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CIRCULATING ANGIOGENIC STEM CELLS IN DIABETES

By

Nagma Zafar

M.B.B.S, Gandhi Medical College, India, 1991

A Dissertation

Submitted to the Faculty of the University of Louisville School of Medicine

in Partial Fulfillment of the Requirements for the Degree of

Doctor of Philosophy in
Physiology and Biophysics

Department of Physiology and Biophysics

University of Louisville,

Louisville, Kentucky

December 2015

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A Dissertation Approved on

November 30, 2015

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DEDICATIONS

This work is dedicated to my caring and supportive husband, Dr. Jawed Nasim, without whose encouragement I would not have been able to endure the complexities of the many different roles that come with balancing a PhD and growing a family. I would also like to thank my beautiful children Urooj, Umar and Aliya for helping me keep my perspective and surrounding me with love, laughter, and grammatical assistance. Additionally, I would like to thank my parents, Drs. Kaiser and Zafar Jamil, who instilled in me the joy and excitement of investigative science as I grew and followed their scientific careers.

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ABSTRACT

ROLE OF CIRCULATING ANGIOGENIC STEM CELLS IN
CARDIOVASCULAR DISEASE

Nagma Zafar

November 30, 2015

Circulating angiogenic stem cells (CACs) are rare cells found in peripheral blood that have been shown to contribute to endothelial repair and new blood vessel formation. These cells could be biomarkers and/or therapeutic targets for the assessment and prevention of cardiovascular disease (CVD), which is the leading cause of mortality globally and in the United States. Diabetes is an independent risk factor for CVD and its prevalence has been increasing in epidemic proportions since the mid 1990s. The vascular pathophysiology associated with diabetes is not completely understood and there are inconsistent reports on the role of CACs in diabetic vasculopathy. To study this further we tested the hypothesis that diabetes depletes circulating levels of CACs, due to hyperglycemia or insulin resistance and that CAC depletion contributes to vascular dysfunction associated with diabetes. It was further proposed that in subjects with diabetes CACs have decreased proliferative and angiogenic potential and that changes in plasma cytokine levels are associated with CAC levels. Studies presented here identify one subgroup of CAC, (CAC-3:

AC133⁺/CD34⁺/CD45^{dim}/CD31⁺/CD14⁻), that is reduced in diabetes and whose levels are negatively associated with hyperglycemia and endothelial function. Furthermore we found that increased plasma levels of soluble ICAM-1 are also associated with decreased CAC-3 levels and VEGFR2 surface expression. Our results also show that subjects with diabetes have CACs with decreased adhesive and proliferative capacity. These studies identify the specific CAC phenotypes that are affected by diabetes and suggest that CAC levels are a robust index of long-term glycemic control and that their levels reflect hyperglycemia rather than insulin levels. These studies also suggest that CAC levels may be monitored by bedside assessment of endothelial function. Overall these findings may help in the prognosis and early identification of CVD risk in patients with diabetes, independent of other risk estimates.

TABLE OF CONTENTS

DEDICATIONS	iii
ACKNOWLEDGEMENTS.....	iv
ABSTRACT.....	v
LIST OF FIGURES	vii
LIST OF TABLES	ix

CHAPTER I

GENERAL INTRODUCTION

CIRCULATING ANGIOGENIC STEM CELLS IN CARDIOVASCULAR HEALTH AND

DISEASE..... 1

Development and Repair of the Vasculature 6

Biology of Circulating Angiogenic Stem Cells 12

Identification of CACs from Peripheral Blood..... 16

CACs as potential biomarkers of CVD 20

Therapeutic potential of CACs 24

PROJECT OBJECTIVE 26

CHAPTER II

DEPLETION OF CIRCULATING ANGIOGENIC STEM CELLS IN TYPE 2 DIABETES
AND ENDOTHELIAL DYSFUNCTION

INTRODUCTION.....	28
METHODS AND MATERIALS	30
RESULTS.....	40
DISCUSSION.....	58

CHAPTER III

CELLULAR AND PLASMA FACTORS CONTRIBUTING TO CAC-3 DEPLETION IN
DIABETES

INTRODUCTION.....	63
METHODS AND MATERIALS	66
RESULTS.....	70
DISCUSSION.....	91

CHAPTER IV

ANGIOGENIC POTENTIAL OF CULTURED CACs

INTRODUCTION.....	96
METHODS AND MATERIALS	98
RESULTS.....	106
DISCUSSION.....	125

CHAPTER V

CONCLUDING DISCUSSION.....	130
----------------------------	-----

REFERENCES	135
------------------	-----

CURRICULUM VITAE	158
------------------------	-----

LIST OF FIGURES

FIGURE	PAGE
1. Ontogeny of Circulating Angiogenic Stem Cells	8
2. The new paradigm in vascular biology	11
3. Scheme for sample processing and Gating Strategy for CAC-3	35
4. Gating strategies for 18 phenotypes of CACs	37
5. Linear association between adjusted CAC-3 counts and glycemic indices	48
6. Linear association between adjusted CAC-3 counts and reactive hyperemia index	53
7. Linear association between reactive hyperemia index and glycemic indices ..	57
8. Summary of associations between CAC-3, endothelial function and diabetes	62
9. VEGFR2, CXCR4 and IR expression in non-diabetics and diabetics	73
10. Mitochondrial membrane potential in circulating mononuclear cells	76
11. Heat map of plasma anlylate levels in diabetics and non-diabetics	80
12. Association of CAC-3 counts with sICAM-1 levels	86
13. Association of sICAM-1 with HbA1c and VEGFR2 expression on CAC-3	90

14. Scheme representing the conclusions of Chapter II and III	94
15. Model for the <i>in vivo</i> assessment of the angiogenic potential of late outgrowth cells.....	104
16. Chi square analysis of late outgrowth cells from diabetics and non-diabetics	108
17. Linear regression analysis of percent CD34 ⁺ /CD31 ⁺ in the late outgrowth cells against HbA1c levels	110
18. Tube-like structure formation in diabetics and non-diabetics	113
19. Laser Doppler perfusion imaging 4 weeks after ischemia	116
20. Foot images of necrotic toes post hind limb ischemia	118
21. Line graph of perfusion recovery.	120
22. Morphology and immunophenotyping of late outgrowth cells.....	123
23. Surface antigen expression from late outgrowth cells	125

LIST OF TABLES

TABLE	PAGE
1. Effect of CVD risk factors on CAC counts and function.....	23
2. Chi square analysis of high and low CAC count groups with diabetes.....	42
3. Cohort demographics, medical diagnosis and laboratory data stratified by high and low CAC-3 counts	44
4. Logistic regression modeling low CAC-3 count group with diabetes and glycemic indices	47
5a and b. Unadjusted and adjusted associations CAC-3 count group with endothelial function, inflammation and oxidative stress	51
6a and b. Unadjusted and adjusted associations of reactive hyperemia groups with CAC-3, diabetes, inflammation and oxidative stress.....	55
7. Levels of plasma analytes measured with reference ranges.....	78
8. Significant associations of plasma analyte groups with diabetes	83
9. Linear regression of HbA1c levels with plasma analytes that had a significant association with diabetes.....	83

10. Linear regression of CAC-3 counts with plasma analytes that had a significant association with HbA1c	84
11a and b. Unadjusted and adjusted associations between CAC-3 count groups and levels of soluble adhesion molecules	88

CHAPTER I

GENERAL INTRODUCTION

CIRCULATING ANGIOGENIC STEM CELLS IN CARDIOVASCULAR HEALTH AND DISEASE

Cardiovascular disease (CVD) is the leading cause of mortality in both men and women not only in the United States but also globally (1). The overall CVD death rate in 2011 was 31.3% in the United States, with coronary artery disease being the most prevalent type of heart disease (2). Although the death rate from CVD has been declining since the mid 1990s the burden of poor cardiovascular health remains high (3; 4). The economic costs of CVD and stroke were estimated at \$475.3 billion in 2009, which included \$313.8 billion in direct medical expenses and \$161.5 billion in indirect costs. (5).

The prevalence of diabetes and obesity, both independent risk factors for CVD has seen a rapid incline since the mid 1990s. According to the National Diabetes Surveillance System, a division of the Centers for Disease Control and Prevention (CDC), USA, the percent of the US adult population with the diagnosis of diabetes in 1990 was 2.5% or 6.2 million. This number has more

than tripled in the past two decades to 9.3% or 29.1 million in 2012. If this trend continues it is projected that by the year 2050, 1 in 3 US adults could have diabetes. This trend could potentially reverse gains made in decreasing CVD mortality rates and decrease gains in life expectancy for the next generation for the first time in 100 years. (6; 7).

Diabetes associated CVD is an expensive and serious cause of morbidity and mortality (8). The current burden of diabetes in terms of direct and indirect costs in the United States is around \$245 billion. Among adults diagnosed with diabetes, mortality from all causes is about 2.5 times higher than among adults without the diagnoses of diabetes. The absolute risk of CVD deaths in diabetics compared to non-diabetics is higher across every stratum of age, race, and risk factor level. (9). The cardiovascular complications of diabetes affect both the macro- and micro-vasculature and include coronary heart disease (CHD), stroke, peripheral arterial disease, nephropathy, retinopathy, neuropathy and cardiomyopathy. Atherosclerotic CHD often affects multiple vessels, is asymptomatic, and patients with diabetes who develop coronary artery disease have outcomes far worse than those who do not have diabetes (10; 11). Mortality from stroke is almost 3-fold higher in diabetics than non-diabetics (12). The leading cause of end stage renal failure, non-traumatic limb amputations and blindness in adults is diabetes (6). Almost 75% of the mortality in diabetics arises from CVD (13).

The pathogenesis of CVD in diabetes is poorly understood and further insight is required to develop interventions, preventive care strategies and

therapeutics for diabetes-associated vascular pathology. All of the major CVD risk factors, such as hypertension, hyperlipidemia, obesity, smoking, age, gender, and genetic factors continue to act as independent contributors to CVD, with the addition of the metabolic risk factors associated with diabetes (9). Specific to type 2 diabetes (T2D) is hyperglycemia and hyperinsulinemia that possibly accelerate vasculopathy by enhanced formation of glycosylated proteins and advanced glycation products and by increasing endothelial dysfunction (14).

The crucial role of the endothelium, the innermost lining of the vasculature, has long been apparent in vascular pathology. The endothelium plays a prominent role in the development of many pathophysiological conditions such as atherosclerosis, hypertension and thrombosis (15; 16). It also participates in many homeostatic processes such as control of vasomotor tone, trafficking of cells and nutrients, maintenance of blood fluidity, permeability regulation and formation of new blood vessels (17). In its quiescent state, the endothelium expresses an anticoagulant, antiadhesive and vasodilatory phenotype. Upon activation, the endothelium has the capacity to perform new functions without evidence of cell injury. The activated endothelium develops procoagulant, proadhesive and vasoconstricting properties in response to physiological and pathophysiological stimuli, with subsequent return to the quiescent state. The persistence of the activated phenotype results in a dysfunctional endothelium that is prone to premature apoptosis setting the stage for vascular disease states (17).

The endothelium displays several homeostatic imbalances in diabetes. Insulin signaling in the healthy endothelium is mediated predominantly through activation of the phosphoinositide-3 kinase (PI3-K)/Akt pathway with minimal activation of mitogen-activated protein kinase (MAPK) /extracellular signal-regulated kinase (ERK) pathway (18; 19). Activation of the PI3-K/Akt enhances endothelial nitric oxide synthase (eNOS) expression and nitric oxide (NO) production, which plays multiple roles in preventing atheroma formation (20; 21). NO diffuses into the surrounding vascular smooth muscle cells and induces vasodilatation. NO also inhibits platelet aggregation, SMC proliferation and nuclear transcription of leukocyte-adhesion molecules including vascular cell adhesion molecule (VCAM) and intercellular adhesion molecule (ICAM) (22). In normal physiological circumstances, the PI3-K/Akt pathway predominates and endothelial function is preserved. However while in T2D that is characterized by insulin resistance there is a selective deficiency of the PI3-K/Akt pathway resulting in decreased NO (18; 23). Signaling via the MAPK/ERK pathway is unaffected. Continued activation of MAPK/ERK pathway enhances secretion of a potent vasoconstrictor endothelin (ET-1), which through its pro-inflammatory and mitogenic effects augments the pathogenesis of CVD (24; 25).

Endothelial dysfunction in diabetes is also mediated via inflammatory factors [such as interleukins, C-reactive protein, ICAM, VCAM and tumor necrosis factor-alpha (TNF- α)] and hyperglycemia [through the receptor for advanced glycation end-products (RAGE)] by the activation of nuclear factor κ B (NF κ B), a transcription factor that further stimulates expression of inflammatory

genes (26; 27). Overexpression of either TNF- α or I κ B kinase stimulates the development of insulin resistance that ultimately down regulates the PI3-K/Akt pathway and decreases NO production resulting in endothelial dysfunction (28).

Oxidative stress from the accumulation of reactive oxygen species (ROS) also decreases NO bioavailability by either direct degradation of NO, through formation of peroxynitrite or by uncoupling of eNOS (29). In the presence of insulin resistance peroxynitrite has also been implicated in the down regulation of PI3-K/Akt pathway (30). In the normal physiological state mitochondrial ROS production is minimal. However during periods of exposure to high glucose, as in diabetes there is an increase in the proton gradient within the electron transport chain that enhances superoxide production and leads to an accumulation of excessive ROS (31).

While endothelial dysfunction can be viewed as an early marker of CVD, however there is a significant bench-to-bedside gap with this approach as there are no convenient blood or bedside tests to assess endothelial function (32). The observation that precursors of the endothelium can be sampled from peripheral blood has expanded the ability to assess early events in vascular pathophysiology. These precursors known as circulating endothelial progenitor cells (EPCs) or circulating angiogenic stem cells (CACs) have been reported to participate in the process of endothelial repair and vasculogenesis (33). Quantitative and qualitative assessments of these cells may serve as a biomarker of CVD initiation and progression. Preserving or enhancing the

number and function of CACs could result in novel approaches to preventive care and therapeutics in cardiovascular medicine.

Development and Repair of the Vasculature

Survival of the vertebrate embryo depends upon the development of a system that delivers oxygen and nutrients to, and removes wastes from growing tissues. This is accomplished by the development of the circulatory and the hematopoietic systems from the embryonic mesoderm. This process is initiated when signals from the visceral endoderm pattern the underlying mesoderm (34-36). Aggregates of mesodermal cells form blood islands in the extra-embryonic yolk sac. This phase of development is known as primitive hematopoiesis and occurs at stage E7.0 to E7.5. Cells within the blood island develop into primitive blood cells, and the surrounding cells flatten to form the primordial endothelial cells (37; 38). Coalescence of blood islands leads to the development of a primitive vascular plexus that subsequently remodels to make mature vascular plexus.

Definitive hematopoiesis is marked by the generation of multi lineage hematopoietic stem cells from a small subset of the primordial endothelial cells at stage E8.25 to E9.5 in the extra embryonic yolk sac and by stage E9.5-10.0 in the embryonic aorta/gonad/mesonephros region, the fetal liver and the fetal bone marrow. As blood and endothelial cells develop concomitantly this led to the suggestion of a common progenitor –the hemangioblast, that gives rise to both hematopoietic and endothelial cells (39-41) (Fig. 1).

FIGURE 1. Ontogeny of Circulating Angiogenic Stem Cells

Based on the location of the cells this scheme is divided into three overlapping components - Embryo, Bone Marrow and Peripheral Blood. The red arrows delineate the pathway to circulating angiogenic stem cells, beginning in the inner cell mass of the embryo and culminating in the endothelium. CACs are mesodermal in origin and differentiate from the hemangioblast, which is also a precursor of hematopoietic stem cells. CACs may also differentiate further downstream from the granulocyte and macrophage progenitor.

