Chemokines and Neovascularization in Coronary Artery Disease

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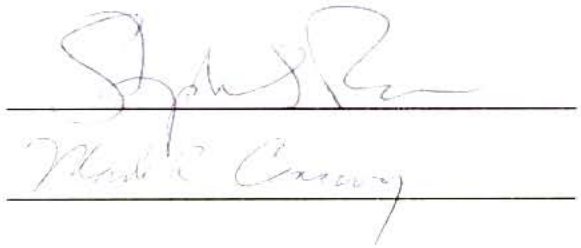

1. PROJECT DESCRIPTION

A. Specific Aims

Coronary artery disease (CAD) remains the leading cause of death and disability worldwide. In patients with chronic stable angina, neovascularization of the myocardium is a compensatory mechanism that occurs in response to repetitive or chronic myocardial ischemia. This process, which consists of both the formation of new capillaries (angiogenesis) and the formation of collateral arterial supply (arteriogenesis), is associated with markedly improved cardiac function and clinical outcomes. **A striking, but mechanistically unexplained, clinical observation is that patients with similar degrees of coronary stenosis and myocardial ischemia have markedly heterogenous extent of myocardial neovascularization**. Chemokines are a superfamily of cytokines originally described for their role in mediating leukocyte recruitment to sites of inflammation, but have subsequently been established as important mediators of angiogenesis and arteriogenesis in diverse disease settings. Many chemokine ligands and receptors are expressed in the ischemic myocardium, where angiogenesis and development of collaterals occur. While chemokines are known to mediate new blood vessel formation in diverse biological settings, less is known regarding their role in the ischemic heart. **My overall hypothesis is that an imbalance of angiogenic and angiostatic chemokines contributes to neovascularization of the myocardium in patients with chronic stable angina.**

I have tested this hypothesis in a highly characterized population of chronic stable angina patients undergoing coronary angiography under the following **Specific Aims:**

1. To correlate the presence and extent of myocardial neovascularization in patients with chronic stable angina undergoing coronary angiography with their circulating levels of angiogenic and angiostatic chemokines.

Hypothesis to be tested: A. Patients with chronic stable angina have higher levels of circulating angiogenic chemokines versus angiostatic chemokines as compared to patients without chronic stable angina. *B.* Among patients with chronic stable angina, there is a correlation between the extent of myocardial neovascularization, as determined by angiographic imaging, and the level of angiogenic versus angiostatic chemokines.

2. To determine the frequency of genetic polymorphisms in angiogenic and angiostatic chemokines associated with myocardial neovascularization in a highly characterized population of chronic stable angina patients undergoing coronary angiography.

Hypothesis to be tested: Patients with chronic stable angina and angiographic evidence of neovascularization carry chemokine polymorphisms resulting in increased angiogenic and reduced angiostatic chemokine levels, as compared to patients with chronic stable angina and no evidence of neovascularization.

I am ideally positioned to perform this work because: **1)** the population referred for coronary angiography at the University of Virginia is phenotypically well-defined and includes a high proportion of patients with chronic stable angina as well as normal controls; **2)** the cardiac catheterization laboratory at the University of Virginia is a high-volume center with resources available to systematically enroll patients into the study; **3)** I have already established a cardiac catheterization laboratory-based database of plasma and genomic DNA samples; **4)** my collaborators, Drs. Robert Strieter, Brian Annex and Stephen Rich, are recognized leaders in the fields of chemokine biology, ischemic neovascularization and population genetics, respectively; and **5)** I am an interventional cardiologist with a track record in clinical research. The data generated thus far is being presented as my thesis for a Masters of Science degree in Clinical Investigation in the Department of Public Health Sciences at the University of Virginia.

The **significance** of this proposal is that it examines the mechanism of the important but incompletely understood clinical problem of neovascularization of the ischemic myocardium. Its **innovation** is that it has the potential to link the well-characterized mechanism of chemokine-mediated neovascularization to ischemic heart disease.

