation of macrophages into the anti-inflammatory M2 subtype, which has been reported to promote arteriogenesis [297] and is necessary in ischemia-induced vessel growth in type 2 diabetic mice [298]. Although we did not examine macrophage subtypes in our in vitro studies, the markers we observed to be present are consistent with the above-described in vivo studies.

Gene Expression for Markers of Endothelial Cell and Macrophage Activation

Gene expression of adhesion molecules (ICAM-1, VCAM-1), growth factors (MCP-1), cytokines (TNF-α) and receptors (RAGE) that are known to be upregulated in activated EC/macrophages was quantified by real-time PCR in order to determine whether direct and/or indirect treatment of cells with AGE or BSA induced activation. When HCAEC were treated directly with AGE or BSA, there was no significant effect on the expression of these genes (Fig 13A-E), showing that AGE itself does not activate quiescent EC. Interestingly, the RAGE receptor did show a non-significant trend ($p=0.08$) towards increase with AGE treatment. This result does confirm that RAGE is expressed in quiescent EC. However, since there was no direct effect of AGE on quiescent EC, the receptor is either not present on the cell surface or its signaling is otherwise inactive in quiescent EC.

When RAW 264.7 cells were treated with AGE, we obtained the surprising finding that RAGE mRNA was downregulated (FIG 17A) while TNFα mRNA was not significantly affected (Fig 17B). Our previous results demonstrates that AGE causes RAW cells to produce TNFα. The gene expression results suggest that 1) the increase in TNFα protein may be post-transcriptional and 2) this effect is not mediated through RAGE signaling. In fact, other groups have shown that AGE signals through TLR4 on monocytes. Interestingly, in those studies, both HCAEC and monocytes were found to express TLR4 and RAGE; however, HCAEC were not responsive to direct stimulation with AGE, (as shown by undetectable TNFα expression) despite having both receptors. TLR4 also has a co-receptor (CD14) and these authors showed that CD14 is expressed on the monocyte/macrophage cell surface but not by EC. They further identified CD14 as

necessary in order for AGE to produce a direct effect [285]. Although we did not analyze CD14 in the present study, our results are consistent with a non-RAGE mediated effect of AGE to induce TNFα production in macrophages, but not EC.

We also assessed mRNA for activation markers in EOMA exposed to BSA- and AGE-treated RAW cell media. Again, there was no significant effect of the AGE-treated RAW media compared to the BSA-treated RAW media (Fig 18A-D). It is interesting to note that MCP-1 was not upregulated by the treatment. It is possible that MCP-1 was not upregulated because PLGF, which acts to promote the function of MCP-1, is downregulated by the AGE-treated RAW media [171].

Possible Mechanism

Diabetes has been established to impair arteriogenesis through inhibition of growth factor production and release, and advanced glycation end products (AGE) have been implicated in this effect. However, the mechanism through which this occurs has not been established. In this study, we provide new insights by showing that PLGF, a key arteriogenic growth factor produced by endothelial cells which is important in the early phase of arteriogenesis, was reduced by AGE via a mechanism involving induction of macrophage inflammatory cytokine secretion.

Decreased macrophage infiltration to sites of arteriogenesis is known to occur in type II diabetes [182]. Under normal conditions, PLGF binds to VEGFR-1 on macrophages [299] and has a chemotactic effect to attract macrophage infiltration. Macrophages then release additional arteriogenic cytokines like TNFα and MCP-1 that allow further macrophage migration into the tissues, smooth muscle cell proliferation and

collateral growth. We speculate that in the setting of type II diabetes, monocytes are activated by AGE and overexpress TNFα, which counters the production of PLGF through a negative feedback mechanism, thereby limiting the monocyte infiltration and subsequent signaling that is induced by PLGF in non-diabetics.