FIGURE 1.

Ontogeny of Circulating Angiogenic Stem Cells

In an adult, the bone marrow continues to harbor multi-lineage stem cells that provide the cellular components of whole blood throughout life. Multi-lineage stem cells that are capable of reconstituting a sublethally ablated bone marrow are known to be present in the peripheral circulation since 1987 (42). The observation that stem cells – endothelial progenitor cells also known as circulating angiogenic stem cells (CACs) are capable of vascular repair and vasculogenesis in peripheral blood was first made in 1997 by Asahara et al (43).

The existence of CACs challenged the concept that postnatal vascular repair occurred only by the proliferation of the local endothelium and that new blood vessels formation occurred only by sprouting from preexisting vessels (angiogenesis) (44; 45). It has now been proven that new blood vessels in adults can be made *de novo* by recruitment of circulating angiogenic stem cells (CACs) to areas of hypoxia, where these cells proliferate and assemble into blood vessels that subsequently anastomose with a preexisting vessel (43) (Fig. 2). This concept was further verified by bone marrow transplantation of genetically labeled cells and their subsequent incorporation into the new vasculature supporting ischemic tissue (46-49). CACs were also found to incorporate in tumor models of angiogenesis, strengthening the evidence that circulating stem cells contribute to neovasculogenesis (50). Some studies have measured the percent contribution of CACs to newly formed blood vessels of ischemic lesions and have estimated that CACs contribute up to 25% of the endothelial cells therein (51).

FIGURE 2. The new paradigm in vascular biology

Circulating Angiogenic Stem Cells transgress from the lumen of the blood vessel and migrate to sites of hypoxia where they assemble and proliferate making blood vessels *de novo* that subsequently anastomose with preexisting vasculature. CACs also repair the existing vasculature by replacing denuded endothelium.

FIGURE 2.

Circulating Angiogenic Cells Contribute to Postnatal Vasculogenesis and Repair of the Endothelium.

However, studies have also reported absence of bone marrow derived stem cells in the endothelial layer and tunica media of blood vessels in areas of angiogenesis in models of ischemia and tumor angiogenesis (52; 53). These and other studies report the presence of bone marrow derived stem cells in the adventitia of growing collaterals and suggest that these cells may represent pericytes, fibroblasts or leucocytes that localize to areas of angiogenesis or arteriogenesis and support the new vasculature by secreting paracrine factors such as growth factors, cytokines and proteases (54-57).

The contribution of CACs in the repair of injured endothelium was confirmed by their identification in denuded endothelium post balloon injury, and their lining on the vascular surfaces of implanted ventricular assist devices and dacron grafts (58-60). Taken together these studies affirm a new paradigm in vascular biology that the formation, repair and maintenance of the vasculature may be in part dependent upon circulating angiogenic stem cells.

Biology of Circulating Angiogenic Stem Cells

Precursors of CACs – hemangioblasts - reside in specific microenvironments called niches that are mostly located mostly in the bone marrow (61-63). The presence of CAC niches at other sites such as: the heart, vascular tissue and spleen, has also been reported (58; 64-68). These environments enable the maintenance, self-renewal, proliferation and differentiation of stem cells. Although each type of stem cell has a unique niche, in general, a niche contains stem cells, stromal cells and extracellular matrix. It

receives neuronal inputs and has blood vessels that bring in systemic signals and provide a conduit for cell egress. The stromal cells may consist of numerous cell types that include reticular cells, fibroblasts, macrophages, osteoblasts, osteoclasts, neuronal cells, adipocytes, and pericytes (69; 70). The interactions of the stem cells with stromal cells, and the extracellular matrix, and the bioavailability of stem cell cytokines, enables the preservation of the stem cell pool for long-term renewal thereby contributing to growth, repair and maintenance of tissues in the adult.

In the steady state, CAC precursors are maintained in the quiescent or G_0 phase of the cell cycle except for a small fraction of stem cells that are in the S or G_2/M phases (71). The interactions between stromal cell derived factor (SDF-1), secreted by the stromal cells and its receptor CXCR4 is responsible for keeping the stem cells within the niche in a quiescent state (72; 73). Stem cell factor (SCF) and its receptor c-kit also play an important role in cycling and reconstituting the stem cell pool in niches (74). Upon stimulation CAC precursors undergo asymmetric cell division, self-renewing and releasing progenitor cells into the circulation (75-77).

A variety of stimuli promote the release of CACs into the blood stream. The most potent physiologic stimuli is hypoxia (78; 79). Growth factors (vascular endothelial growth factor, placental growth factor, basic fibroblast growth factor), cytokines (granulocyte colony stimulating factor, granulocyte monocyte colony stimulating factor, erythropoietin, stromal cell derived factor-1), elastases, proteases (matrix metalloproteinase 9), hormones (estrogen) and exercise also

are reported to stimulate or be critically involved in the mobilization of CACs into circulation (80-82).

Tissue ischemia induces CAC mobilization through the activation of the transcription factor, hypoxia-inducible factor-1 (HIF-1), which is a heterodimer composed of α and β subunits. While HIF-1 β is constitutively expressed and HIF-1 α expression is regulated by cellular oxygen concentration. HIF-1 α is rapidly degraded under normoxic conditions, however degradation is inhibited in the presence of hypoxia allowing dimerization of the two subunits and binding of the now active HIF-1 to sites in the nucleus that promote transcription of genes such as VEGF, SDF-1 and erythropoietin (83; 84). The gradients created by these factors causes changes in the stromal cell-stem cell interactions and in the extracellular matrix leading to the egress of CACs into circulation (78; 79).

One well described stromal cell-stem cell interactions in c-kit positive stem cells that enables mobilization of CACs occurs via a protease, matrix-metalloproteinase-9 (MMP-9). Kit Ligand (KitL) is expressed on the surface membrane of stromal cells and is cleaved by MMP-9 to release soluble KitL (sKitL). This sKitL binds to the c-kit receptor on the stem cells and initiates stem cell cycling, differentiation and mobilization. MMP-9 release is regulated by VEGF, G-CSF and SDF-1 (85).

The survival of CACs in the peripheral circulation is crucial to enable repair or formation of new vasculature. As CACs home to environments that are ischemic or have tissue injury they, like other stem cells must have a high resistance to oxidative stress. Studies have demonstrated increased levels of

antioxidant enzymes such as catalase, glutathione peroxidase and manganese superoxide dismutase (MnSOD) in human CACs (86; 87). Increased sensitivity to apoptosis and decreased expression of Flk-1 was found to be involved in decreased CAC counts seen in mice deficient in glutathione peroxidase (GPx-1) that were subjected to ischemia (88). In humans there is evidence that VEGF acts via the VEGFR2 to promote CAC survival, proliferation and migration (89). Inflammation or inflammatory cytokines may reduce CAC survival, increase apoptosis and reduce angiogenesis (90). Accelerated senescence through telomerase inactivation is another mechanism that could decrease survival of CACs. Decreased bioavailability of nitric oxide, increased exposure to reactive oxygen species, hyperglycemia, high levels of ox-LDL and angiotensin-II have also been implicated in decreased survival of CACs (90-95).

Attachment to sites of vascular repair or migration to sites of new vessel formation is dependent on a variety of factors that enable stem cell rolling, adhesion and transmigration. These include: P-selectin and E-selectin interactions for rolling (96), VCAM and ICAM interactions with integrins for adhesion (97), and matrix degrading enzymes (MMP-2, MMP-9, cathepsin, urokinase) for migration (98-100).

Homing of CACs to sites of repair or ischemia involves the CXCR4/SDF-1 interaction (101-103). In the bone marrow the disruption of this interaction is required for the egress of CACs into the peripheral circulation. Decreased levels of SDF-1 accompanied by decreased activation of CXCR4 are required for stem cell mobilization, while the reverse is required for homing. Hypoxic tissues, as

generated during exercise up regulate SDF-1 resulting in CAC homing to these sites and new vessel formation (83; 104).

The coordination of this entire process maintains the pliability of the vasculature and cardiovascular health. Besides contributing to vascular integrity CACs have been show to secrete growth factors such as: VEGF-A, VEGF-B, SDF-1, and insulin-like growth factor-1 (105). The paracrine effect of these factors enables local angiogenesis by promoting migration and differentiation of the surrounding mature endothelium. In addition, factors secreted by CACs stimulate cardiac resident c-kit progenitor cells and mature cardiomyocytes and promote recovery or remodeling of cardiac tissue post injury (46; 106).

Identification of CACs from Peripheral Blood

CACs have been identified from peripheral blood by two methods: 1) based on surface antigen expression and 2) based on their ability to adhere, proliferate and form colonies *in vitro*. Both methods have yielded multiple phenotypes of cells that have the ability to contribute to new vessel formation. Yet neither of these methods can reliably predict the fate of these cells in an *in vivo* model. Additionally, it is not known if the phenotypes generated by culture conditions exist in the blood stream (107).

Selection based on Surface Antigens

In peripheral blood CACs are identified as heterogeneous populations due to their changing surface antigen profile, as they are in the process of losing stem cell characteristics and acquiring properties of the local endothelium (108). This has made it difficult to arrive at a single and uniform definition for a

circulating angiogenic stem cell (109-111). The difficulty in converging on a single unifying definition also arises due to the hierarchical proximity of these cells to hematopoietic stem cells resulting in commonality of some surface antigens.

Stem cells capable of reconstituting the bone marrow after myeloablative therapy have been identified from the CD34 positive fraction of peripheral blood, cord blood and bone marrow. This stem cell pool and the AC133 positive pool are reported to contain stem cells capable of differentiating into endothelial cells (112-114). The CD34 antigen, sialomucin is a cell surface glycoprotein is found on hematopoietic stem cells, angiogenic stem cells and on capillary endothelial cells (33; 115; 116). Although the function of this antigen is not well characterized, its expression is used for the immuno-purification of stem cells and its expression is considered to be one of the characteristics of a CAC despite the fact that it is also expressed on hematopoietic stem cells and on certain fractions of the mature endothelium (117).

The AC133 antigen, prominin coexists on a subpopulation of hematopoietic stem cells that are bright for CD34 surface expression. AC133 expression is not found on mature endothelial cells, hence the dual expression of AC133 and CD34 represent CACs and not circulating mature endothelial cells. AC133 expression is lost from stem cells as they differentiate and commit to a specific lineage (toward the mature cell type). Hence dual expression of AC133 and CD34 is used to identify early and highly proliferative stem cells compared to the AC133 low or negative/CD34⁺ cells (118).

The surface antigens VEGFR2 and CD31 are expressed on stem/progenitor and mature endothelial cells. Their coexistence with AC133 defines a population of stem cells capable of vasculogenesis. The AC133+/VEGFR2+ cells have been found in the neointima of ventricular assist devices. These cells have also been reported to be rapidly mobilized 6 -12 h following vascular injury and they have been shown to give rise to endothelial cells (119; 120). Thus combinations of surface antigens present on stem cells (CD34, AC133) and endothelial cells (KDR/VEGFR2, CD31) are thought to more accurately define CACs (121; 122).

CACs may be distinguished further as cells of hematopoietic and endothelial lineages by the expression of CD45 and CD14. Wherein, cells with a low (CD45dim) or deficient expression of CD45 (CD45 negative), and negative for CD14 (CD14 negative) are of endothelial lineage rather than of hematopoietic origin (123; 124).

Selection based on Cell Culture

The three most commonly described cell culture methods for characterizing CACs from peripheral blood mononuclear cells are as follows:

- 1) Colony Forming Assay – Endothelial Cell (CFU-EC or CFU-Hill) (125), wherein mononuclear cells were initially plated on fibronectin-coated plates, and after 48 h the non-adherent cells were collected and replated again on fibronectin coated plates. The appearance of clusters or colonies with an endothelial cell phenotype was noted on days 4-7. These colony counts were reported to correlate to endothelial function and cardiovascular risk (125). Although these CFU-EC

express endothelial markers, subsequent analysis revealed that these cells also express myeloid markers such as CD3. The core of the CFU-EC was made of angiogenic T cells and monocytes that have acquired an endothelial phenotype due to a high concentration of endothelial growth factors in the media (126). It has been convincingly demonstrated that early outgrowth cells are predominantly of monocytic lineage (127).

2) EPC culture assay (128; 129) – where peripheral mononuclear cells were plated on gelatin or fibronectin-coated plates and the non-adherent cells were discarded on day 3, and the culture was continued with the adherent cells. Colonies that appeared from the adherent cells between days 4–10 were called early EPCs, and outgrowth cells that appeared after day 14 were referred to as late EPCs. These early and late EPCs differed in their ability to release cytokines, as the late EPCs had lower cytokine release. Both phenotypes were able to incorporate into capillaries. The early and late EPCs isolated by this method were also reported to have myeloid and hematopoietic markers. It is also reported that in the early EPCs endothelial gene promoters were silenced (130)

3) Endothelial Colony Forming Cells (ECFC) assay (127; 131) - where peripheral blood mononuclear cells were plated on collagen-1 coated plates and non-adherent cells were removed on day 2, and the culture was continued with the adherent cells. Endothelial cells with a high proliferative capacity appeared between days 14 and 21. These cells differed from those of the above methods in that they lacked myeloid and hematopoietic cell markers. These outgrowth cells were capable of forming vascular networks in matrigel.

Regardless of the methodology used for CAC quantification the conclusion from numerous studies investigating their role in vascular pathology suggests that these cells are depleted and/or have defective functional capabilities. To be applicable in a clinical setting, CAC quantification must be a simple, rapid and reproducible procedure, making flow cytometry the choice method. However CAC are rare in peripheral blood, and if only 1 or 2 antigenic markers are used to identify these cells and increase yield then specificity is lost. While increasing the number of antigenic markers will increase specificity this leads to a low yield with higher variability. While the culture methods allow for functional assessment of CACs, they are more time consuming and the exact origin of the cultured cells is yet to be identified.

CACs as potential biomarkers of CVD

Leucocyte, neutrophil and lymphocyte counts are used routinely as cellular biomarkers in the assessment of the etiology of febrile events. The utility of CACs in CDV risk estimation has been studied extensively as their levels are reported to vary in acute and chronic CVD. These cells seem to reflect the state of the underlying vasculature and could hence serve as a biomarker for CVD.

Tabulated below are results from some studies that compared levels of CACs in subjects with and without CVD and its risk factors (Table 1). The overall trend is a reduction in the number and function of CACs with aging and presence of CVD risk factors. Increased levels have been reported during physiological states such as, the follicular stage of the menstrual cycle and exercise (132; 133), and pathological states such as acute ischemia (134).

The number of CD34⁺ cells in peripheral blood under steady state conditions ranges from 1-5/mm³ or 1000–5000/ml blood (135; 136). It is estimated that about 80% of CD34⁺ cells are hematopoietic stem cells, about 15% CACs, and the remainder express antigens for other lineages such as smooth muscle cells (137). The number of circulating CD34⁺ cells is very small compared with the other cellular components. In one cubic millimeter of peripheral blood there are, ~ 5 x 10⁶ red blood cells, ~ 4-10 x 10³ white blood cells, and ~ 110 x 10³ platelets. CACs are subsets of the CD34⁺ fraction and hence they are fewer in number. It is estimated that the number of CD34⁺KDR⁺ cells in 1ml of whole blood is around 350-750 [or 50-100/ 1 million WBC (0.005-0.01%)] and the AC133⁺CD34⁺KDR⁺ subset of cells are 20 fold lower (107). However due to the non-uniform methodologies used for measuring and characterizing CAC, and the heterogeneity in their identity, normal values of their levels in the steady physiological state have not yet been established.