B. Background and Significance

1. Chronic ischemic heart disease is a significant public health problem.

Neovascularization: a continuum of vasculogenesis, angiogenesis, arteriogenesis, and collateral formation. **Angiogenesis**: formation of new capillaries from preexisting ones; triggered by ischemia, tissue acidosis and oxidative stress. **Arteriogenesis**: stabilization of newly formed vessels by smooth muscle cells and extracellular matrix; triggered by shear stress. **Collateral formation**: anastomotic conduits bridging severe arterial stenoses; triggered by changes in shear stress.
Table 1: Definitions of key terms used in this proposal

Coronary artery disease remains the leading cause of death and disability worldwide [1, 2]. The improved survival of patients with acute coronary syndromes, including unstable angina and acute myocardial infarction, has resulted in a growing patient population with chronic symptoms. Chronic myocardial ischemia is due to the narrowing of the arterial lumen by atherosclerotic plaque leading to diminished myocardial blood flow. In some patients with CAD, this chronic ischemic process leads to myocardial dysfunction, congestive heart failure and lethal arrhythmias. In others, however, robust neovascularization (Table 1) promotes myocardial salvage [3-8]. This is important because patients with myocardial neovascularization have significantly improved clinical outcomes [9-14]. The pathophysiology underlying the heterogeneity of neovascularization in patients with myocardial ischemia, even with similar angiographic patterns of CAD, remains unclear.

2. *Chemokines are major mediators of neovascularization.*

Chemokine ligands are a superfamily of structurally homologous heparin-binding cytokine molecules that were originally described for their

chemotactic properties for leukocytes, but have subsequently been recognized as mediators of diverse biological processes including neovascularization [15]. Chemokines share 4 conserved cysteine residues at their amino-terminus. Chemokines are subdivided into CC, CXC, C, and CX_3C families based on the sequence of amino acids in relation to the first two cysteine residues: in the CC chemokine family, these two cysteine residues are adjacent, whereas in the CXC family, the first 2 cysteine residues are separated by a non-conserved amino acid. The CXC family ligands are further divided on the basis of presence or absence of a glutamic acid-leucine-arginine (Glu-Leu-Arg or ELR) sequence immediately adjacent to the CXC motif [16-18]. The presence of the ELR motif is functionally important: the ELR+ CXC chemokines are potent promoters of angiogenesis, whereas a subset of the non-ELR CXC chemokines are potent inhibitors of angiogenesis (Table 2).

Systematic name	Prior name	Receptor
Angiogenic		
CXCL1	Gro-α	CXCR2
CXCL2	Gro-β	
CXCL3	Gro-γ	
CXCL5	ENA-78	
CXCL6	GCP-2	
CXCL8	IL-8	
CCL2	MCP-1	CCR2
Angiostatic		
CXCL4	PF-4	CXCR3
CXCL9	Mig	
CXCL10	IP-10	
CXCL11	I-TAC	

Table 2. Chemokine ligands and receptors involved in angiogenesis.

The role of chemokines in mediating neovascularization has been extensively documented in diverse biological settings, with the notable exception of ischemic heart disease [19]. Specifically, chemokines are critical mediators of

angiogenesis in many cancers, including bronchogenic carcinoma, breast cancer, gastrointestinal malignancies, prostate carcinoma, melanoma, renal cell carcinoma, ovarian cancer, glioblastoma and head and neck cancers [20-26], as well as non-malignant diseases including animal models of corneal neovascularization and wound healing [27-31], human pulmonary fibrosis and its animal counterparts [15, 32-34], solid-organ transplant rejection [35] and acute respiratory distress syndrome [36].

In the context of heart disease, chemokine-mediated angiogenesis has been demonstrated within atherosclerotic plaques [37-39]: the angiogenic ELR+ CXC chemokine, CXCL8, is over-expressed in human coronary artery plaque samples, as compared to control samples from internal mammary arteries without atherosclerosis, where it co-localized with Factor VIII-related antigen expression on endothelial cells in the coronary atherectomy specimens, and is the major mediator of net angiogenic activity of the plaque [40]. A large number of chemokines are induced in the context of myocardial ischemia and failure [41, 42], but the specific contribution of these mediators to angiogenesis has not been clearly established. Studies of mediators of myocardial neovascularization have primarily focused on the role of growth factors, specifically, vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF) [5, 44-52].

Of relevance to this proposal, angiogenic chemokines mediate a greater role in neovascularization in a number of settings than growth factors [36, 53, 54]. In addition, the arteriogenic CC chemokine, CCL2, has been shown to be involved

in infarct-associated inflammation and healing in a mouse model of myocardial infarction [56], and in arteriogenesis in a mouse model of hind-limb ischemia [43]. Chemokines are critical mediators of angiogenesis and arteriogenesis in diverse biological settings, but their role in neovascularization of the ischemic myocardium has not been studied in detail.