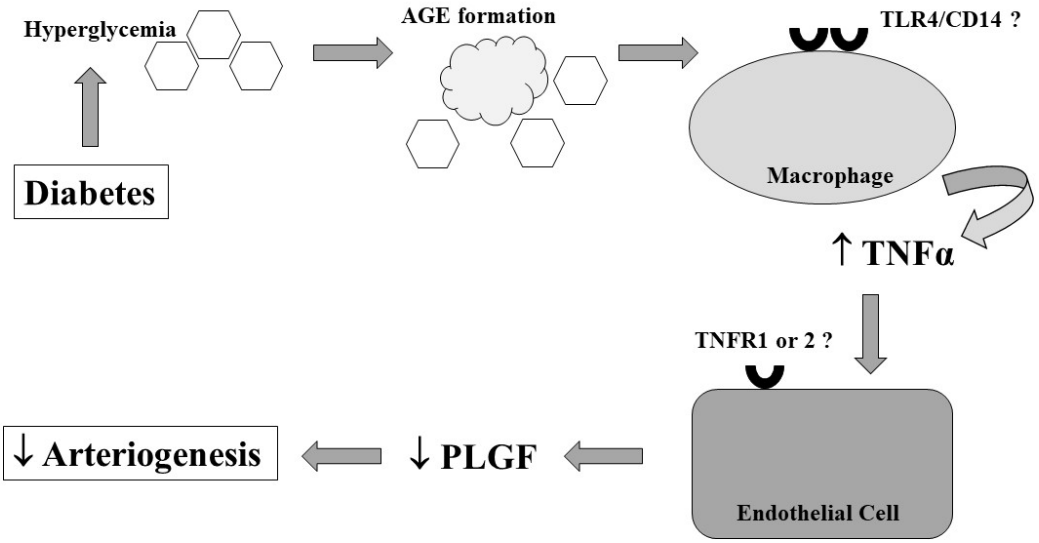

Fig. 19

Future Studies

In order to confirm that TNFα is the mediator of the effect of AGE-treated RAW cell media on PLGF expression in EC, studies with TNFα and TNFR inhibitors are necessary. The effect of BSA-treated RAW cell media on PLGF was unexpected and not consistent with the hypothesis that TNFα is the only macrophage-derived cytokine influencing PLGF expression. Therefore, further studies are necessary to identify how other cytokines such as IL-10 and IFN-γ can contribute to activation of HCAEC and regulation of PLGF. Studies on receptors other than RAGE (TLR4, CD14) which may be

mediating the effects of AGE on both the macrophage and the endothelial cells are also necessary.

Limitations of the Study

The BSA and AGE products used in this study were synthesized in the laboratory. We found that BSA itself had an effect on macrophage cytokine secretion, confounding our results and possibly reflecting an immune response of the murine cells to the bovine protein. Therefore, future studies should avoid this effect by either 1) using commercially synthesized AGE compounds which do not promote such a reaction, 2) matching the species of the albumin to the cell type, and/or 3) de-glycosylating albumin by treatment with PNGase before use, which some researchers believe decreases its antigenicity.

Another limitation of the study is that only healthy (non-diabetic) cell lines were used in this study. Therefore, our experiments may not accurately model how cells would respond to AGE in the diabetic environment. Studies of aged cells/animals would also increase the clinical relevance of the research, since peripheral vascular disease and coronary artery disease primarily affect the elderly.

Clinical Implications

Our results, along with those from other groups, suggest that targeting the innate immune response might be one effective way to clinically improve arteriogenesis. Transplantation of healthy monocytes into an area of collateral growth is currently being explored. Another strategy could be to block the effects of AGE on monocytes, e.g. by application of an inhibitor that targets steps in the pathway to suppress the production of

cytokines. Anti-inflammatory drugs are another possibility. AGE breakers that degrade AGE before it could exert negative effects could also potentially promote and restore arteriogenesis in people living with type II diabetes.

CHAPTER V

CONCLUSION

These studies demonstrate that AGE can reduce PLGF expression in endothelial cells by inducing the secretion of inflammatory mediators such as TNFα by macrophages. Therefore, AGE/TNFα signaling via macrophages may be a possible mechanism for reduced PLGF expression and reduced collateral growth in people with type 2 diabetes. These findings provide novel insights into diabetic vascular disease and may lead to future therapies that can stimulate collateral growth in these patients

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