CAC levels have also been reported to be predictive of outcomes such as all cause mortality, CV mortality and major CV events in subjects with CVD; atherosclerotic disease progression in subjects with stable CAD (138-140); and all-cause survival and cumulative cardiovascular event-free survival in subjects with chronic renal failure on hemodialysis (141; 142). These studies support the role of CACs as a biomarker for CVD, however they are based in high risk subjects and have a short term follow up period. Longitudinal studies with long term follow up, and studies in low risk subjects are required to validate the use of CAC counts as a biomarker for CVD (143).

TABLE I. Effect of CVD Risk factors on CAC counts and function

Selected studies that report the effects of CVD risk factors on CAC counts in human subjects are listed. This table reveals that despite the heterogeneity in identifying CACs their depletion and decreased function are prominent.

TABLE 1.

CVD Risk Factor or Disease	Study Cohort (n)	Effect on CAC Number	Effect on Function	Reference
Age (43-80 years)	Stable CAD patients (50)	↓CD34 ⁺ /AC133 ⁺		Scheubel,RJ. 2003 (144)
Hyperlipidemia (Total-Cholesterol and LDL -C)	Diagnosis of Hypercholesterolaemia (20) and matched controls (20). No statin use	↓CACs +ve for DII-LDL uptake and Lectin binding	↓Proliferation, migration, adhesion	Chen,JZ 2004 (145)
Hyperlipidemia TG, TC/HDL-C ratio, 10 year FRS	Males (24) with 10 yr average CVD risk of 20 (FRS range 2-36). No statins	↓CFU-Hill (no association with CD34 ⁺ /AC133 ⁺ cells in PB)	-	Pellegatta,F 2005.
HDL-C	Males (24) with 10 yr average CVD risk of 20 (FRS range 2-36). No statins	↑CFU-Hill (no association with CD34 ⁺ /AC133 ⁺ cells in PB)	-	Pellegatta,F 2005.
Hypertension	Newly diagnosed untreated subjects with essential hypertension (30), age and sex matched controls	↓CD34 ⁺ /KDR ⁺	-	Pirro,M 2007 (146)
Hypertension	135 consecutive hospitalized patients with CVD and 25 controls	↓CD34 ⁺ /AC133 ⁺ /CD45 ^{low}	-	Umemura,T 2008 (147)
Type 2 Diabetes	Subjects ranging in their glucose tolerance from NGT(205), IGT(43), IFG, new onset of DM(32) to >20 years of DM	↓CD34 ⁺ and not CD34 ⁺ /KDR ⁺ in those with IGT and new onset DM	-	Fadini,GP 2010 (148)
Type 2 Diabetes	Subjects with T2D (20) and age-matched controls (20)	↓EPC clusters in culture		Tepper,OM 2002 (149)
Smoking	Smokers (15) and matched nonsmokers (14), all healthy.	↓CD45 ^{low} /CD34 ⁺ /CD133 ⁺ ↓CD45 ^{low} /CD34 ⁺ /CD133 ⁺ /VGEFR2 ⁺		Kondo,T 2004 (150)
Smoking	Healthy (15) and CAD patients (45)	↓CD34 ⁺ /KDR ⁺	↓Migration in CAD patients	Vasa,M 2001 (151)
Obesity	General population 20 men who met criteria for obesity and metabolic syndrome, and 10 age matched healthy controls	↓CD34 ⁺ /KDR ⁺		Westerweel,PE 2008 (152)
Sum of risk factors (age, sex, HTN, DM, Smoking, FH of CAD, LDL-C level)	Healthy (15) and CAD patients (45)	↓CD34 ⁺ /KDR ⁺	↓Migration in CAD patients	Vasa,M 2001 (151)
FRS score	Males with CVD risk (45)	↓CFU-Hill	↑Senescence	Hill,JM 2003 (125)

Therapeutic potential of CACs

The potential of CACs in treating conditions such as myocardial ischemia and peripheral limb ischemia has been investigated ever since these cells were first reported. In animal models of limb ischemia, CAC biology has been approached from numerous angles to investigate their effect on vascular regeneration. These approaches have included the use of injections of freshly isolated CACs, culture-expanded CACs, genetically modified CACs, and/or use of factors that improve mobilization, survival or homing of CACs (33; 46; 65; 78; 103; 128; 153-155). These experiments have demonstrated various levels of incorporation of CACs into the vascular structures, improvements in tissue function and perfusion, and reduced scarring.

In humans, initial clinical trials using CAC therapy in ischemic conditions have shown promising results. The Therapeutic Angiogenesis using Cell Transplantation (TACT) trial was a randomized trial and the first report on the use of bone marrow-derived mononuclear cells to treat critical limb ischemia due to peripheral arterial disease. Intramuscular injection of autologous CACs lead to significant improvements in leg pain, pain-free walking distance, and reduced ulcer size, that were maintained for at least 2 years (156). At the three years follow-up assessment the TACT trial showed significantly lowered amputation rates in the treatment group (157).

Similar positive outcomes have been reported in the first randomized study investigating the effects of intracoronary infusion of CACs -The Transplantation of Progenitor Cells and Regeneration Enhancement in Acute

Myocardial Infarction (TOPCARE-AMI) trial- that has now published a five year follow up report (158-160). Improvements in cardiac function included reduced functional infarct size, improvement of LV ejection fraction, reduced diagnosis of heart failure and no hospitalization for heart failure.

Currently there are several clinical trials using a variety of CAC phenotypes with different modes of delivery that mostly demonstrate beneficial effects in patients with critical ischemia (161-164). Because of the heterogeneity among these trials, it is difficult to identify which CAC phenotype has the most benefit for CVD. Furthermore, these trials mostly utilize very high-risk individuals where it is easier to show benefits. Trials in lower risk populations are required to confirm benefits of CAC therapy. What has been learned from clinical trials so far is that CAC therapy is feasible with no reports of unexpected adverse outcomes such as the possibility of tumor development, calcification of vasculature, or arrhythmias. Overall the safety profile of CAC therapy is promising.

PROJECT OBJECTIVE

A dysfunctional endothelium is the primary determinant of many pathophysiological states especially CVD. Circulating angiogenic stem cells (CACs) play an important role in maintaining endothelial health and in new blood vessel formation. Levels of CACs have been found to be predictive of cardiovascular events and mortality. Although the levels of these cells reflect overall CVD risk, studies assessing their association with major CVD factors such as hypertension, dyslipidemia and diabetes have yielded inconsistent results and the mechanisms contributing to their depletion remain unknown. We hypothesized that CAC depletion in diabetes is mediated in part by hyperglycemia or insulin resistance and that this depletion contributes to vascular dysfunction. We further proposed that in diabetics CACs have decreased proliferative and angiogenic potential and that altered plasma cytokine levels impact CAC levels. To test these hypotheses we conducted a cross sectional human study and addressed the following specific aims 1) quantify phenotypically distinct circulating levels of angiogenic cells in subjects with and without diabetes and analyze the association of these cells with diabetes, hyperglycemia, insulin resistance and endothelial dysfunction (Chapter II);

2) measure and analyze cellular and plasma factors that contribute to changes in CAC levels (Chapter III); 3) assess the angiogenic potential of the cultured CACs from subjects with and without diabetes using *in vivo* and *in vitro* assays (Chapter IV).

We found that CACs expressing surface antigens AC133, AC133/CD31, CD34, CD34/CD31, CD34/KDR, CD34/AC133/CD45 and CD34/AC133/CD31/CD45^{dim} are lower in subjects with diabetes. CD34⁺/AC133⁺/CD31⁺/CD45^{dim} cells (CAC-3) were found to have an inverse relationship with HbA1c and fasting plasma glucose, but not with insulin levels or the HOMA-IR score. Peripheral endothelial function was found to have a strong positive correlation with CAC-3. In diabetics, decreased levels of CAC-3 were associated with decreased surface expression of VEGFR2 and CXCR4 in diabetics and soluble ICAM-1 levels had a negative correlation with CAC-3 counts and VEGFR2 expression. These novel new associations could potentially reduce the bench-to-bedside gap in assessing early endothelial dysfunction in subjects with diabetes.

CHAPTER II

DEPLETION OF CIRCULATING ANGIOGENIC STEM CELLS IN TYPE 2 DIABETES AND ENDOTHELIAL DYSFUNCTION

INTRODUCTION

Cardiovascular mortality is the leading cause of death worldwide (1). Identifying early and specific indicators of declining vascular health allows a greater window of opportunity for institution of preventive care measures that delay progression to the disease state. Among those non-communicable diseases of enormous economic and clinical significance is diabetes related CVD. With increasing obesity and other risk factors, the global prevalence of diabetes is estimated at 8.3% or 387 million (<http://www.idf.org/diabetsatlas/>), and in the United States it is estimated to be at 9.3% or 29.1 million in 2012. Diabetes-associated CVD is an expensive and serious cause of morbidity and mortality (8). The current burden of diabetes in terms of direct and indirect costs in the United States is around \$245 billion. Among adults diagnosed with diabetes, mortality from all causes is about 1.5 times higher than among adults without the diagnoses of diabetes.

The role of CACs in diabetes-related vasculopathy has been an area of intensive research since CACs were first reported in 1997 as endothelial progenitor cells (EPCs) (43; 107). CACs are heterogeneous populations of stem cells in peripheral blood that are in the process of losing their stem cell characteristics and acquiring properties of the local endothelium (108). They are defined as cells that have a combination of stem cell surface antigens such as, CD34, AC133 and endothelial antigens such as KDR/VEGFR2 or CD31 (121; 122). Most studies have reported CACs as cells that express either only CD34 or CD34/KDR antigens, without accounting for the pan-leukocyte antigen CD45 or the monocyte antigen CD14. This omission may not adequately discriminate the hematopoietic stem cells from CACs (123; 124).

The impact of hyperglycemia, prediabetes and overt diabetes on CACs has been extensively studied (165; 166), more so in relation to CVD outcomes such as acute myocardial infarction (167-169). Most studies have reported a depletion of CACs as defined by only CD34 surface antigen or CD34/KDR expression. Diabetes is also reported to adversely impact all stages of CAC biology including mobilization, survival, homing and proliferation (170; 171). Studies reporting the impact of common anti-diabetic medications and related interventions on CACs (172-174) have also shed some light into this very complex area of diabetic vascular therapeutics. However there are very few reports on the association of CAC levels and clinical measures of endothelial function. One study reported a negative correlation of CAC colony outgrowth

cells and endothelial function as measured by flow mediated dilation (FMD) of the brachial artery (125).

Hence we examined the association of CACs with Type 2 Diabetes (T2D) and indices of glycemic control, CVD risk and with markers and measures of endothelial function that will help improve effective bench to bedside screening and intervention in subjects with T2D.

Using a comprehensive definition of a CAC CD34⁺/AC133⁺/CD31⁺/CD45^{dim}/CD14⁻; CAC-3 we hypothesized, that the depletion of this phenotype occurring in Type 2 Diabetes is mediated in part by hyperglycemia or insulin resistance and is associated with endothelial dysfunction. To address this we studied a cohort of subjects with and without Type 2 Diabetes. Other measurements included peripheral arterial tonometry, levels of 17 other CAC phenotypes and inflammation and oxidative stress markers. In this study we assessed only one aspect of endothelial dysfunction which is reactive hyperemia as measured by change in pulse wave amplitude following a period of ischemia.

METHODS AND MATERIALS

Study Cohort

The cohort was selected from the University of Louisville Endocrinology and Primary Care Clinics during the period between June 2011 and May 2013. Subjects were prescreened and those with conditions known to affect peripheral blood cell counts and bone marrow function were excluded from the study. These conditions included malignancies, organ transplant, renal replacement therapy,

type 1 diabetes, untreated thyroid disease, anemia, acute infections, HIV infection, hepatitis, and unhealed wounds. Subjects taking hormone replacement therapy, or medications affecting bone marrow function or peripheral blood cell counts, those that received transfusion of blood products within the past year, or hospitalization within past 5 months were also excluded from the study. Other exclusion criteria included subjects unwilling or unable to provide informed consent, pregnant or lactating women, prisoners and other vulnerable populations. Subjects were between the ages of 22 and 65 years. The study was approved by the Institutional Review Board at the University of Louisville (IRB 10.0350), and all individuals gave a written informed consent. A total of 108 subjects enrolled in the study but in the final analysis 3 subjects were eliminated due to processing errors with their samples.

Clinical procedures

Subjects enrolling in the study had a morning appointment and were requested to fast for a minimum of 8 h. All menstruating female subjects were in the luteal phase of their cycle. At the clinic, subjects filled out a Health and Diabetes Questionnaire, biometrics were recorded, and urine and venous blood samples were collected.

Peripheral endothelial function was measured using the EndoPAT, instrument, according to the guidelines recommended by the manufacturer (Itamar Medical). The EndoPAT uses fingertip plethysmography to measure the changes in pulse wave amplitude observed before and after a period of reactive hyperemia, which is quantified as a score and reported as the Reactive

Hyperemia Index (RHI). A score of 2.00 or higher indicates good endothelial function and a score lower than 1.67 indicates poor endothelial function. RHI has been reported to correlate with flow-mediated dilation of the brachial artery in subjects with coronary artery disease (CAD) (175). It is also reported to indicate early endothelial dysfunction in subjects with CAD (176).

Waist and hip was measured in accordance with WHO 2008 Expert Consultation Report. Body fat percentage was measured using a hand held bioelectrical impedance instrument (Omron, model BF306) and expressed as a percent of the body weight.

Circulating Angiogenic Stem Cell Quantification

Eight ml of venous blood was collected in a Mononuclear Cell preparation tube (CPT: Becton Dickinson) and processed within 4 h of collection. Mononuclear cells were separated by centrifugation at 1700g for 30 min. The buffy coat layer was collected and washed twice with PBS+1%BSA. Cells were pelleted by centrifugation at 400g for 5 min, and incubated with FcR block (Miltenyi Biotec) for 10 min. Aliquots of 50ul were then used for immunophenotyping. The following fluorescently-tagged anti-human monoclonal antibodies were incubated mononuclear cells for 30min in the dark: CD41a-FITC (BD Biosciences), CD235a-FITC (eBioscience), CD16-FITC (eBioscience), CD14-q655 (Invitrogen), CD34-PE-CF594 (BD Biosciences), AC133-APC (Miltenyi Biotec), CD31-V450 (BD Biosciences), VEGFR2-Alexa Fluor 700 (R&D Systems) CD184 (CXCR4)-PE-Cy7 (eBioscience), and CD45-V500 (BD Biosciences). In addition a live-dead green stain (Invitrogen) was used to

discriminate cell viability. The cells were washed and re-suspended in PBS+1%BSA at a cell concentration of 2 million cells/ml. A 50ul aliquot of counting beads (Accucount; Spherotch) was added and sample then run on an LSR II (Becton Dickinson) set to acquire 20,000 beads.

Collected events were analyzed using FlowJo software. Initial gating selected a mononuclear cell population that was negative for CD235a, CD41a, CD16, CD14 and the dead cell marker, thus removing red blood cells, platelets, granulocytes and monocytes. This non-monocytic mononuclear population was then gated for CD31⁺ and CD45^{dim} cells. Finally, CACs were defined within this population as those cells positive for CD34 and AC133. Positive gating was accomplished using an unstained sample from the same subject (Figure 3A and B). CAC numbers were quantified and normalized to 1 ml of whole blood using bead counts. Gating strategies for the other CAC phenotypes differed after the selection of the non-monocytic mononuclear population and are depicted in Figures 4A and B.

FIGURE 3. Scheme for sample processing and Gating Strategy for CAC-3

(A). Buffy coat layer from the CPT tube was collected, washed and aliquots used for surface staining. Scatter plot for the initial gating scheme is depicted, showing the gates around the lymphocyte (or mononuclear) population and counting beads.