3. Heterogeneity in myocardial neovascularization may be genetically mediated.

Clinically, there is a striking degree of heterogeneity in the presence and extent of myocardial neovascularization among patients with chronic stable angina and similar degrees of epicardial coronary stenoses. The biological basis for this observation is has not been established: the only factor postulated to play a role in the extent of neovascularization of the ischemic myocardium is diabetes mellitus; its effect, however, remains controversial [46, 57-60] and does not explain the heterogeneity in myocardial neovascularization observed in non-diabetics or within diabetic populations. In the absence of recognized *acquired* predictors, an *inherited* basis for this heterogeneity has been postulated [52, 61]. In relation to this, a number of functional single nucleotide polymorphisms (SNPs) of angiogenic and angiostatic chemokines have been reported: these SNPs which can also exist as haplotypes (a set of closely linked SNPs inherited as a unit) are associated with increased or decreased levels of chemokines, and play an important role in diverse diseases [62, 63]. For example, the CXCL8 -251 (A/T) SNP is associated with

increased CXCL8 levels, and has been associated with higher risk of prostate, colorectal, gastric, and squamous cell carcinoma; and the CCL2 -2518 (G/A) SNP is associated with increased CCL2 production, and has been associated with diabetes mellitus, coronary atherosclerosis, left ventricular dysfunction, and myocardial infarction. Genetic factors that affect levels of angiogenic and angiostatic chemokines may provide a biological explanation for the observed heterogeneity in neovascularization of the ischemic myocardium.

C. Preliminary Data

I have performed a pilot study to determine whether chemokine levels were similar when sampled from the coronary sinus and from the peripheral blood in patients with significant left anterior descending or left circumflex CAD. The levels of CXCL5, CXCL11, and CCL2 (representative angiogenic, angiostatic, and angiogenic/arteriogenic chemokines) in the paired samples did not differ significantly in the patients studied (Figure), demonstrating the feasibility of the proposed experiments to assess these chemokines in the peripheral blood.

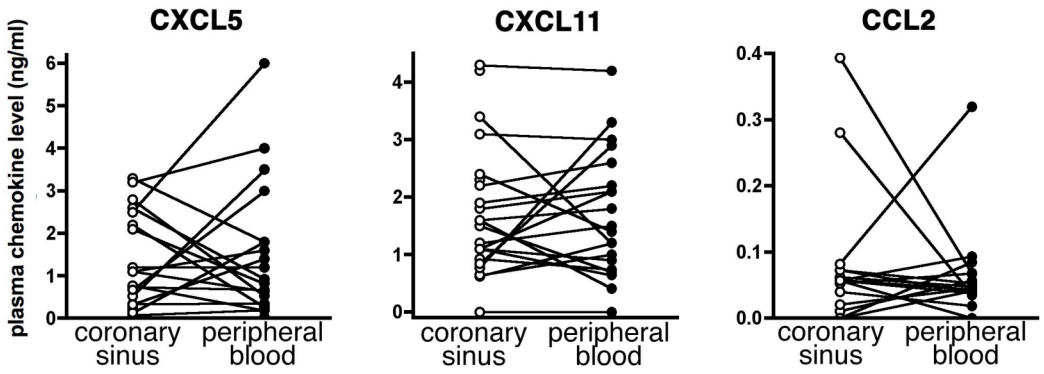

Figure. Comparison of simultaneous coronary sinus and peripheral blood samples collected from 23 patients with LAD or circumflex disease undergoing coronary angiography. There was no difference in chemokines levels measured in paired samples (p=0.65, p=0.71, and p=0.60 for CXCL5, CXCL11 and CCL2, respectively, by paired t tests).

Of the projected 525 patients needed for the study, I have enrolled 334 thus far. Complete demographic information has been obtained, and all angiograms have been reviewed for the presence and extent of CAD and myocardial neovascularization. All data has been entered into the database. The data is summarized in (Table 3). Plasma samples from the 334 patients are being analyzed for the angiogenic and angiostatic chemokines listed in Table 2. DNA has been isolated from the blood samples in all patients and chemokine polymorphisms are being measured.

Demographic data	
Men	220 (66%)
Women	114 (34%)
Age (median, years)	58 +/- 11
Race	
Caucasian	263 (79%)
African-American	59 (18%)
Hispanic	8 (2%)
Asian	4 (1%)
Cardiac risk factor data	
Diabetes	104 (31%)
Hypertension	267 (80%)
Hyperlipidemia	282 (84%)
Tobacco use	117 (35%)
Angiographic data	
No CAD	54 (16%)
Non-obstructive CAD*	110 (33%)
Obstructive CAD without neovascularization*	83 (25%)
Obstructive CAD with neovascularization*	87 (26%)

Table 3. Baseline characteristics of 334 patients enrolled to date. CAD= coronary artery disease; *, obstructive CAD is defined as the presence of ≥90% lesion

D. Experimental Design and Methods

Specific Aim 1: To correlate the presence and extent of myocardial neovascularization in patients with chronic stable angina undergoing coronary angiography with their circulating levels of angiogenic and angiostatic chemokines.

