Evaluation Of Advanced Glycosylation End Products With Advanced Glycosylation End Product-Specific Elisa In Diabetic Patients And Correlation With Severity Of Coronary Artery Disease

Alfonso F. J. Prieto

A. Statement of Study Purpose Rationale

Coronary artery disease, especially myocardial infarction, is the major cause of mortality in diabetic patients. Furthermore, the adjusted mortality rates for coronary artery disease in diabetic men is two to three times greater, and for diabetic women is three to seven times greater when compared to non-diabetics MAYO IMR 1994-1995). The DCCT research group concluded that strict glycemic control can significantly delay diabetic complications, but the number of cardiac events in this young, Type I diabetic study group was small. In contrast, Abraira et al studied intensive insulin therapy for type II diabetics, and found an increased number of cardiac events in their initial pilot study of older diabetics, suggesting, as others have as well, that insulin itself may promote atherogenesis. Consequently, there is limited, inconclusive data regarding glycemic control and development of coronary artery disease in diabetics.

The dramatic increase in number of coronary artery reperfusion procedures such as angioplasty and stent placement in diabetics is of concern, especially since it has been shown in multiple trials that diabetes is a consistent clinical predictor for restenosis after angioplasty with restenosis rates from 49-71% (nondiabetics restenosis rate 24-46%) (Doron 1996). Also, diabetics have been found to have a higher restenosis rate after coronary artery stent placement (Carrozza 1993). Better understanding of the evolution of coronary artery disease in diabetics is needed to improve clinical outcomes from these interventions.

The pathogenesis of coronary artery disease in diabetics appears to be multifactorial, involving well known factors for atherogenesis in nondiabletics such as smoking and hypercholesterolemia, as well as other mechanisms including: hyperinsulinemia (Sober 1996); decreased heparin sulfate (Vernier 1992); platelet hypersensitivity to agonists (epinephrine, collagen, thrombin, arachnidonic acid) (Winocour 1992); increased thromboxane A2 production (Davi 1992); endothelial dysfunction with decreased EDRF (Bucala 1991); PG12 (Umeda 1989); increased ET-1 production (Takahashi 1990); and dysregulation of growth factor expression producing increased amounts of IGF-I(Murphy 1991), bFGF (Linder 1991), TGF-alpha (McLain 1992), and TGF-beta (Yamamoto 1991).

Advanced glycosylation end products (AGEs) are special glycated proteins formed nonenzymatically in diabetics as a function of time and glucose concentration, via a chemical reaction known as the Maillard reaction in which an Amadori intermediate (I- amino -I- deoxyketose) is produced. AGE formation for the most part is an irreversible reaction. Therefore, the amount of AGEs produced represents a long term cumulative marker of glycemic control, distinct from glycosylated hemoglobin (reversible reaction) which gives an estimate of glycemic control over several months. Brownlee has described three mechanisms by which AGEs can cause tissue damage in diabetes, and they include: alteration of signal transduction pathways by binding to the extracellular matrix; alternating the level of cytokines, hormones, and free radicals by acting with AGEspecific receptors; and by intracellular formation of AGEs. AGEs appear to play a paramount role in diabetic complications in animals and in humans including tissue damage to connective tissue, nerves, blood vessels, and retina. Furthermore, aminoguanidine which inhibits AGE formation by reacting with the AGE precursor 3-deoxyglucosone (Hirsch J) has been noted to prevent diabetic retinopathy, neuropathy, and nephropathy in animal models and in humans. Currently, a multicenter drug trial is in progress to evaluate the effectiveness of

aminoguanidine in retarding diabetic nephropathy. Glycosylation inhibition may become an effective novel way to prevent diabetic complications.

Beisswenger et al have described that collagen-linked AGEs measured via enzyme-linked immunosorbant assay (ELISA) from skin biopsies correlate with preclinical stages of diabetic retinopathy and nephropathy. Evaluating the relationship between AGE formation and severity of coronary artery disease in diabetics may provide crucial information to refine our understanding of the progression of coronary artery disease in diabetics, and possibly a novel way to clinically evaluate these patients and facilitate clinical decision making in regards to the most appropriate therapy.