(B). Representative image of gating strategy used for CAC-3. Selection of the non-monocytic mononuclear population is used for all CAC and KDR phenotypes. Subsequent gating for CAC-3 was selected from the CD31⁺ and CD45^{dim} population, from where CD34⁺ followed by AC133⁺ cells were selected.

FIGURE 3.

A.

B.

Gating Strategy Based on Surface Markers

FIGURE 4. Diagram showing gating strategies for 18 phenotypes of CACs

(A). Flow chart of gating strategy used on 105 subjects. Represented in black lettering are the initial parent gates. The red lettering represents the 14 phenotypes of CACs that were quantified to study the influence of diabetes on their circulating levels. The purple lettering describes CAC-3 from figure 3B.

(B). Flow chart of gating strategy used on a subset of 87 subjects where surface expression of KDR (VEGFR2) was also measured.

FIGURE 4.

A.

B.

Quantification of blood and urine analytes

Plasma was separated from EDTA anticoagulated blood by centrifugation at 1500g for 10 min and stored at -80°C until further use. Plasma glucose, insulin levels, lipid profile, and creatinine were analyzed using the Cobas Mira Chemistry System (Roche Diagnostic Systems, Inc.). Glucose was measured by the hexokinase assay (Sekisui Diagnostics, Charlottetown, Canada), insulin by the immunoturbidimetric assay (Kamiya Biomedical Company, Seattle, WA), and LDL-C, HDL-C and triglycerides were measured by enzyme assays using calibrators from Wako Diagnostics (Richmond, VA). Total cholesterol was measured similarly with calibrators and controls from Thermo Electron. Creatinine was measured by the Jaffe reaction using alkaline picarate obtained from Thermo Electron. hsCRP was measured using the VITROS Chemistry Products at a CLIA certified lab. HbA1c was measured by using the Bayer A1C-Now kit according to the instructions by the manufacturer. Complete blood cell counts were quantified on the Abbott Cell Dyne.

Aliquots of urine samples were frozen at -80°C until further use. Urinary F2 isoprostane metabolite was measured at the Division of Clinical Pharmacology, Vanderbilt University School of Medicine, Nashville, TN (177).

CVD Risk Score Calculations

Ten-year atherosclerotic cardiovascular disease (ASCVD) risk scores for heart disease or stroke were calculated using the algorithm published in 2013 ACC/AHA Guideline on the Assessment of Cardiovascular Risk (178)

The sum of CVD risk factors was calculated by assigning a score of +1 for each of the following features: age > 40 years, male gender, diagnosis of hypertension or on medications for hypertension, LDL cholesterol of >130 mg/dl, current smoker, and diabetes (151).

Measures of Obesity

Three methods were used to account for body size and fat distribution – body mass index (BMI), waist to hip ratio (WHR) and percent body fat. BMI was calculated using the standard formula: $\text{weight (lb)} / [\text{height (in)}]^2 \times 703$, with BMI of ≥ 30 reported as obese. Waist and hip measurements were in accordance with WHO 2008 Expert Consultation Report (www.who.int). The cut off for high waist-to-hip ratio (WHR) was taken from the recommendations in the same report, where abdominal obesity is defined as a WHR above 0.90 for males and 0.85 for females. Body fat percentage was measured using a hand held bioelectrical impedance instrument (Omron, model BF306) and expressed as a percent of the body weight.

Statistical Analysis

Descriptive statistics are presented as mean $\pm$ SD for continuous variables and as frequencies and percentages for categorical data. Subjects were divided into high and low CAC count groups using the median value, where cells below the median value were in the low cell group and cells equal to and above the median value were in the high cell group. Cohort demographics and CVD risk factors were compared across the high and low CAC strata using independent sample t-tests, Wilcoxon rank sum test and chi-squared tests. Continuous

variables that were not normally distributed were analyzed using the Wilcoxon rank sum test, a non-parametric test whereas the categorical variables were analyzed using the chi-squared test. The factors significantly associated with the cell groups in the univariate analysis were later used in the multivariate analysis.

Logistic regression was used to examine the relationship between CAC-3 levels and diabetes, HbA1c, fasting plasma glucose, HOMA-IR, fasting plasma insulin, reactive hyperemia index, plasma hsCRP and urinary F2 isoprostane metabolite independently. Each of the models was adjusted for ethnicity and body fat percentage.

Furthermore, the association of reactive hyperemia index with CAC-3, diabetes, HbA1c, fasting plasma glucose, inflammation and oxidative stress was investigated independently using logistic regression. The reactive hyperemia index was divided into a low and high group, where a value below two was in the low group and a value of two and greater was in the high group.

Linear associations between the mean predicted CAC-3 counts and HbA1c, Fasting Plasma Glucose and Reactive Hyperemia Index were analyzed.

Data management and statistical analyses were performed using SAS 9.4 and IBM SPSS Statistics version 21.0. P values < 0.05 were considered statistically significant.

RESULTS

Cohort Characteristics

The study population included 105 subjects with a mean age of 48 years. The cohort was mostly obese with 59% of the cohort having a BMI of 30 or

greater, and 72% of the cohort having a high waist to hip ratio. There was a higher but not statistically significant representation of Caucasians (59%) and females (58%). Self-reported current smokers constituted 20% of the cohort and 13% were reported as former smokers. The diagnosis of hypertension was held by 52% of the cohort, of T2D by 45%, and of hyperlipidemia by 39%. The mean HbA1c levels among the diabetics was 8.6% with an average duration of diabetes of 10 years. The use of metformin, statins and diuretics in the cohort was 35%, aspirin and ACE Inhibitors use was reported by 31%, and insulin use by 30% (Table 2).

Association of Diabetes across CAC strata

Cell counts of each of the eighteen phenotypes of CACs measured were dichotomized into high and low cell groups based on their median value. A chi square analysis was conducted to test the association of diabetes with the cell count groups (Table 2.). Subjects with diabetes were predominantly in the low cell count group with a significant association for six of the eighteen phenotypes – $CD34^+$, $CD34^+/KDR^+$, $CD34^+/31^+$, $AC133^+$, $AC133^+/CD31^+$, and $CD45^+/AC133^+/CD34^+$. The association of CAC-3 with diabetes had a p value of 0.09. However these associations do not account for confounders in the demographics. CAC-3 was further analyzed in a multivariate logistic regression model.

Table 2. Chi square analysis of high and low CAC cell count groups with Diabetes.

CAC Phenotypes	Diabetics n (%) of Total n=105	Diabetics n (%) in High Count Group n=52	Diabetics n (%) in Low Count Group n=53	P Value
CAC-1	47 (45)	20 (39)	27 (51)	0.198
CAC-2	47 (45)	24 (46)	23 (43)	0.776
CAC-3	47 (45)	19 (37)	28 (53)	0.093
CAC-4	47 (45)	21 (40)	26 (49)	0.372
CAC-5	47 (45)	16 (31)	31 (59)	0.004*
CAC-6	47 (45)	17 (33)	30 (57)	0.014*
CAC-7	47 (45)	21 (40)	26 (49)	0.372
CAC-8	47 (45)	25 (48)	22 (42)	0.499
CAC-9	47 (45)	18 (35)	29 (55)	0.038*
CAC-10	47 (45)	20 (39)	27 (51)	0.198
CAC-11	47 (45)	17 (33)	30 (57)	0.014*
CAC-12	47 (45)	20 (39)	27 (51)	0.198
CAC-13	47 (45)	21 (40)	26 (49)	0.372
CAC-14	47 (45)	18 (35)	29 (55)	0.038*
CAC-15	47 (45)	21 (40)	26 (49)	0.372
CAC-16	35 (40)	15 (34)	20 (47)	0.238
CAC-17	35 (40)	13 (30)	22 (51)	0.040*
CAC-18	35 (40)	18 (41)	17 (40)	0.896

* denotes $p < 0.05$.

Demographic comparison across CAC-3 strata

The mean, median and range of CAC-3 counts per ml of blood for the cohort were 86, 46, and 0 – 919 respectively. The mean $\pm$ SD of the low cell group was 19 ± 13 , and of the high cell group was 154 ± 144 per ml blood. Demographics between the two groups differed only in ethnicity and body fat percentage, wherein a higher proportion of African Americans were represented in the high cell group, as were subjects with a higher body fat percentage (Table 3).

Table 3: Cohort demographics, medical diagnosis and laboratory data stratified by high and low CAC-3 cell counts.

Variable – n (%) or Mean ± SD	Total n = 105	High Count n = 52	Low Count n = 53	P Value
Age (years)	47.9 ± 10.6	48.2 ± 10.5	47.6 ± 10.8	0.77
Gender				0.06
Female	61 (58)	35 (67)	26 (49)	
Male	44 (42)	17 (33)	27 (51)	
Ethnicity				0.02*
Caucasian	62 (59)	28 (54)	34 (64)	
African American	31 (30)	21 (40)	10 (19)	
Asian	4 (3.8)	0 (0.0)	4 (7.5)	
Latino	3 (2.9)	2 (3.8)	1 (1.9)	
Others	5 (4.8)	1 (1.9)	4 (7.5)	
Caucasian and all others	62 (59)	28 (54)	34 (64)	0.28
African American and all others	31 (30)	21 (40)	10 (19)	0.02*
CVD Risk Factors				
Diabetes	47 (45)	19 (37)	28 (53)	0.09
Hyperlipidemia	41 (39)	21 (40)	20 (38)	0.78
Hypertension	55 (52)	26 (50)	29 (55)	0.63
Body Mass Index ≥ 30	62 (59)	34 (65)	28 (53)	0.19
High Waist-Hip Ratio	76 (72)	38 (73)	38 (72)	0.75
Body Fat Percentage	34 ± 9	37 ± 8	32 ± 10	0.02*
Current Smokers (self report)	21 (20)	14 (27)	7 (13)	0.08
Former Smokers (self report)	14 (13)	4 (8)	10 (19)	0.09
Never Smokers (self report)	70 (67)	34 (65)	36 (68)	0.78
Medication				
Insulin	31 (30)	14 (27)	17 (32)	0.56
Metformin	37 (35)	16 (31)	21 (40)	0.34
Statins	37 (35)	19 (37)	18 (34)	0.78
Beta Blockers	23 (22)	13 (25)	10 (19)	0.49
ACE Inhibitors	32 (31)	19 (37)	13 (25)	0.18
Diuretics	37 (35)	16 (31)	21 (40)	0.34
Aspirin	33 (31)	12 (23)	21 (40)	0.07

Table 3: Continued

Variable – n (%) or Mean ± SD	Total n = 105	High Count n = 52	Low Count n = 53	P Value
Glycemic Indices				
HbA1c (%)	6.87 ± 2.1	6.71 ± 2.0	7.03 ± 2.2	0.76
Fasting Glucose mg/dl	140 ± 74	136 ± 69	144 ± 80	0.79
Fasting Insulin (μIU/ml)	24 ± 20	24 ± 19	23 ± 22	0.36
HOMA-IR Score†	3.4 ± 3.5	3.4 ± 2.6	3.4 ± 4.3	0.51
Fasting plasma lipids (mg/dl)				
Total Cholesterol	191 ± 46	190 ± 45	192 ± 49	0.94
LDL-Cholesterol	103 ± 31	101 ± 28	105 ± 35	0.55
HDL-Cholesterol	48 ± 16	49 ± 19	47 ± 11	0.73
Triglycerides	145 ± 129	142 ± 128	149 ± 133	0.76
CVD Risk Scores				
10 year ASCVD Risk Score	7.7 ± 10.2	8.7 ± 12.0	6.5 ± 7.3	0.72
Sum of CVD Risk Factors	2.7 ± 1.5	2.6 ± 1.4	2.9 ± 1.6	0.31
Plasma Creatinine (mg/dl)	0.98 ± 0.2	0.99 ± 0.2	0.96 ± 0.2	0.74
White Blood Cell Count (K/μL)	5.5 ± 1.9	5.9 ± 2.0	5.2 ± 1.7	0.09
Social and Economic factors				
Alcohol Use	51 (49)	28 (54)	23 (43)	0.33
Exercise				0.34
None	23 (22)	14 (27)	9 (17)	
Mild	35 (33)	18 (35)	17 (32)	
Vigorous	47 (45)	20 (39)	27 (51)	
Education level ≥ College	79 (75)	37 (71)	42 (79)	0.42
Income ≥ \$20,000	60 (57)	28 (54)	32 (60)	0.49

High and low cell count groups were assigned by the median value of the CAC-3 cell count. Abbreviations: HbA1c – Hemoglobin A1c, HOMA-IR – Homeostatic Model Assessment-Insulin Resistance, LDL – Low Density Lipoprotein, HDL – High Density Lipoprotein, ASCVD – Atherosclerotic Cardiovascular Disease.

* denotes $p < 0.05$.

Adjusted Associations of CAC-3 levels with Diabetes and Glycemic Indices

In the unadjusted association the diagnosis of diabetes influenced CAC-3 levels at a significance level of less than ten percent ($p = 0.09$). Also, there was no significant association between CAC-3 levels and HbA1c, fasting plasma glucose, fasting plasma insulin levels and HOMA-IR scores. However in the multivariable analysis, using the logistic regression model (Table 4) and adjusting for the covariates that were significantly different in the two groups (African American ethnicity and body fat percentage) diabetes and HbA1c significantly influenced CAC-3 levels: $p = 0.004$ and 0.033 respectively. Subjects with diabetes were 4.4 times more likely to belong to the low CAC-3 group, and with every unit increase in HbA1c the odds of belonging to the low CAC-3 group increased by 28%.

Adjusted CAC-3 counts had significant negative correlations with HbA1c and fasting plasma glucose levels, $p = 0.006$ and $p = 0.004$ respectively. This decrease in cell count was quantified as 6.5%, for every 1 unit increase in HbA1c and at 6% for every 30 unit increase in plasma glucose level (Figure 5). Fasting insulin levels and HOMA-IR scores did not influence CAC-3 levels even after adjusting for known confounders.

Multivariable analysis confirmed the depletion of the 6 phenotypes found to have significant univariable associations. However these cell types did not have significant associations with RHI and were hence not analyzed further.

Table 4: Logistic Regression modeling low CAC-3 count group with each of the variables listed below.

Variable	P Value	Odds Ratio	95% CI
Diabetes	0.004*	4.407	1.609 – 12.071
HbA1c	0.033*	1.282	1.020 – 1.612
Fasting Plasma Glucose	0.074	-	-
HOMA-IR Score	0.421	-	-
Fasting Plasma Insulin	0.739	-	-

All models are adjusted for African American ethnicity and body fat percentage. * denotes $p < 0.05$.

Figure 5. Linear association between adjusted CAC-3 counts and glycemic indices. Illustrated are scatter plots of log transformed adjusted CAC-3 counts against HbA1c (A) and fasting plasma glucose (B).

Plot (A): $R^2 = 0.077$, and for every 1 unit change in HbA1c, cell count decreases by 6.5%. Plot (B): $R^2 = 0.095$ and for every 30 unit change in plasma glucose, cell count decreases by 6%.

FIGURE 5

Associations of CAC-3 levels with Endothelial Function, Inflammation and Oxidative Stress

Endothelial function, as measured by RHI significantly influenced CAC-3 levels in the unadjusted (Table 5) and adjusted analysis (Table 6). In the adjusted analysis subjects with RHI scores of < 2 were 2.8 times more likely to belong to the low CAC-3 group. Adjusted CAC-3 counts had a significant positive correlation with RHI ($p = 0.004$; Figure 6). The increase in CAC-3 count was quantified to be 11.25% for every 0.5 unit increase in RHI.

Inflammation as measured by plasma hsCRP, and oxidative stress as measured by urinary F2 isoprostane metabolite were not significantly associated with CAC-3 with or without adjustments (Table 5a and 5b).