Patient selection and enrollment: All men and women age > 18 years referred for coronary angiography are eligible for enrollment. Patients *with* symptoms of angina pectoris and angiographic evidence of CAD but *without* recent

progression or acceleration of symptoms, are defined as patients with **chronic stable angina**. Patients with angiographically normal coronary arteries serve as the control group. Exclusion criteria include: acute coronary syndrome, elevated troponin, hematocrit < 30, and known blood-transmittable, inflammatory or infectious disease.

Sample collection: Informed consent for the study is being obtained prior to the catheterization procedure. Following vascular access, and prior to coronary angiography a 30ml peripheral blood sample is drawn from the sidearm of the sheath: half of the blood sample is being used for DNA isolation and genotyping, and half is centrifuged, and the plasma is collected for measurement of angiogenic and angiostatic chemokines. Samples are labeled using a unique study code (no patient identifiers), and are stored in a -80 freezer. The unique study code key is kept in my office in a locked cabinet. Angiogenic and angiostatic chemokines (Table 2) are being measured by ELISA or Luminex in the laboratory of my collaborator, Dr. Robert Strieter.

Angiographic analysis: The following detailed angiographic data is being collected: (1) presence and extent of non flow-limiting and flow-limiting lesions of the epicardial arteries (lesions compromising < 70% or ≥ 70% of the lumen, respectively); (2) antegrade epicardial flow according to Thrombolysis in Myocardial Infarction classification [64]; (3) presence of collateral formation assessed by the Rentrop classification [65]; (4) presence and extent of microvascular blood flow assessed by myocardial blush grade [66]; and (5) left ventricular

function assessed by left ventriculography. Angiograms are bing analyzed in a blinded manner to the chemokine data by two interventional cardiologists (a third interventional cardiologist is the final arbiter in the event of disagreement).

Database: Demographic (age, gender, race, co-morbidities, cardiac risk factors, social history, family history of CAD, medical therapy) laboratory (lipid profile, renal and liver function, hematologic profile), angiographic, and chemokine data are being prospectively collected, and entered into a computerized database compliant with HIPAA regulations.

Statistical consideration and projected enrollment: In Specific Aim 1, I planned to enroll 150 patients with chronic stable angina and CAD (75 with and 75 without angiographic evidence of neovascularization), and 75 patients without CAD. I have based the projected enrollment on published studies that have successfully measured peripheral blood chemokine levels in small numbers of patients with lung disease and cancer (< 25 patients per study) [67, 68]. I have successfully enrolled 170 patients with chronic stable angina (87 with and 83 without angiographic evidence of myocardial neovascularizaton). The final model will adjust for age, co-morbidities, and left ventricular systolic function.

Anticipated results: My hypothesis predicts that in patients with chronic stable angina: (1) the presence of neovascularization will correlate with high levels of angiogenic/arteriogenic chemokines, and low levels of angiostatic chemokines, and (2) the absence of neovascularization will be associated with low levels of angiogenic/arteriogenic chemokines, and high levels of angiostatic chemokines.

In patients with angiographic evidence of neovascularization, I anticipate that the extent of neovascularization will correlate with peripheral blood concentrations of angiogenic/arteriogenic chemokines.

Specific Aim 2: To determine the frequency of genetic polymorphisms in angiogenic and angiostatic chemokines associated with myocardial neovascularization in a highly characterized population of chronic stable angina patients undergoing coronary angiography.

Patient selection and enrollment: This study compares the two groups of CAD patients with chronic stable angina: (1) those with angiographic evidence of neovascularization, and (2) those without angiographic evidence of neovascularization. The inclusion and exclusion criteria are otherwise identical to Specific Aim 1.

SNP selection and genotyping: The following functional SNPs that have been shown result in increased or decreased levels of angiogenic and angiostatic chemokines are being measured [63]: (1) CXCL2 Tandem repeat -665 (AC)n; (2) CXCL8: -251 (A/T), -845 (C/T), +781 (C/T), +1633 (C/T), +2767 (A/T), and Haplotypes; (3) CXCL10 Haplotypes; (4) CXCL11 DIP -599del5; and (5) CCL2 -2518 (G/A), and Haplotypes. Variation in regions of high inter-marker linkage disequilibrium is being evaluated by HapMap data [69].Genotyping assays are being conducted in the Center for Public Health Genomics in the laboratory of

Dr. Stephen Rich.