The purpose of this study is to elucidate the relationship between collagen-linked AGE formation in the skin and severity of coronary artery disease in diabetics.

Hypothesis: There will be a significant difference between AGE formation and the severity of the coronary artery disease.

Null Hypothesis: There will be no significant difference between AGE formation and the severity of coronary artery disease.

B. Description of Study Design and Statistical Analysis:

a. Study design

This will be a cross-sectional descriptive study in which eligible subjects (see section B.2.) will be interviewed and an encounter form (see addendum #1) will be filled out to compile demographic as well as medical data. Laboratory tests to be drawn at this time included fasting glucose, lipid profile, PAI-1, hemoglobin Al-C, chem-7, insulin level and C-peptide. Afterwards subjects will be consented (see addendum #2) and a skin biopsy will be performed, and the sample will be sent to determine collagen AGE level (person performing AGE determination will be blinded to study subject). Patients will then have previously scheduled coronary angiography and results will be noted, and reviewed by a cardiologist blinded to the patient's diagnosis of diabetes.

b. Subject Selection

Subjects will be selected from Columbia Presbyterian Medical Center Catheterization Lab schedule who fill the following inclusion criteria:

1. Diagnosis of diabetes mellitus according to National Diabetes Data Group
2. Patients scheduled for non-emergent coronary catheterization
3. Normal kidney function as defined as creatinine .6-1.5
4. Age greater than and including 18

Exclusion criteria:

1. No diagnosis of Diabetes Mellitus according to National Diabetes Data Group criteria
2. Patients going to emergent coronary catheterization
3. Patients with abnormal kidney function defined as creatinine greater than 1.5
4. Ageless than 18 Control subjects will be considered those diabetics enrolled in this study who have no identifiable coronary artery disease by cardiac angiography.

c. Cross Over

none

d. Randomization

none

e. Duration of the entire study

The data will be collected until the estimated number of study patients is reached, which we approximate to be one year. The patient's participation, which will include informed consent, data acquisition via interview, blood samples, skin biopsy, and scheduled cardiac catheterization will all take approximately 2 hours, but this depends on technical aspects of the catheterization itself.

f. Statistical Analysis

We will use standard one-way ANOVA to compare mean levels of AGEs among patients with none, one, two, and three coronary artery disease and to test the null hypothesis that there is no difference between mean AGE levels. We also will adjust for possible confounding effects of age, sex and duration of diabetes by using ANCOVA to determine mean AGE levels for each level of coronary artery disease. We also will analyze the level of correlation between the degree of coronary artery disease and AGE levels by using Spearman's correlation coefficient. According to the reviewed literature, AGE collagen determination with AGE-specific ELISA is a test that follows a Gaussian distribution with an estimated standard deviation of 10.

The prevalence of coronary artery disease is higher for diabetics, but there are conflicting studies regarding the characterization of the increased coronary artery disease in diabetics; but it is generally agreed that diabetics have more triple vessel disease and diffuse coronary artery disease when compared to non-diabetics. After review of literature (Dortimer, 1978), it is estimated that the incidence of no coronary disease is 11%, one vessel coronary disease is 14%, two vessel coronary disease is 32%, and three vessel coronary disease is 43% for diabetic patients. Using a power of 80% and a $p<0.05$, the number of patients necessary to obtain statistical significance is estimated to be 240.