Table 5a: Unadjusted association of the independent variables listed below with high and low CAC-3 count groups.

Variable n (%) or Mean $\pm$ SD	Total	High Count	Low Count	P Value
Endothelial Function				
Reactive Hyperemia Index	2.1 $\pm$ 0.6	2.3 $\pm$ 0.6	2.0 $\pm$ 0.7	0.033*
Reactive Hyperemia Index > 2.0	49 (49)	31 (63.3)	18 (36.7)	0.018*
Inflammation				
Plasma hsCRP $\geq$ 3 mg/L	30 (29)	20 (39)	10 (19)	0.15
Oxidative Stress				
Urinary F2 Isoprostane Metabolite ng/mg Cr	1.0 $\pm$ 0.6	1.0 $\pm$ 0.6	0.9 $\pm$ 0.6	0.12

* denotes $p < 0.05$.

Table 5b: Logistic regression modeling low CAC-3 count group with each of the variables listed below.

Variable	p value	Odds Ratio	95% CI
Reactive Hyperemia Index < 2.0	0.023*	2.84	1.16 – 6.95
Plasma hsCRP	0.902	-	-
Urinary F2 Isoprostane Metabolite	0.914	-	-

All models are adjusted for African American ethnicity and body fat percentage.

* denotes $p < 0.05$.

Figure 6. Linear association between adjusted CAC-3 counts and reactive hyperemia index (RHI). Scatter plot of log transformed adjusted CAC-3 counts against RHI: $R^2 = 0.089$ and for every 0.5 unit increase in RHI CAC-3 count increases by 11.25%.

FIGURE 6

Associations of Endothelial Function with CAC-3, Diabetes, Glycemic Indices, Inflammation and Oxidative Stress

Official reference values for RHI are not available, but in general RHI values below 2 are categorized as endothelial dysfunction, whereas higher RHI values are considered normal endothelial function (Itamar product information). To analyze of the influence of the above variables on endothelial function, the cohort was divided into high and low RHI groups, wherein subjects with a score greater than two were assigned to the high RHI group. The univariable associations showed significant associations with CAC-3, diabetes, HbA1c and fasting glucose levels (Table 6a). Subjects with low CAC-3 counts mostly belonged to the low RHI group, $p = 0.02$. This association persisted in the multivariable analysis after adjusting for diabetes (Table 6b), wherein subjects with low CAC-3 counts are 2.5 times more likely to belong to the low RHI group.

The diagnosis of diabetes had a significant negative effect on endothelial function, $p < 0.001$. Both HbA1c and fasting glucose levels were negatively associated with RHI (Figure 7), $p = 0.02$. For every 1 unit increase in HbA1c, RHI decreased by 3.4%; and for every 30 unit increase in plasma glucose, RHI decreased by 3%. Insulin levels, HOMA-IR scores, inflammation and oxidative stress did not have a significant influence on endothelial function.

Table 6a: Unadjusted association of CAC-3 count groups, diabetes, glycemic indices, inflammation, and oxidative stress with high and low reactive hyperemia groups. High index includes values > 2.

Variable n (%) or Mean $\pm$ SD	Total n=100	High Index n=49	Low Index n=51	P Value
CAC-3 Groups				0.02*
High Count Group	51 (51)	31 (63)	20 (39)	
Low Count Group	49 (49)	18 (37)	31 (61)	
Diabetes	44 (44)	12 (25)	32 (63)	<0.001*
HbA1c %	6.79 $\pm$ 2	6.36 $\pm$ 2	7.21 $\pm$ 2	0.03*
Fasting Plasma Glucose	138 $\pm$ 69	120 $\pm$ 55	155 $\pm$ 78	0.04*
Inflammation				
Plasma hsCRP level mg/L	3.5 $\pm$ 4.4	3.4 $\pm$ 4.3	3.7 $\pm$ 4.6	0.88
Oxidative Stress				
Urinary F2 Isoprostane Metabolite ng/mg Creatinine	0.94 $\pm$ 0.61	0.88 $\pm$ 0.42	0.99 $\pm$ 0.74	0.83

* denotes $p < 0.05$

Table 6b. Logistic regression modeling low reactive hyperemia group with the variable listed in each row.

Variable	P value	Odds Ratio	95% CI
Low CAC-3 counts	0.0431*	2.452	1.028 – 5.846
Diabetes	0.0004*	4.938	2.043 - 11.934
Plasma hsCRP	0.653	-	-
Urinary F2 Isoprostane Metabolite	0.593	-	-

All models are adjusted for diabetes, and no adjustment is used in the model with diabetes. * denotes $p < 0.05$

Figure 7. Linear association between reactive hyperemia index and glycemic indices. Illustrated are scatter plots of log transformed reactive hyperemia index against HbA1c (A), and fasting plasma glucose (B). For plot (A): $R^2 = 0.057$ and for every 1 unit increase in HbA1c RHI decreases by 3.4%. For plot (B): $R^2 = 0.058$ and for every 30unit increase in plasma glucose, RHI decreases by 3%.

FIGURE 7.

DISCUSSION

Cross sectional analysis of this relatively low risk cohort that is mostly obese, middle aged and contains subjects with the mean duration of diabetes for ten years reveals that non-monocytic circulating angiogenic stem cells (CAC-3) are depleted in diabetes. We also observed that other phenotypes ($CD34^+$, $CD34^+/KDR^+$, $CD34^+/31^+$, $AC133^+$, $AC133^+/CD31^+$, and $CD45^+/AC133^+/CD34^+$) were also depleted.

Previous reports of CAC depletion in diabetes compared cell numbers between diabetic to non-diabetic cohorts. However this is not appropriate as the two populations are inherently distinct and have many confounders such as medications (148; 179-181). In this study, the cohort was divided into high and low CAC count groups, and covariates that were different in the two groups were accounted for in the multivariate analysis. These covariates were African-American ethnicity and body fat percentage. Medication use and disease states were no different in the two groups. This method confirmed that subjects with diabetes predominantly had low circulating counts of CACs. A similar analysis was reported by Werner et al (138) where EPCs were stratified into low, medium and high groups in subjects that had the diagnosis of CAD by angiography. This cohort included 507 male patients with a mean age 66 years of which 147 were diabetics and these diabetics did not have a predilection to the low EPC count group. However, this study was not designed to assess the influence of diabetes on EPC levels but to assess the ability of EPCs to predict CVD outcomes in subjects with CAD.

Our data suggests that a specific phenotype of CAC (AC133⁺/CD34⁺/CD45^{dim}/CD31⁺/CD14^{neg}: CAC-3) has a linear and negative correlation with glucose level and a positive correlation with endothelial function. Identifying specific populations of CACs that are depleted and impact endothelial function is crucial for the development of therapeutics and in assessing disease progression. A similar CAC (AC133⁺/CD34⁺/CD45^{dim}) population is reported to be depleted in conditions of CVD risk such as aging, hypertension and smoking (147; 150) suggesting that these CACs may be useful in monitoring early vascular dysfunction.

The association of CAC counts and endothelial function found in this study has not been reported yet, although, Hill *et al* reported a positive correlation between FMD and CFU-Hill outgrowth cells in subjects with varying degree of CVD risk (125). RHI is strongly correlated with FMD and is reported to be low in subjects with diabetes and has a negative correlation with glucose levels (175; 182; 183). This finding is important in reducing the bench-to-bedside gap in assessing endothelial function and the reparative capacity of the vasculature.

We were unable to find associations between CAC count groups and inflammation (hsCRP), and oxidative stress (isoprostane F2 metabolite) in our cohort. Possible as the population studied is relatively young and has an average duration of diabetes of 10 years that these effects were not detectable. The average hsCRP and isoprostane F2 metabolite levels in the cohort are relatively low at 3.51 mg/L and 1.0 ng/mg creatinine respectively.

Hyperglycemia could decrease CAC counts by decreasing mobilization of CACs from the bone marrow, or increasing senescence or apoptosis of CACs. The vasculature in diabetes is reported to have decreased eNOS and increased NADPH oxidase activity both effectively decreasing the bioavailability of NO (184), and signaling through NO is required for the bone marrow release of stem cells (185). Hyperglycemia enhances senescence of bone marrow cells by via activation of p38 mitogen activated protein kinase (MAPK) or Akt/p53/p21 pathways (93; 186), and apoptosis via activation of p38 and ERK 1/2 MAPKs pathways with the activation of nuclear factor (187).

In conclusion we report that both diabetes and endothelial dysfunction contribute to the depletion of CACs independently. The effect of diabetes on CAC-3 counts was greater than the effect of endothelial dysfunction on CAC-3 counts (Figure 8a; $p = 0.004$ and 0.043 respectively). Depletion of CAC-3 correlated with the level of hyperglycemia and the level of endothelial dysfunction (Figure 8b). This work suggests that CAC counts may be monitored in an office setting by measuring RHI and that a combination of RHI scores and HbA1c levels could be indicative of early vascular pathology in diabetes.

FIGURE 8. Summary of associations between CAC-3, endothelial function and diabetes.

Illustrated are the adjusted associations of diabetes and low RHI group with low CAC count group (A), and the association of HbA1c and RHI with CAC counts (B). Figure (A): The arrowheads point to the dependent (Y) variable. The CAC model is adjusted for ethnicity and body fat percentage and the Reactive Hyperemia Model is adjusted for diabetes. Figure (B): Illustrates the association between CAC counts, HbA1c and RHI; r = Spearman correlation coefficient. Also depicted are the percent changes in CAC counts and RHI.

FIGURE 8.

A.

B.

CHAPTER III

CELLULAR AND PLASMA FACTORS CONTRIBUTING TO CAC-3 DEPLETION IN DIABETES

INTRODUCTION

In our previous study we found that the circulating levels of CAC-3 were depleted in subjects with diabetes. Hence, to probe the mechanisms underlying this depletion, we focused on the role of cellular and plasma factors. Previous studies have shown that the levels of circulating stem cells in the peripheral blood depend upon the ability of these cells to proliferate in niches and to be mobilized and survive in the peripheral blood as well as to home to sites of endothelial repair or form new blood vessels. Therefore, we examined four key factors regulating these processes, i.e. the expression of vascular endothelial growth factor receptor 2 (VEGFR2), C-X-C chemokine receptor type 4 (CXCR4), and insulin receptor (IR), and mitochondrial membrane potential ($\Delta\Psi$).

The VEGF receptors, VEGFR-1, -2 and -3, are a family of receptor tyrosine kinases that play a key role in angiogenesis and vasculogenesis (188; 189). Of the 3 receptor types, signaling through VEGFR2 has been found to be most critical for vasculogenesis. The absence of VEGFR2 is embryonic lethal in knock out mice that die due to the lack of vasculogenesis (190). The receptor is

mostly expressed on endothelial cells, hematopoietic stem cells, and megakaryocytes (191; 192). The peptide VEGF-A is the primary ligand for VEGFR2 although other VEGF isoforms VEGF-C and -D also bind to VEGFR2 as well. Stimulation of VEGFR2 by VEGF-A has been studied extensively in endothelial cells where it is known to mediate endothelial proliferation, survival, migration, tubular morphogenesis and sprouting (193; 194). Inhibition of VEGF signaling leads to capillary regression (195). VEGF has also been found to play an important role in mobilizing CACs during ischemia (134; 196). In subjects with T2D early outgrowth cells from peripheral mononuclear cells (CFU-Hill) were characterized as having decreased proliferation capacity, reduced adhesiveness and reduced tube-forming capacity *in vitro* (197). However whether signaling through VEGFR2 plays a role in these functions and indeed even VEGFR2 density on CAC-3 have not been studied.

CXCR4 is a G-protein coupled receptor that is expressed in several tissues including hematopoietic stem cells, lymphocytes and endothelial cells. During embryonic development, interactions of CXCR4 with its ligand SDF-1 is important for the formation of vascular, hematopoietic, cardiac, and nervous systems (198; 199). In adults, this interaction is vital for leucocyte trafficking, and the mobilization and homing of hematopoietic stem cells (200; 201). The activation and down-regulation of CXCR4 are important for the release and homing of stem cells (79). A number of growth factors, inflammatory mediators, and cytokines influence the transcription and expression of CXCR4 (202-204).

Thus we analyzed the expression of CXCR4 on CAC-3 and its associations with hyperglycemia, inflammation and oxidative stress.

In addition to VEGFR2 and CXCR4, we also studied the insulin receptor, which is a receptor tyrosine kinase that mediates the biological effects of insulin and whose activation on healthy endothelial cells is coupled with the release of nitric oxide. Extensive evidence suggests that abnormalities in insulin receptor signaling are critical events underlying the development of T2D (205; 206). Individuals with insulin resistance have reduced expression of the insulin receptor in peripheral tissues, particularly the liver, muscle, and adipose tissues (207). Insulin receptor abundance is an important determinant of the specificity of insulin action (208). Hence we quantified the expression of insulin receptor on CAC-3 and its associations with hyperglycemia, inflammation and oxidative stress.

Hyperglycemia in diabetes negatively impacts tissue function through several well described mechanisms. These include: (1) increased flux of glucose and other sugars through the polyol pathway; (2) increased intracellular formation of advanced glycation end products (AGEs); (3) increased expression of the receptor for AGEs and its activating ligands; (4) activation of protein kinase C (PKC) isoforms; and (5) enhanced signaling through the hexosamine pathway (209). It has been proposed all of these mechanisms are secondary to a single upstream event the mitochondrial overproduction of reactive oxygen species (ROS) (210). Several studies have reported the association of mitochondrial membrane potential ($\Delta\Psi$) with ROS production, wherein an increase or decrease

in $\Delta\Psi$ is associated with increased ROS production (211-215). During periods of high glucose concentrations, as in diabetes there is an increase in the proton gradient within the electron transport chain that enhances superoxide production and leads to an accumulation of excessive ROS (31). To determine whether diabetes affects mitochondrial function in CACs we measured change in $\Delta\Psi$ as an index of the overall metabolic activity, using a flow cytometry method developed in our laboratory.

Cytokines regulate immune responses, hematopoiesis, and the differentiation, migration, activation and proliferation of a variety of cell types (216). Changes in cytokine profiles from the healthy to the disease state could be considered as potential surrogates of disease activity. Thus we measured plasma levels of eighteen cytokines and analyzed their associations with the diabetes and CAC-3.

METHODS AND MATERIALS

Flow cytometric analysis of receptor density on CAC-3

Mononuclear cells from peripheral blood were processed and stained with fluorescently tagged anti-human monoclonal antibodies using methods described in Chapter II. The antibodies used here were AlexaFluor 700 anti-VEGFR2 (KDR; R&D Systems), PE-Cy7 anti-CXCR4 (CD184; eBioscience) and PE anti-Insulin Receptor (IR or CD220; eBioscience). Gating strategy for CAC-3 was applied as also described in Chapter II, and the median fluorescence intensity from each sample calculated using LSR II (Becton Dickinson) software FACS Diva and expressed in arbitrary units.

Flow cytometric analysis of $\Delta\Psi$ in circulating cells

Initial sample collection, preparation and processing were similar to that described in Chapter II. For incubation with FcR block, and all subsequent steps, cells were re-suspended in a solution containing Dulbecco's Modified Eagle's Medium Base (DMEM; Sigma Aldrich D 5030) and 1%BSA. After staining for CAC surface antigen stains, the cells were washed and incubated with 50nM tetramethylrhodamine methyl ester perchlorate (TMRM; Sigma Aldridge T 5428) to assess $\Delta\Psi$. Three separate tubes at a cell concentration of 2×10^6 /ml were used. One of the tubes contained TMRM only, the second tube contained TMRM and 1 μ g/ml Oligomycin A (Sigma Aldridge 75351) to measure the maximum $\Delta\Psi$ (TMRM max), and the third tube contained TMRM and 1 μ M carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone (FCCP; Sigma Aldrich C2920) to measure the basal or background TMRM fluorescence (TMRM min). These tubes were incubated for 20 min in the dark at 37°C, 50ul of counting beads (Accucount; Spherotch) were added and sample then analyzed on an LSR II (Becton Dickinson) set to acquire 20,000 beads.