Data management and error checking: For each SNP, maximum likelihood estimates of allele frequencies are being tested for departures from Hardy Weinberg Equilibrium (HWE) (indicating genotyping errors) using the chi-square goodness of fit test. If a departure from HWE is not resolved, only genotypic association analysis is being performed.

Association analyses: Allele frequencies of each SNP in each race/ethnicity group are being assessed with χ^2 testing of HWE, and pair-wise linkage disequilibrium by the D′ and r^2 statistics. To test for an association between neovascularization and each SNP, a logistic regression analysis is being performed. Haplotype analyses is being performed in the models providing evidence of at least one SNP attaining an independent significant association. The final model will adjust for age, co-morbidities, and left ventricular systolic function.

Statistical consideration and projected enrollment: In Specific Aim 2, using a log-additive model with 80% power, I will need a total of 500 patients with chronic stable angina (250 with and 250 without angiographic evidence of neovascularization). Allowing for a 5% withdrawal rate, I will enroll 525 patients. I am confident I will be able to achieve my sample size calculation because the University of Virginia performs > 2,000 coronary angiograms a year, the vast majority of whom are potential study candidates, and I have already enrolled 334 patients which is 64% of my final sample size.

Anticipated results: My hypothesis predicts that patients with robust angiogenesis will disproportionately carry chemokine SNPs associated with increased levels of angiogenic chemokines relative to angiostatic chemokines.

2. ETHICAL ASPECTS OF STUDY

A. Informed consent

Potential research subjects are being asked to participate in this study after they have provided informed consent for the cardiac catheterization procedure. Patients are initially asked if they are interested in learning about this research study. If so, the research study is discussed in detail, and questions are answered. At that time they are invited to participate in the research study. If they agree to participate, they sign the research consent form. All men and women who fulfill the inclusion/exclusion criteria are eligible to participate.

B. Identity protection

Demographic, laboratory, angiographic, chemokine and polymorphism data are being prospectively collected and entered into the patient database. The database is housed in a desktop computer that is not connected to the internet, is password protected, and is locked to a desk. No patient identifiers are being used. All patients are given a unique study code. A hardcopy of the log which contains the patients' names, medical record numbers, and unique study codes in

a locked file cabinet in my office: this information will not being entered into a computer. Signed research consent forms are also being secured in a locked file in my office. No identifying information is being entered into the patient database. All HIPAA regulations regarding personal health information are being followed.

C. Removal from study

At any time the patient may request to withdraw from the study. If this occurs, the patient's plasma and DNA samples will be destroyed, and all data entered into the database pertaining to that patient will be deleted. Thus far, only one patient has requested to be removed form the study.

D. Reports of test results

All data is being collected for research purposes only and is not available to the patient, legal representatives, private physicians, family members, medical or life insurance providers, or the patient's employer. No information that could possibly be linked to the patient will be published.

E. Risks to patient

The main risk to the patient is that associated with blood collection. Blood collection is being obtained via the sidearm of the sheath placed in order to perform the coronary angiogram. The total volume of blood that is being obtained (30 cc's) is small, and does not cause a decrease in the overall blood count. There is also a risk that someone other than the PI will gain access to the genetic data. However, this is highly unlikely due to the stringent methods that are being followed to ensure patient confidentiality. There is no psychological, social, economic or legal risks related to participation in this research, because the information obtained is not being released to anyone, including the patient.

F. Possible benefits

This research will not provide direct, immediate benefit to either the patient or to society. However, it is hoped that the information obtained will result in novel therapies that will benefit others with chronic myocardial ischemia in the future.

3. SIGNIFICANCE OF PROBLEM TO CARDIOVASCULAR RESEARCH

The presence of well-developed myocardial neovascularization is associated with dramatically improved clinical outcomes in patients with chronic ischemic heart disease. The mechanism of this neovascularization is therefore an

important clinical question, but it has not been studied extensively. The proposed research has the potential to advance the field of cardiovascular investigation by implicating chemokine-mediated angiogenesis and arteriogenesis to ischemic heart disease and by identifying genetic determinants that may influence this important process. Ultimately, this work has the potential to lead to novel therapeutic applications of chemokines in promoting myocardial neovascularization in patients with chronic ischemic heart disease.

4. POTENTIAL EXTENSION OF PROPOSED RESEARCH

Generating a database of a large number of highly phenotyped patients undergoing coronary angiography with a repository of plasma and DNA samples will serve as a unique and valuable resource for future translational and genetic studies of myocardial neovascularization. Importantly, the availability of detailed patient-level data will enable me to assess the effect of specific phenotypes on an individual's potential for myocardial neovascularization.

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