C. Description of Study Procedures:

Possible subjects for this study will be selected from the cardiac catheterization lab (Milstein Hospital 2nd floor) scheduling list. Charts will be reviewed to identify patients with a history of diabetes mellitus. After possible diabetic patients have been identified, the primary physician for that patient will be asked if the patient is a possible candidate for this protocol. After approval from the primary physician, the patient then will be approached by a physician associated with the research group and the protocol will be described in lay terms, and then they will be asked for their participation. If the patient agrees, inclusion and exclusion criteria will be reviewed to ensure that the patient is eligible for this protocol. If patient is eligible and consents (see addendum #2) to participate, then the encounter form (see addendum #1) will be filled out which will included demographic data of the patient such as name, age, sex, address, telephone number, medical record number, and also medical information including current medications, duration of diabetes, hypertension, hypercholesterolemia, smoking, prior myocardial infarction, prior coronary artery bypass grafting, prior catheterization results if known, and left ventricular ejection fraction. After encounter form is filled out, the patient will have the skin biopsy taken, then several blood samples will be taken from the venous sheath at the time of catheterization for the following tests: glycosylated hemoglobin, PAI-1, fasting lipid profile, insulin level, C-reactive protein, and a chemistry seven panel. After these blood samples are collected, the patient will have the skin biopsy taken.

a. Skin Biopsy Procedure

Skin samples will be obtained from the right upper buttock via a 4-rnm punch biopsy (after area has been sterilized with betadine), and in 30 minutes they will be snap frozen in liquid nitrogen and then immediately frozen at -70 degrees Celsius until all samples are collected and AGE determination will be performed. Hemostasis will be achieved at biopsy site.

b. Determination of AGEs in Skin Collagen: (as per Makita)

After all samples are collected and frozen at -70 degrees Celsius, they will be thawed slowly at room air. The epidermis will then be removed by sharp dissection under a dissecting microscope. The sample will then be minced and homogenized with a tissue homogenizer for 60 seconds. Sample then will be placed in chloroform-methanol (2:1) for 12 hours at 4 degrees Celsius (lipid extraction), washed, and suspended in 0.02-M HEPES buffer with pH 7.5 with AM calcium chloride. Then, the sample will be digested with HEPES buffer with 280 U of type VII collagenase (Sigma pharmaceuticals) for 48-72 hours at 37 degrees Celsius with constant shaking. The pellet is expected to have less than 3% of the total tissue collagen in all subjects. The digested samples will then be centrifuged at 15,000 x g, and the supernatants of the samples will be used to determine AGE content. An immunochemical method using polyclonal antiserum prepared to AGE-RNase will react with samples in a competitive ELISA. AGE content will be

defined as one AGE unit which will be equivalent to the amount of antibody reactive material equivalent to lug of an AGE-BSA standard.

c. Determination of the Severity of Coronary Artery Disease

The study subjects have been already scheduled for coronary anglography at the Columbia Presbyterian Medical Center Cardiac Catheterization Laboratory. After the cardiac anglography is performed, the cine will be obtained.

The catheterization results from the performing cardiologist will be noted (in terms of no, one, two, or three vessel disease, dominance of circulation, presence of diffuse disease, and comment on ejection fraction). A cardiologist who will be blinded to the patient's diagnosis of diabetes, will review the cine and each fill out a cine review form (see addendum #3) in which he/she will comment on the number of coronary vessels affected and severity of vessel disease, presence of diffuse disease, and determination of visually estimated ejection fraction. For the purpose of this study, any lesion with greater than 75% stenosis of the coronary artery lumen of a given vessel when compared to the maximal luminal diameter of the affected vessel, will be considered a diseased vessel. The Cine review form will include a diagram of the coronary arteries adapted from Dortimer et al (1978) in which the coronary vessels will be divided into segments so as to duplicate the coronary anglogram findings. A scoring system to determine the severity of coronary disease was adapted from Dortimer et al (1978) in which lesions will be graded as follows: 0 = no disease, 1 intimal disease to less than 50% stenosis, 2 = 50-74% stenosis, 3 = 75-95% stenosis, 4 = 95-99% stenosis or subtotal occlusion, and 5 = 100% stenosis or total occlusion. The maximum score for a given vessel would be the sum of the stenosis score of all the segments of that coronary artery. Diffuse disease will be defined as more than three of five segments of the left anterior descending artery were considered to have significant stenosis (a score of three or greater); if three of five segments of the left dominant circumflex coronary artery were graded three or higher; if two of four segments of the left non-dominant circumflex artery were graded greater than three, or if two of four segments of the right dominant right coronary artery were graded three or higher. Dominance of the coronary circulation will be classified as right or left dominance. Also, as per the CASS investigators, left main coronary disease with right dominance will be considered two vessel disease and left main coronary artery disease with left dominance will be considered three vessel disease. Visually estimated left ventricle ejection fraction of the ventriculogram will be classified as normal, mild, moderate, and severely reduced ejection fraction. The blinded cardiologists cine review form will be considered as the official catheterization result for the purpose of this study.