Gating strategies for the collected events were according to the procedure described in Chapter II. In the final CAC-3 gate, the median fluorescence intensity of TMRM in the three tubes was determined and the percent maximum TMRM intensity (% Max TMRM) was calculated using the formula [(TMRM only – TMRM min) / (TMRM max– TMRM min)] x 100.

Quantification of Cytokines

Cytokines were quantified using a subset of 87 enrolled subjects. Eight ml of venous blood was collected in a mononuclear cell preparation tube (CPT: Becton Dickinson) and processed within 4 h of collection. Plasma was separated by centrifugation at 1700g for 30 min and aliquots were stored at -80°C until further use.

Premixed Magnetic Luminex multiplex kits (R&D Systems) were used to quantify cytokines in three panels: (1) A six plex human angiogenesis panel (FCSTM02-06) measuring angiogenin, angiopoietin-1, FGF- basic, PDGF-BB, VEGF-A and VEGF-D; (2) a 10 plex screening panel (LXSAHM-10) measuring angiopoietin-2, EGF, EPO, HGF, ICAM-1, PIGF, SCF, E-Selectin, VCAM-1, and VEGF-C; (3) human MMP performance assay (FCSTM07-02) measuring MMP-2 and MMP-9. Only one freeze thaw cycle of the plasma was allowed and samples were analyzed in duplicate. Procedures were performed according to the manufacturer's instructions and quantified using the Luminex MAGPIX® CCD Imager. Concentrations were interpolated from a standard curve generated by log-transformed values of recombinant human proteins and their respective median fluorescent intensities. Seven subjects did not have duplicate values and were eliminated from further analysis.

In brief, the principle of this assay is similar to an ELISA wherein analyte-specific antibodies are pre-coated onto color-coded magnetic microparticles. Standards and samples are pipetted into wells where the immobilized antibodies bind the analytes of interest. After washing away any unbound substances, a

biotinylated antibody cocktail specific to the analytes of interest is added to each well. Following a wash to remove any unbound biotinylated antibody, streptavidin-phycoerythrin conjugate (Streptavidin-PE), which binds to the biotinylated antibody, is added to each well. A final wash removes unbound Streptavidin-PE, the microparticles are resuspended in buffer and fluorescence analyzed using the Luminex MAGPIX Analyzer. A magnet in the analyzer captures and holds the superparamagnetic microparticles in a monolayer. Two spectrally distinct Light Emitting Diodes (LEDs) illuminate the beads. One LED identifies the analyte that is being detected and the second LED determines the magnitude of the PE-derived signal, which is in direct proportion to the amount of analyte bound.

STATISTICS

Analysis of receptor density and $\Delta\Psi$

Data are reported as mean $\pm$ SEM, and groups were compared using the non-parametric Mann Whitney test and ANOVA. Linear regression was used to examine the relationship between receptor expression and HbA1c, hsCRP, and urinary isoprostane F2 metabolite.

Cytokine analysis

Cytokine values are reported as mean $\pm$ SD. As the data had extreme values it was transformed via winsorisation, a transformation method used for limiting extreme values to reduce the effect of possibly spurious outliers.

The heat maps shown in Figure 11 are based on log transformed data, where the values ranging from low to high are color coded from red to blue respectively. They represent the data in a visual format, where the Y-axis represents the cytokine measured and the X-axis represents individual samples.

The association between diabetes and cytokine levels was examined using the Chi-squared test or the Fishers exact test. Cytokine levels were dichotomized based on the median split where cytokines below the median value were in the low cytokine level group and cytokines equal to and above the median value were in the high cytokine level group. Linear regression was used to analyze continuous associations. A generalized linear model was used to confirm the association between soluble adhesion molecules with CAC-3 levels, after adjusting for covariates that were significantly different in the 2 groups - ethnicity, body fat and diabetes.

All data management and statistical analyses were performed using SAS 9.4 and IBM SPSS Statistics version 21.0. *P* values < 0.05 were considered statistically significant.

RESULTS

Influence of Diabetes on VEGFR2, CXCR4 and IR expression on CAC-3

As CAC-3 counts were decreased in diabetics, we investigated the properties of this cell type further to delineate possible causal factors for its depletion. We measured the surface density of VEGFR2, CXCR4 and IR on CAC-3 and found that the expression of IR was not different between diabetics and non-diabetics. However in comparison with non-diabetics the expression of

VEGFR2 and CXCR4 was significantly lower in diabetics ($p = 0.033$ and 0.001 respectively; Figure 9A). Of the three receptors studied only the expression of VEGFR2 had a significant negative correlation with HbA1c, hsCRP and isoprostane F2 metabolite (Figure 9B).

FIGURE 9. VEGFR2, CXCR4 and IR expression in non-diabetics and diabetics. (A) Illustrated are the expression levels of VEGFR2, CXCR4, and IR in diabetics, and non-diabetics. (B) Illustrated are regression plots demonstrating the negative associations of VEGFR2 with HbA1c, hsCRP and urinary isoprostane F2 metabolite.

FIGURE 9

A.

B.

Mitochondrial membrane potential in circulating mononuclear cells

Next we measured $\Delta\Psi$ as an indicator of the proton gradient in circulating cells. Cell populations lacking stem cell markers (mature + stem cells) had a higher $\Delta\Psi$ than stem cells, and CAC-3, the most primitive cell type analyzed, had the lowest $\Delta\Psi$. This observation parallels the metabolic requirements during cell maturation where the energy currency for stem cells is mostly derived through glycolysis, while mature cells predominantly use mitochondrial respiration, and indicating an increase in mitochondrial biogenesis with differentiation and acquisition of the mature phenotype.

We found no difference in the $\Delta\Psi$ measured in CAC-3 from diabetics and non-diabetics despite the increased plasma glucose level in the former group. However, the $\Delta\Psi$ in stem cells negative for AC133 (CAC 7), which have been suggested to be more mature than CAC-3, was significantly higher in diabetics than non-diabetics (118).

FIGURE 10. Mitochondrial membrane potential in circulating mononuclear cells. Illustrated is the % Max TMRM staining (mitochondrial membrane potential) for various cell populations as determined by FACS in diabetics and non-diabetics.

FIGURE 10.

Delta psi in circulating cells

Plasma analytes levels in diabetics and non-diabetics

We next measured plasma analytes. Listed in Table 7 are our determined values (R&D Systems) and defined reference values. Our measured values are within these reported reference values. We then generated heat maps of plasma analyte levels from the cohort (Figure 11). Subjects were classified by the diagnosis of diabetes and graded for changes in cytokine levels by color where low is red and high is blue. Plasma analyte variations were more apparent in diabetics than non-diabetics

Table 7. Levels of plasma analytes measured in the cohort with reference ranges.

Plasma Analytes (References for published values)	Measured Level (Citrate, platelet poor plasma)		Reference value range (single plex R&D)		
	Mean	SD	EDTA	Heparin	Citrate
Angiogenin ng/ml (217)	121	16			160-370
Angiopoietin-1 pg/ml (218)	615	190			39-20,000
Angiopoietin-2 ng/ml (218)	3.3	1.7	1.1-4.4	1-5	
VEGF-A pg/ml (217)	14.7	3.4	ND-115	ND-55	
VEGF-C pg/ml (219)	103	48	185-1231	222-819	
VEGF-D ng/ml (220)	223	16	ND-437	134-508	
PLGF pg/ml (221)	3.4	1.2			ND-17
SCF pg/ml (222)	51	15			629-1187
EGF pg/ml (223)	2.2	1.1			ND-11
PDGF-BB pg/ml (224)	44	21	ND-129	ND-32	
bFGF ng/ml (225)	123	12	ND-15		
HGF pg/ml (226)	65	25			251-742
EPO mIU/ml (227)	1.1	0.11	3.1-15		
sE-selectin ng/ml (228)	26	10			13-51
sVCAM-1ng/ml (177)	581	01	341-897	301-875	
sICAM-1 ng/ml (177)	194	83	100-307	106-337	
MMP-2 ng/ml (177)	198	59	141-278	155-342	
MMP-9 ng/ml (177)	18	8		13-105	

Non-standard abbreviations: ND = not detected.

FIGURE 11. Heat map of levels analytes from plasma of diabetics and non-diabetics. Illustrated are log transformed plasma analyte levels measured from each subject, where the values ranging from low to high are color coded from red to blue respectively. Data are represented in a visual format where the analytes are on the Y-axis and cohort subjects are on the X-axis: (A) non-diabetics, and (B) diabetics.

FIGURE 11.

A. Heat map of levels of analytes from plasma of non-diabetics

B. Heat map of levels of analytes from plasma of diabetics

Plasma analytes are elevated in diabetics

Each measured analyte was stratified into high and low level groups as determined by its median value. The association of all analyte level groups with diabetes was analyzed by chi square analysis and it was found that most cytokines were higher in diabetics than non-diabetics. Those that were significantly associated with diabetes include growth factors (Ang-1, Ang-2, VEGF-A, PlGF, HGF, EPO), inflammatory markers (sICAM-1, sE-selectin) and a protease (MMP-9; Table 8).

Diabetes-associated analytes were then analyzed for their association with HbA1c levels. All cytokines except Epo and MMP-9 had a significant positive correlation with HbA1c (Table 9). As HbA1c levels were associated with low CAC-3 counts (Chapter II), we next analyzed the association of these cytokines with this cell type (Table 10). Amongst these cytokines only sICAM-1 levels had a significant negative correlation with CAC-3 counts (Figure 12).

Table 8. Significant associations of plasma analytes groups with diabetes, dichotomized by their median value into high and low groups.

Plasma Analytes	Total n = 80 Diabetics n (%)	High Analyte Level Diabetics n (%)	Low Analyte Level Diabetics n (%)	P Value
Angiopoietin-1	30 (38)	20 (50.0)	10 (25.0)	0.021
Angiopoietin-2	30 (38)	21 (52.5)	9 (22.5)	0.006
VEGF-A	30 (37.5)	24 (60.0)	6 (15.0)	<.001
PIGF	30 (37.5)	21 (51.2)	9 (23.1)	0.009
EPO	30 (37.5)	23 (47.9)	7 (21.9)	0.018
HGF	30 (37.5)	26 (65.0)	4 (10.0)	<.001
sICAM-1	30 (37.5)	21 (52.5)	9 (22.5)	0.006
sE-selectin	30 (37.5)	22 (55.0)	8 (20.0)	0.001
MMP-9	30 (37.5)	19 (47.5)	11 (27.5)	0.065

P Values are calculated based on Fishers Exact test.

Table 9. Linear regression of HbA1c levels with plasma analytes that had a significant association with diabetes.

Plasma Analytes	Beta	R square	P Value
Angiopoietin-1	0.003	0.089	0.007*
Angiopoietin-2	0.421	0.117	0.002*
VEGF-A	0.263	0.195	<.001*
PIGF	0.723	0.194	<.001*
EPO	2.394	0.017	0.243
HGF	0.044	0.304	<.001*
sICAM-1	0.007	0.079	0.011*
sE-selectin	0.096	0.211	<.001*
MMP-9	0.051	0.037	0.085

* denotes $p < 0.05$.

Table 10. Linear regression of CAC-3 counts with plasma analytes that had a significant association with HbA1c.

Plasma Analytes	Beta	R square	P Value
Angiopoietin-1	0.057	0.014	0.303
Angiopoietin-2	-2.502	0.002	0.695
VEGF-A	1.528	0.003	0.620
PIGF	-8.990	0.014	0.289
HGF	-0.069	0.000	0.868
sICAM-1	-0.275	0.060	0.028*
sE-selectin	-0.201	0.000	0.852

* denotes $p < 0.05$.

FIGURE 12. Association of CAC-3 counts with sICAM-1 levels. Illustrated is the linear, negative association between sICAM-1 levels and CAC-3 counts.

FIGURE 12.

High sICAM-1 levels are associated with decreased CAC-3 counts and decreased expression of VEGFR2 on CAC-3.

As soluble adhesion molecules are a measure of endothelial activation we expanded our analysis to include sICAM-1, sVCAM-1 and sE-selectin. The plasma levels of these molecules in the high and low CAC-3 count groups were different in the univariable analysis (Table 11a). For the multivariate analysis using generalized linear model, the model was adjusted for confounders that were previously identified in the cohort in Chapter II (African American ethnicity and body fat percentage, diabetes). We found that only sICAM-1 levels had a significant negative association with CAC-3 counts (Table 11b). The association of sICAM with CAC-9 and CAC-11 was also analyzed and found not to be significant, suggesting specificity for CAC-3 (data not shown).

sICAM-1 levels had a positive correlation with HbA1c and a negative correlation with VEGFR2 expression on CAC-3 suggesting that hyperglycemia induced endothelial activation may play a role in the depletion of CACs in diabetes (Figure13).

Table 11a. Unadjusted association between CAC-3 count groups and the levels of soluble adhesion molecules.

Variables	Total (n=80)	High CAC-3 Count Group (n=40)	Low CAC-3 Count Group (n=40)	P Value
sICAM-1 ng/ml	194 ± 83	176 ± 81	211 ± 82	0.056
sVCAM-1 ng/ml	581 ± 201	567 ± 209	595 ± 195	0.389
sE-selectin ng/ml	26 ± 10	26 ± 10	25 ± 10	0.470

Table 11b. Multivariable analysis using generalized linear models for CAC-3 counts and the independent variable in each row.

Variable	P Value	Beta Estimate	95% Confidence Limits
sICAM-1	0.008*	-0.0033	-0.0058 - -0.0009
sVCAM-1	0.743	0.0002	-0.0009 – 0.0013
sE-selectin	0.297	-0.0129	-0.0113 – 0.0113

Models are adjusted for African American ethnicity, body fat percentage and diabetes. * denotes $p < 0.05$.

FIGURE 13. Association of sICAM-1 with HbA1c and VEGFR2 expression on CAC-3. Illustrated are the relationships between sICAM-1 and HbA1c (A), and VEGFR2 (B). While HbA1c increases as sICAM-1 levels increase, VEGFR2, decreases as sICAM-1 increases.

FIGURE 13.

A.

B.

DISCUSSION

The experiments described in this Chapter investigated the possible mechanisms by which hyperglycemia could play a role in decreasing CAC-3 counts. We found that the expression of VEGFR2 and CXCR4 on CAC-3 is diminished in diabetics. It is known that transcription factor HIF and the peptide VEGF-A upregulate VEGFR2 expression (229). In our cohort despite higher levels of VEGF-A in diabetics VEGFR2 expression was decreased. Furthermore we also found that HbA1c, hsCRP and isoprane F2 metabolite had negative correlations with VEGFR2 expression, suggesting that hyperglycemia, inflammation and oxidative stress may play a role in the regulation of VEGFR2 expression or they may alter the activity of VEGF-A resulting in decreased expression of VEGFR2. It is known that the survival, proliferation, and migration of CACs is influenced by downstream signaling through VEGFR2, and its decreased expression in diabetics may have contributed to the depletion of CAC-3 (230-232).

CXCR4 signaling plays a prominent role in homing of CACs, and its expression is positively regulated by the transcription factor NRF-1. Several signaling molecules, cytokines and growth factors including VEGF-A are also reported to enhance CXCR4 expression, while inflammatory factors attenuate CXCR4 expression (203; 204; 233; 234). Our finding of decreased CXCR4 expression suggests that decreased mobilization also contributes to CAC-3 depletion, despite the presence of higher levels of growth factors in diabetics. Reduced mobilization of CACs has been proposed by others as a possible

causative mechanism for low CAC counts in peripheral blood (78; 235). A reduction in CXCR4 expression on peripheral blood mononuclear cells has also been reported in patients with CVD (236; 237). Yet another study reported a positive correlation of CXCR4 expression on CACs with levels of progenitor cells in control individuals, while this correlation was absent in diabetic patients (181). The reduced expression of key surface antigens involved in vascular health in diabetics directs us to further investigate the regulation of expression of these antigens, and this may improve our understanding of CAC biology and in the development of novel therapeutics.

We were unable to detect a difference in $\Delta\Psi$ in CAC-3, suggesting that the presence of increased substrate and diabetes does not affect mitochondrial activity in early circulating stem cells. However, hyperglycemia has been reported to increase $\Delta\Psi$ in mature non-insulin sensitive tissues such as kidney and retinal cells of STZ diabetic rats (238; 239). Other studies have reported an increase in uncoupling protein 2 in cells exposed to hyperglycemia and postulated this to be an adaptation to the high $\Delta\Psi$ to protect against resulting increased ROS generation (240). A wider array of tests will be necessary to clearly detect and define the mitochondrial changes in CAC-3 in diabetics.

Increase in $\Delta\Psi$ in diabetics was detected in slightly more mature circulating stem cell, CAC 7, whose cell levels were not affected by diabetes. This suggests that mitochondrial effects of diabetes may be apparent in more mature CACs, and that changes in cellular function may not manifest with a corresponding decrease in cell number. Several studies report changes in

mitochondrial function, number, biogenesis and dynamics in mature insulin sensitive and non-insulin sensitive tissues (241-244).

Cytokine analysis revealed that most growth factors are increased in diabetics, and despite this increase CAC counts tend to be lower. Our studies suggest that decreased expression of receptors on CACs precedes the decline in growth factors that reported in diabetes of longer duration. Among the inflammatory analytes measured in plasma, only high sICAM-1 levels were associated with low CAC-3 counts and with decreased expression of VEGFR2, suggesting a role for inflammation in the regulation of VEGFR2 expression. The increase in sICAM-1 was also positively associated with glycemic control. An inverse correlation of sICAM-1 levels has been reported with CD133⁺/CD34⁺ cells in subjects with early ischemic stroke (245). High levels of sICAM-1 are also reported to be predictive of myocardial infarction in healthy men (246). Investigating the role of sICAM-1 in T2D, particularly in the regulation of VEGFR2 expression on CACs could improve our understanding of diabetic vasculopathy.

A summary of our analysis thus far is illustrated in Figure 14. Low CAC-3 counts are associated with diabetes, hyperglycemia, endothelial dysfunction and high sICAM-1 levels. This suggests that hyperglycemia-induced endothelial activation leads to an increase in inflammatory factors that negatively impact CAC-3 levels. Although both diabetes and high sICAM-1 were associated with decreased expression of VEGFR2 we found no direct association between VEGFR2 and CAC-3 counts.

FIGURE 14. Scheme representing the conclusions of Chapter II and III. Low CAC-3 counts are associated diabetes, hyperglycemia, endothelial function and high sICAM-1 levels.

FIGURE 14.

CHAPTER IV

ANGIOGENIC POTENTIAL OF CULTURED CACs

INTRODUCTION

CACs play an important role in the maintenance, and repair of existing blood vessels and in the formation of new blood vessels. Thus autologous CAC therapy could have potential for treating vascular disorders. However subjects with vascular disease are reported to have decreased number and function of CACs (235; 247; 248). These defects could be overcome by enhancing bone marrow mobilization of CACs, or by ex-vivo expansion of CACs with or without modifications to enhance their functionality. Several clinical trials have demonstrated that such therapy is feasible, does not cause harm and has potential benefits (156-158; 160). However several aspects such as the mode of delivery of CACs, number of CACs needed for revascularizations, the ability of CACs to survive and engraft in ischemic zones, need to be addressed prior to the use of CACs for regenerative cardiovascular therapy.

Although there have been significant advancements in understanding CAC biology, the phenotypic identity of the CAC subpopulations that are critical for vascular regeneration remains unclear. There is also uncertainty

about the origin of cultured CACs and whether these cultured cells are functionally identical to those in found circulation (249). Identifying a CAC precursor that can be easily cultured and is also involved in vascular regeneration could help in advancing the diagnostic and therapeutic potential of CACs.

Several methodologies have been reported for ex-vivo expansion of CACs of which the 3 most common ones are described in Chapter I. CACs may have different properties and function depending upon culture conditions (250). The current consensus is that there are two types of outgrowths: the early and late outgrowths.

The early outgrowth cells or CFU-Hill appear as clusters with central rounded cells surrounded by spindle shaped cells. The number of clusters are quantified as colony counts. These colony counts have been shown to correlate with CVD risk and cells from these colonies improve revascularization in animal models of ischemia but do not form tube like structures *in vitro* (33; 128; 251). They express both myeloid and endothelial markers and it is proposed they promote vasculogenesis in a paracrine manner by secreting angiogenic factors (127; 252-254).

Late outgrowth cells have a morphology similar to mature endothelial cells forming a monolayer with a cobblestone appearance. They express endothelial markers such as CD31, Von Willebrand factor (vWF), VE-cadherin and endothelial nitric oxide synthase (eNOS) but do not express myeloid cell markers (127; 131; 255). They have a high proliferative capacity and readily form tube like

structures (129; 256). There is concern that these cells may not originate from the bone marrow and represent shed proliferative endothelial cells (131; 257; 258). However some studies have confirmed their bone marrow origin and their mobilization from bone marrow in response to stimuli such as exercise, G-CSF, and VEGF (127; 259; 260). Late outgrowth cells are phylogenetically similar to a true CAC and a detailed functional characterization of these cells is necessary for their therapeutic application. Hence, we assessed the angiogenic potential of late outgrowth cells cultured from subjects with and without diabetes using both *in vitro* and *in vivo* methods.

METHODS AND MATERIALS

Culture of late outgrowth cells from human peripheral blood

Venous peripheral blood was collected in four 6ml EDTA (Vacutainer) tubes from the cohort described in Chapter II. A total of 20ml of blood was collected for cell culture, and these samples were processed within 4h of collection. Plasma was separated by centrifugation at 1500g for 10min. The non-plasma fraction of blood was collected, diluted with 20ml of PBS and layered atop of 15ml of ficoll (Ficoll-Paque, GE Healthcare) and the tubes centrifuged at 740g for 30min at room temperature. The buffy coat layer was collected and washed twice with EBM-2 basal medium (Lonza CC-3156) containing 10% fetal bovine serum (FBS, Atlanta Biologicals), penicillin and streptomycin. Cells were pelleted by centrifugation at 515g for 10min, and a cell count was performed prior to the last wash. Cells were resuspended in complete medium [EGM-2 (Lonza CC-3202) containing 10% FBS] and seeded into 6-well fibronectin coated plates

(Corning 354402), at a cell concentration between $2-5 \times 10^7$ cells in 4ml complete media. The cells were cultured at 37°C with 5% CO₂. Two days after seeding the media was gently removed, the wells washed once with fresh complete media and replaced with 3ml of fresh media. Subsequently, media was changed every day for 5 days, then every other day until the outgrowth cells were ~80% confluent. Cobblestone-like colonies appeared between 1 – 4 weeks. Upon confluence, the cells were detached with 0.25% trypsin-EDTA (Life Technologies) and replated to scale up in collagen-1 coated plates (BD Labware 354400).

Immunophenotyping of late outgrowth cells

Late outgrowth cells were characterized prior to their use in the tube-forming assays and for injection in the hind limb ischemia experiment. At ~ 80% confluence, cells were detached from the plate using 0.25% trypsin-EDTA, neutralized in media and pelleted at 400g for 5min at 4°C. The cells were resuspended in 100µl of PBS+1% BSA containing 10µl of FcR (Miltenyi Biotec) block and incubated for 10min. At this time 50 µl aliquots of were left unstained (negative control) or were incubated with a panel of fluorescently-tagged anti-human monoclonal antibodies [PE-CF594-CD34 (BD Biosciences), APC-AC133 (Miltenyi Biotec), V450-CD31 (BD Biosciences), Alexa Fluor 700-VEGFR2 (R&D Systems) and V500-CD45 (BD Biosciences)] for 30 min in the dark. In addition a live-dead green stain (Invitrogen) was used to discriminate cell viability. Then 1ml of PBS+1% BSA was added and the samples spun by centrifugation at 400g for 5min. The supernatant was discarded and the pellet was reconstituted in 300µl

PBS+ 1% BSA and a total of 10,000 cells were then analyzed on an LSR II flow cytometer (Becton Dickinson).

Data analysis was accomplished using FlowJo software. After initially selecting live cells, we identified cells positive for each of the individual antigens (CD34, AC133, VEGFR2, CD31 and CD45). From the CD34 gate, cells were further sorted for CD31 and VEGFR2, and their expression was reported as a percent of the parent gate.

Tube forming assay

Late outgrowth cells from both diabetics and non-diabetics were cultured in normal media containing 5mM of glucose. To mimic a sustained hyperglycemic milieu, cells from diabetics were also cultured in high glucose (25mM). Diabetic cells were incubated with an equivalent amount of an osmotic control (mannitol). Cells between passages 3-6 were used for all functional assays.

For the tube formation assay, we chilled the matrigel (BD Biosciences), pipette tips, and a 96 well plate (Corning) by placing them at -20°C overnight. The next day, 50µl of matrigel was pipetted into each well while keeping the plate, pipette tips and matrigel cold. The matrigel-coated plate was then incubated at 37°C for 60min. The cultured EPCs were detached from tissue culture plates with 0.25% Trypsin-EDTA, washed and counted. Each matrigel well was seeded with 1.2×10^4 cells in 200µl of complete media, and incubated at 37°C. Each sample was seeded in duplicate. Images were captured at 8, 16 and 24 hours using an EVOS FL microscope (Thermo Fischer Scientific). Completely formed tubes were counted from the images using NIH Image-J software.

Hind limb ischemia and CAC injections

All procedures were performed in accordance with the University of Louisville's Institutional Animal Care and Use Committee. Four animal groups were used: sham with vehicle (PBS) injection, sham with cell injection, hind limb ischemia (HLI) with vehicle injection, and HLI with cell injection (Figure 15A). Unilateral hind limb ischemia was induced by ligation of the femoral artery in 12 week old, female SCID-beige mice (C.B-Igh-1b/GbmsTac-Prkdcscid-Lystbg N7; Taconic). Cells from diabetics or non-diabetic subjects were cultured and harvested as described above and reconstituted in PBS to contain 0.5×10^6 cells in 40ul. We then injected 20ul (0.25×10^6 cells) at 2 sites, intramuscular and medial to the site of femoral artery ligation (Figure 15B). Control mice received injections of PBS. Anesthesia was induced by isoflurane inhalation (3% isoflurane mixed with 100% O₂) and maintained by continued isoflurane inhalation at a lower dose (1-2%). The left groin area was shaved and a small incision made through the skin. The femoral artery and vein exposed, sterile 7.0 sutures were threaded under and around the femoral artery and vein, knotted for permanent ligation and both blood vessels bisected. Skin was closed using sterile nylon 6.0 sutures and tissue adhesive sealant. Sham HLI surgery was conducted in the same manner, except that blood vessels were not ligated. Body temperature was maintained at 37°C for the procedure.

Supportive care involved antibiotic prophylaxis with Baytril 0.1mg/ml in drinking water started 1 week prior to surgery and was discontinued on post-op day 10. Analgesic support was provided with Ketoprofen 5 mg/kg subcutaneous

(SQ) prior to surgery and at 24 h post-op. Hydration was supported by 1ml of normal saline SQ prior to surgery and subsequently with Napa-Nector (Systems Engineering) until post-op day 7. Nutritional supplementation with Diet Gel Boost (Clear H₂O) was provided from post-op day 1 to 7.

FIGURE 15. Model for the *in vivo* assessment of the angiogenic potential of late outgrowth cells. Illustrated is the experimental design for hind limb ischemia experiment (A), and the anatomy of the femoral artery (B). Depicted in white are the sites of femoral artery ligation and cell injections.

FIGURE 15.

A.

B.

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Laser Doppler perfusion imaging

Mice were anesthetized as above and blood flow measurements from both ischemic and non-ischemic feet and hind limbs were obtained weekly for 4 weeks using the Laser Doppler Perfusion Imaging scanner (Moor LDI2 Instrument, Wilmington, DE). LDPI values scale linearly with the product of red blood cell velocity and the number of blood cells within the tissue, and are listed in arbitrary units. The images are recorded as digital color-coded images with the red hue indicating regions with maximum perfusion; yellow indicating medium perfusion and blue representing the lowest perfusion values (261). Two images were taken for every mouse, and analyzed for return of blood flow (recovery) to the ischemic leg as a percentage of blood flow in the non-ischemic leg.

STATISTICS

Categorical data are reported as a frequency and analyzed using chi square or Fishers exact test. Continuous data are reported as mean $\pm$ SEM and, comparison between groups was analyzed using the non-parametric Mann Whitney test or ANOVA. Linear regression was used to examine the relationship between percent CD34⁺/CD31⁺ cells and HbA1c levels. $P < 0.05$ was considered statistically significant.

RESULTS

Late outgrowth cells are decreased in diabetics

The initial appearance of a colony was seen in 76 of 105 subjects, and there was no difference in colony appearance between diabetics and non-diabetics (Figure 16A). However, not all of colonies continued to expand and, at week 3 outgrowth cells from 57 subjects continued to grow which allowed for phenotype characterization. The number of diabetics demonstrating late outgrowth cells was significantly lower than that from non-diabetics ($p = 0.03$; Figure 16B). The dual expression of CD34 and CD31 in the outgrowth cells was quantified as a percent of the parent population of live cells. Based on the median value (50th percentile), subjects were dichotomized as lying above or below the 50th percentile. We found that subjects with diabetes mostly belonged below the 50th percentile group (lower CD34/CD31 expression; Figure 16C). Linear regression of CD34⁺/CD31⁺ expression and HbA1c levels showed a significant negative correlation (Figure 17).

FIGURE 16. Chi square analysis of late outgrowth cells from diabetics and non-diabetics. Illustrated are subjects with and without initial outgrowths (A), subjects with persistent outgrowth cells at three weeks (B), and outgrowth cells containing greater or less than the 50th percentile of CD34/CD31 positivity (C).

FIGURE 16.

A.

B.

C.

FIGURE 17. Illustrated is the linear regression analysis of percent CD34⁺/CD31⁺ in the late outgrowth cells against HbA1c levels (A). Also illustrated are representative images of the morphology of late outgrowth cells based on the percentile of CD34⁺/CD31⁺ expression (B).

FIGURE 17.

A.

B.

***In vitro* assessment of angiogenic potential of late outgrowth cells**

Late outgrowth cells from diabetics and non-diabetics formed tube-like structures on matrigel. The morphology of the tube forms among the diabetics had a greater variability. While some diabetics had small tube forms, others had open or broken tubes. However, overall there was no difference in the number of tube forms between the groups (Figure18).

FIGURE 18. Tube-like structure formation in non-diabetics and diabetics.

Illustrated are the representative images of tube-like structures from individual subjects (A), and the quantification of these structures (B) reported as mean $\pm$ SEM.

FIGURE 18.

A.

Non Diabetics

Diabetics

B

***In vivo* assessment of angiogenic potential of late outgrowth cells**

Cultured CACs were tested for their ability to enhance revascularization following acute induction of hind limb ischemia. A murine model of hind limb ischemia was used and either vehicle or an equal number of cultured cells from diabetics and non-diabetics were injected.