D. Study Drugs

None.

E. Medical Devices:

None.

F. Study Questionnaires

See addendum #1.

G. Study Subjects and Methods of Recruitment

a. Inclusion Criteria

1. Diagnosis of Diabetes Mellitus according to National Diabetes Data Group
2. Patients scheduled for non-emergent coronary catheterization
3. Normal kidney function as defined as creatinine 0.6-1.5
4. Age greater than and including 18

b. Exclusion Criteria

1. No diagnosis of Diabetes Mellitus according to National Diabetes Data Group criteria
2. Patients going to emergent coronary catheterization
3. Patients with abnormal kidney function defined as creatinine greater than 1.5
4. Age less than 18

c. Subject Identification and Approach

Possible subjects for this study will be identified from scheduled visits to the cardiac catheterization lab (Milstein Hospital 2nd floor). Charts will be reviewed to identify patients with a history of diabetes mellitus.

d. Subject Recruitment

After diabetic patients have been identified, the primary physician for that patient will be contacted, will have the protocol explained, and asked if the patient is a candidate for this protocol. After approval from the primary physician as required by Columbia Presbyterian Medical Center policy, the patient then will be approached by a physician associated with the research group and the protocol will be described in lay terms, and then they will be asked for their participation. The patient will be allowed to ask questions at any time, and to refuse participation. If the patient agrees to participate, inclusion and exclusion criteria will be reviewed to ensure patient is eligible for this protocol. If patient is eligible and consents to participate (see consent forms, one for participation in the study, and the other will indicate that the patient gives permission to review their medical records), then the encounter form will be filled out (see encounter form).

e. Gender or Race Restrictions

None.

H. Confidentiality of Study Data

No names, hospital medical record numbers or other identifying information will be in the data set. Patients will be assigned a separate identification number for purpose of blinding investigators to the patient. Identifying information will be kept by the principal investigator in a separate locked file. Only members of the research group will be allowed access to identifying information. No subject will be individually identified by name or medical record number in any reports of this research.

I. Location of the Study

The study will be conducted in the Cardiac Catheterization Laboratory of the Milstein Hospital Building at Columbia Presbyterian Medical Center. Determination of AGE content will be performed at Dr. Anne Marie Schmidt's Laboratory located at PS 17-501. Data analysis and review will be performed at the Irving Center for Clinical Research Administrative Offices located on the 10th floor of the Harkness Building at Columbia Presbyterian Medical Center.

J. Risks and Benefits

The benefits of this study are multiple. By elucidating the relationship between AGE formation and severity of coronary artery disease in diabetics, we may be better able to manage these patients clinically, especially in view of their high degree of restenosis after coronary artery reperfusion techniques. Furthermore, if a significant relationship is found between AGE formation and coronary artery disease, this would imply therapeutic benefit from glycosylation inhibitors, which may be able to slow progression of coronary artery disease in diabetics.

The risks of this study consist of the risk for cardiac catheterization for which the patient has a clinical indication and therefore this study would pose no additional risk. The patient is to have a 4mm skin punch-biopsy, a procedure which has minimal risks such as bleeding, infection, and pain. We believe that the benefits of this study outweigh the risks, and therefore are justified.

K. Alternative Therapies

None.

L. Compensation and Costs to Subjects

None.

M. Minors and Research Subjects

None.

N. Radiation or Radioactive Substances

None.