The recovery of perfusion in the ischemic limb was compared with the perfusion in the non-surgical limb from the same animal (Figure 19). An equal proportion of animals from all 4 groups had necrotic toes, and only one animal in the vehicle treated group had a loss of all toes. These animals were excluded from the LDPI analysis (Figure 20). An enhanced recovery of perfusion (20 – 35%) was detected in animals that received cells from non-diabetics compared to animals that received vehicle or cells cultured from diabetics, at weeks 3 and 4 post-ischemia (Figure 21).

FIGURE 19. Laser Doppler perfusion imaging (LDPI). Four weeks after induction of ischemia, LDPI images were acquired. Illustrated are representative images and a graphical representation of percent recovery.

FIGURE 19.

FIGURE 20. Foot images. Illustrated are representative images of necrotic toes in the vehicle and cell treated groups after induction of hind limb ischemia by ligation of femoral vessels.

FIGURE 20.

FIGURE 21. Line graph of perfusion recovery. Illustrated is the calculated percent recovery of perfusion 4 weeks after HLI.

FIGURE 21.

Morphology and immunophenotyping of late outgrowth cells.

Cultured cells from all non-diabetic patients had a uniform cobblestone morphology. The outgrowth cells from diabetics were heterogeneous, and included a mixture of cobblestone cells and spindle shaped cells. In these cells we quantified the expression of CD34, CD31, KDR, CD34⁺/CD31⁺ and CD34⁺/KDR⁺ (Figure 22). The expression of AC133 and CD45 was also measured and found to be less than 1% (data not shown). A comparative analysis of the antigens expressed on cells from non-diabetics and diabetics demonstrated that cells from diabetics had a decreased expression of CD31 and CD34⁺/CD31⁺ (Figure 23).

FIGURE 22. Morphology and immunophenotyping of late outgrowth cells.

Illustrated are representative cells from non-diabetics (A), and diabetics (B). The numericals on the images are subject ID numbers. Tabulated below the images are quantifications of the expression of surface antigens from the outgrowth cells for each subject.

FIGURE 22.

A.

	#71	#91	#99	#68	#69
KDR+(%)	98	54	79	97	96
CD31+(%)	89	98	98	87	82
CD34+(%)	26	43	82	36	56
CD34+/KDR+ (%)	99	78	86	99	99
CD34+/CD31+ (%)	100	100	99	97	93

B.

	#65	#94	#37	#70	#38
KDR+(%)	85	59	31	93	0
CD31+(%)	34	81	73	63	0
CD34+(%)	49	61	50	48	0
CD34+/KDR+ (%)	92	61	36	96	0
CD34+/CD31+ (%)	68	91	92	88	0

FIGURE 23. Surface antigen expression. Cells from diabetic (DM) and non-diabetic (NDM) subjects were analyzed for the expression of CD31, CD34⁺ and KDR by flow cytometry and a percent positive for various population determined. CD31 expression is decreased in diabetics compared to non-diabetics.

FIGURE 23.

DISCUSSION

In this study we investigated the differences in angiogenic potential of culture-expanded CACs from non-diabetics and diabetics. We found that diabetics were less likely to have late outgrowth cells from peripheral blood mononuclear cells, than non-diabetics, and suggesting decreased adhesive and proliferative capacities of CACs in diabetics. Similar impairments in the function of cultured CACs are reported from subjects with both T1D and T2D (149; 262). Furthermore we found that the phenotype of outgrowth cells differed, wherein the percentage of CD34⁺/CD31⁺ cells was decreased in diabetics. Finally we found a negative correlation between HbA1c levels and the percentage of CD34⁺/CD31⁺ cells in the outgrowth, suggesting that hyperglycemia alters the properties of late outgrowth cells that may contribute to endothelial dysfunction and poor collateral vessel formation in diabetics.

The *in vitro* assessment of angiogenic potential by formation of tube-like structures was analyzed in both normoglycemic and hyperglycemic conditions. Tube formation was no different between diabetics and non-diabetics regardless of glucose levels in the media. This could be a result of selection bias where only the diabetics that had cells capable of angiogenesis had outgrowths, or that if supported by exogenous growth factors as contained in matrigel, diabetic outgrowth cells are capable of making tube forms. Evidence that hyperglycemia impairs CAC function *in vitro* is unclear Schatteman *et al*, reported results similar to ours where supplementing the culture media with high glucose did not impair

CAC proliferation and differentiation, while Ingram *et al* reported reduced tube like structure formation when neonatal endothelial colony-forming cell were cultured in hyperglycemia (153; 263). Late outgrowth cells are also reported to have decreased migration, proliferation, secretion of angiogenic growth factors, and increased apoptosis when cultured in hyperglycemia or in the presence of AGEs (264; 265). The mechanisms by which hyperglycemia affects the function of mature endothelial cells has been studied extensively and potentially similar pathways may affect hyperglycemia-induced CAC dysfunction. Krankel *et al* demonstrated decreased proliferation, and survival of cultured CACs due to reduced NO production, and Seeger *et al* demonstrated decreased survival of CACs due to hyperglycemia induced activation of the p38 MAP-kinase pathway (266; 267).

Next we assessed the *in vivo* angiogenic potential of diabetic and non-diabetic late outgrowth cells in a murine model of hind limb ischemia. We show that outgrowth cells from non-diabetics enhanced revascularization by 25 - 30% when compared to diabetic outgrowth cells and vehicle, suggesting that diabetic cells had a decreased ability to enhance collateral vessel growth. Improved revascularization using *ex vivo* expanded early and late outgrowth cells from healthy donors is also reported by other investigators in animal models of hind limb ischemia and myocardial ischemia, (46; 78; 128; 153; 268). Some of these studies have reported the incorporation CACs into capillaries, while others have reported a paracrine effect with the secretion of growth factors enabling further

recruitment of CACs or differentiation of local cells as possible mechanisms for this effect.

Reduced post-ischemic revascularization in diabetic animals, and a reduced ability of outgrowth cells from diabetic animals to improve revascularization in healthy mice have also been reported (269-271). These studies report decreased mobilization, migration, adhesiveness, proliferation and increased apoptosis of CACs as mechanisms for reduced vascularization.

A clinical trial using intramuscular injection of autologous CACs to treat critical limb ischemia due to peripheral arterial disease, The Therapeutic Angiogenesis using Cell Transplantation (TACT) trial, reported significantly improved leg pain, reduced ulcer size, and pain-free walking distance that were maintained for at least 2 years (156), and significantly lowered amputation rates in the in the treatment group in the 3 year follow up (157). Similar positive outcomes were reported in a randomized study investigating the effects of intracoronary infusion of CACs, The Transplantation of Progenitor Cells and Regeneration Enhancement in Acute Myocardial Infarction (TOPCARE-AMI) trial, that has now published a five year follow up report (158-160). Improvements in cardiac function included reduced functional infarct size, improvement of LV ejection fraction, reduced diagnosis of heart failure and no hospitalization for heart failure.

It is speculated that autologous stem cell therapy in diabetics may not improve vascular function unless CAC dysfunction associated with diabetes is mitigated and/or CACs are made resistant to the diabetic environment. Animal

studies using genetically modified CACs such as cells that over-express VEGF, eNOS, and telomerase reverse transcriptase have been tested in models of ischemia and have enhanced revascularization (154; 272; 273). Human mesenchymal stem cells transduced with the pro-survival gene Akt-1 also showed enhanced viability and greater functional repair in a mouse infarct model (155). However at this time reports of clinical trials that specifically evaluate autologous angiogenic stem cell therapy in diabetics are yet to be published.

CHAPTER V

CONCLUDING DISCUSSION

This project was designed to obtain new understanding of vascular pathobiology with particular attention to the role of circulating stem cells as key determinants of CVD risk and vascular dysfunction in T2D. Through the course of this project the following questions were addressed: 1) Are circulating CACs depleted in T2D? 2) What specific phenotypes are depleted? 3) Do hyperglycemia, insulin resistance, inflammation, and oxidative stress play a role in this depletion? 4) Are CACs counts decreased in early endothelial dysfunction? 5) What mechanisms contribute to CAC depletion? 6) Does the angiogenic potential of CACs from T2D subjects differ from non-diabetic subjects?

These questions were addressed in a cohort of 105 subjects recruited from the Endocrinology and Primary Care Clinics of the University of Louisville. The cohort comprised of a relatively young adults with a mean age of 48 years who were mostly obese and with high prevalence of hypertension, diabetes and hyperlipidemia. The average duration of diabetes reported was 10 years. None of the subjects in the study were acutely ill, they did not received transfusions of any blood products in the past year, and were not hospitalized in the past 5

months. Among the 47 diabetics the prevalence of vasculopathy was as follows: 16 subjects were diagnosed with micro-vasculopathy only, 17 subjects with both micro- and macro-vasculopathy, 2 subjects with only macro-vasculopathy, and 12 subjects did not have the diagnosis of vasculopathy.

To assess the depletion of CACs in diabetes we used an approach that differed from previous work, i.e., instead of comparing CAC counts from diabetics and non-diabetics, two groups that have many differences and are essentially non-comparable, we stratified CAC counts into high and low levels and then analyzed for the predilection of subjects with diabetes in either group. We found that diabetics had greater representation in the low cell count group, which affirmed that CACs are depleted in subjects with T2D. Our analysis was extensive and included 18 phenotypes of CACs based on the inclusion and exclusion of stem cell antigens (CD34, AC133), endothelial antigens (CD31, VEGFR2), and leukocyte antigens (CD45, CD14). We found seven phenotypes to be depleted in diabetics: (CD34⁺, CD34⁺/KDR⁺, CD34⁺/31⁺, AC133⁺, AC133⁺/CD31⁺, CD45⁺/AC133⁺/CD34⁺, and CD34⁺/AC133⁺/CD31⁺/CD45^{dim}).

We continued our analysis using one phenotype, CAC-3, that meets the most comprehensive definition of an early CAC. We found that depletion of CAC-3 in diabetes was associated with hyperglycemia (HbA1c) but not insulin resistance, inflammation (hsCRP) or oxidative stress (isoprostane F2 metabolite). All of which are known to contribute to diabetic vasculopathy, The selective association of hyperglycemia among the other variables measured may suggest that hyperglycemia is the antecedent event and that maintaining euglycemia

could delay the onset or progression of vasculopathy by CAC mediated vascular regeneration. This delineation was possible as our cohort contained subjects with and without vasculopathy, and the average duration of diabetes was about a decade.

We also found that CAC-3 counts had a positive correlation with reactive hyperemia, a measure of endothelial function. This finding suggests that the levels of CAC-3 may be an important biomarker or a physiological correlate of endothelial injury, dysfunction or inability to regenerate. This association, if confirmed in larger clinical studies, may allow for bedside assessment of the reparative capacity of the vasculature and may have prognostic value in instituting preventive care measures to improve endothelial health in T2D patients.

Additional studies provided further evidence for the role of hyperglycemia in mediating CAC-3 depletion. Specifically we found a negative association of HbA1c levels with VEGFR2 expression on CAC-3, which suggests decreased survival and proliferation of CAC-3. Further, we also found decreased expression of the homing receptor CXCR4 on CAC-3 in diabetics, which may further impede repair and regeneration of the vasculature in diabetes.

As an indicator of mitochondrial activity we measured $\Delta\Psi$ and found that it was no different in the diabetics and non-diabetics in CAC-3. Measures of oxidative stress (isoprostane F2 metabolite) in our cohort did not affect CAC-3 levels but did have a negative association with VEGFR2 expression on CAC-3. It

is possible that effects of oxidative stress will be more evident in older subjects and in subjects with diabetes of longer duration.

Hyperglycemia was also found to have a positive association with plasma sICAM-1 and sE-selectin levels, suggesting that hyperglycemia leads to activation of inflammatory pathways and endothelial activation. While the biological functions of soluble adhesion molecules in plasma are not clearly understood, it has been suggested that they could decrease the adhesion of circulating cells to the activated endothelium and modulate cell trafficking across the endothelium. It is also suggested they could bind to circulating cells and deliver a stimulus (274). Our finding that sICAM-1 levels bear a negative association with CAC-3 counts suggests that it may play a role in CAC survival. Further *in vitro* and *in vivo* studies are needed to understand the mechanisms that underlie this association. It has been reported by Ridker *et al* that high sICAM-1 levels predict myocardial infarction, while Werner *et al* reported that low CAC counts predicted mortality in CAD patients. Our finding of the negative association of CAC-3 and sICAM-1 levels reinforces that the biologic function of sICAM-1 needs to be investigated further (246; 275).

The results presented in Chapter 4 wherein we found decreased ability to culture late outgrowth cells from diabetics support the finding in Chapter 2 that CACs are depleted in diabetes. In addition this result suggests that diabetes is associated with a decrease in the adhesive and proliferative capacities of CACs. We also found that despite similar culture conditions, outgrowths from diabetics had a greater variability in their phenotype and surface antigen expression. The

in vitro assessment of CAC function by tube formation in matrigel was no different in the non-diabetic and diabetic outgrowth cells. However the *in vivo* assessment of angiogenic potential showed that outgrowth cells from diabetics did not enhance revascularization and were no different from vehicle. This suggests that when supplied with external growth factors as in matrigel, these cells are capable of tube-like structure formation, however in and ischemic environment the cells fail to support revascularization, suggesting deficiencies in paracrine function or differentiation.

Overall this project supports the observation that diabetes results in CAC depletion. It further suggests that the depletion of CAC-3 is associated with poor long-term glycemic control and endothelial dysfunction. We report that both diabetes and endothelial dysfunction contribute to the depletion of CACs independently. The positive association of reactive hyperemia and CAC-3 may reduce the bench-to-bedside gap in assessing endothelial function and the reparative capacity of the vasculature. This project also adds to our knowledge of the possible mechanisms by which diabetes induces vascular injury and dysfunction. However because of the cross-sectional design of the study, we were unable to establish cause-effect relationships between diabetes and CAC dysfunction. Further investments in understanding CAC biology, and in methodologies that identify early vascular dysfunction could lead to the institution of preventive care measures or therapeutics directed to improving the number and function of CACs that could improve CVD outcomes, and reduce the burden of CVD in subjects with diabetes.

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PUBLICATIONS & ABSTRACTS

Residential Proximity to Major Roadways Is Associated With Increased Levels of AC133+ Circulating Angiogenic Cells. Arterioscler Thromb Vasc Biol. 2015 Aug 20. DeJarnett**Zafar N**, ...**Bhatnagar A**.

Metabolic remodeling of white adipose tissue in obesity. AJP-Endocrinology and Metabolism, June 2014. Cummins TD, Holden CR, Sansbury BE, **Zafar N**, Tang Y, Hellmann J, Spite M, Bhatnagar A, and Hill BG.

Improving Survival of Vulnerable Infants Increases NICU Nosocomial Infection Rate. Arch. Pediatr Adolesc Med/Vol 155, Oct. 2001. **Zafar N**, Hamvas A.

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Nutrient Excess promotes accumulation of bone marrow-derived progenitor cells in adipose tissue. Abstract at the Experimental Biology Meeting, 2014. Holden CR, Wysoczynski M, Sansbury BE, Hellmann J, **Zafar N**, Gibb A, Bhatnagar A, Hill BG

Type 2 diabetes decreases cardiac stem cell mitochondrial metabolism and prevents myocardial repair. The Cardiovascular Forum for Promoting Centers of Excellence and Young Investigators 2013. Cummins T., Guo Y., Du J., Book M, Zhu X, Wu W, McCracken J, Salabei J, **Zafar N**, Habertzettl P, Holden C, Gibb A, Jones S, Li Q, Bolli R, Bhatnagar A, and Hill BG.

Prostacyclin Does Not Inhibit Pulmonary Vascular Neointimal Formation. Abstract at The American Thoracic Society Meeting, 1998. **Zafar N**, Botney M.

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