

Evaluation of RAGE expression in human diabetes and its relation to inflammatory markers of atherosclerosis

Edwin Lee

A. Study Purpose and Rationale

Type 2 diabetes characterized by insulin resistance and inadequate beta cell insulin secretion represent more than 90% of those with diabetes. The complications of atherosclerosis cause most of the morbidity and mortality in type 2 diabetes.

The metabolic abnormalities caused by diabetes induce vascular dysfunction that predisposes the patient to atherosclerosis. Clinical manifestation of atherosclerosis occurs primarily in 3 vascular beds: coronary arteries, lower extremities, and extra-cranial arteries. Diabetes markedly increases and accelerates the clinical course of each thus increasing the risk of myocardial infarction, stroke, amputation and death. Although diabetic patients undergo revascularization procedures due to acute coronary syndromes or critical limb ischemia, the outcomes are less favorable than in non-diabetic cohorts. Summarized in review (1).

a. RAGE and Diabetes

The consequence of long term hyperglycemia in diabetes causes the formation of Advanced Glycation End products (AGEs). The increased expression of the Receptor for AGEs (RAGE) in the diabetic vasculature overlaps with that of its ligand, AGE. In rodents, blockade of RAGE suppresses vascular hyperpermeability, reduces atherosclerotic lesion in diabetic apoE null mice, decreases the rate of restenosis after vascular injury. In parallel, indices of endothelial cell activation and macrophage migration/function were also suppressed in the presence of RAGE blockade. In humans, an association between increased expression of RAGE and inflammatory cells in atherosclerotic plaques from diabetic patients undergoing carotid endarterectomy for extracranial high-grade (>70%) internal carotid artery stenosis was observed. These animal and human data implicate RAGE in the pathogenesis of accelerated diabetic atherosclerosis

RAGE is expressed in a variety of cell types such as endothelial cells, peripheral blood monocytes, lymphocytes, vascular smooth muscle cells. Besides diabetes, RAGE is also overexpressed in cancers, Alzheimer's disease, immune and inflammatory conditions. These pleiotropic consequence of RAGE activation led to the hypothesis that RAGE is a progression factor amplifying immune and inflammatory responses as proposed by the two-hit model of vascular perturbation mediated by RAGE and its ligands. The hypothesis is that the diabetic vessel wall displays increased expression of RAGE ligands and the receptor itself (first hit). In the presence of additional perturbation (second hit), such as ischemic stress, immune/inflammatory stimuli, physical stress, or modified lipoproteins, there is an exaggerated cellular response promoting formation of vascular lesions (rather than restitution of vascular homeostasis). Summarized in review (2).

b. Human RAGE data limited

RAGE is polymorphic with recent studies reporting multiple genetic variants. The existence of pseudogenes (non-functional gene copy) within RAGE genetic variants makes it more difficult to characterize. Prior studies of RAGE in human diseases were done by immunohistochemistry staining of RAGE in human samples. No commercial RAGE ELISA assay is available. Quantification of RAGE by real time PCR were commonly done on cultured cell lines with very little in-vivo relevance. Recent developments have allowed for real time PCR quantification of RAGE in human tissues such as monocytes. See (3).

c. Atherosclerosis and inflammation

Atherosclerosis formerly considered a lipid storage disease actually involves an ongoing inflammatory response. Recent advances have established a fundamental role for inflammation in

mediating all stages of this disease from initiation through progression and, ultimately, the thrombotic complications of atherosclerosis. Clinical studies have shown that this emerging biology of inflammation in atherosclerosis applies directly to human patients. Summarized in review (4). Elevation in markers of inflammation predicts outcomes of patients with acute coronary syndromes, independently of myocardial damage. In addition, low-grade chronic inflammation, as indicated by levels of the inflammatory marker C-reactive protein, prospectively defines risk of atherosclerotic complications, thus adding to prognostic information provided by traditional risk factors. Summarized in review (5).

This proposal is a pilot study evaluating RAGE expression in monocytes from human diabetics and non-diabetics and its relation with inflammatory markers of atherosclerosis.

B. Study Design and Statistical Analysis

This study will be a case-control study of RAGE expression and its relation with inflammatory markers of atherosclerosis in human diabetics and non-diabetics.

a. Outcomes

The primary outcome will be RAGE expression measured quantitatively by real time PCR from RNA extracted from peripheral blood monocytes. The secondary outcome will be the correlation of RAGE levels to atherosclerotic inflammatory marker levels.

This case-control study requires a total of 60 subjects with 30 subjects in each arm. This allows for an 80% likelihood of detecting a difference ($\beta = 0.80$) in RAGE expression between cases and controls when $\alpha = 0.05$.

C. Study Procedure

a. RAGE expression

This assay will be performed on a one time collection of blood sample obtained by venipuncture. In brief, 10cc of blood samples will be collected in tubes containing EDTA. The tubes will be spun at 3000g at 4°C for 20 minutes. The plasma will be divided equally into six 1.5-mL Eppendorf tubes and then frozen and stored at -70°C. Total RNA will be extracted from the peripheral blood monocytes and real time PCR performed as described elsewhere (6). Assays will be performed blinded as to the diabetic status of the subjects.

b. Inflammatory markers

These markers will be measured at the ICCR core laboratory. In brief, the inflammatory marker levels will be measured in batched samples with the use of enzyme-linked immunosorbent assay (ELISA) utilizing monoclonal antibodies to C-reactive protein (CRP), Lp (a) lipoprotein, soluble intercellular adhesion molecule type 1 (sICAM), soluble vascular intercellular adhesion molecule type 1 (sVCAM-1), soluble E-selectin, interleukin-6 (IL-6) and tumor necrosis factor-alpha (TNF- α) as described elsewhere. Total cholesterol, HDL cholesterol, directly obtained LDL cholesterol and homocysteine levels will be measured on an analyzer. Assays will be performed blinded as to the diabetic status of the subjects.

The likely duration of the study is approximately 1-2 years, depending on the number of appropriate subjects and the rate of recruitment into the study.

D. Study Drugs

No drugs are being studied.

E. Medical Device

No devices are being studied.

F. Study Questionnaires

No questionnaires are currently planned for this study.

G. Study Subjects

a. Cases

Diabetic patients with clinical diagnosis of Type II diabetes who are receiving standard of care but who have less than optimal control of diabetes with a glycosylated hemoglobin (HbA1c>9.0) in the last 6 months. Diabetic patients will be excluded if any signs of overt infection assessed clinically are seen.

b. Controls

Non-diabetic patients defined as fasting (8 hour no caloric intake) plasma glucose<126 mg/dl (7.0 mmol/L) in the last 6 months, will be matched to cases on the basis of age, sex, ethnicity, BMI and smoking status (current, former, non-smoker). Non-diabetic patients will be excluded if any signs of overt infection assessed clinically are seen.

H. Recruitment of Subjects

Potential subjects (both cases and controls) will be identified through contact with housestaff and faculty and through chart review with permission of primary physician.

The patient will not be included in the study unless that patient's primary physician is in agreement and discusses participation before study investigators approach the patient.

The patient must have the capacity to sign informed consent, HIPPA compliant patient authorization and release of medical information forms

Assessment of potential subjects will be conducted in English or Spanish depending on the primary language of the subject. Potential subjects who meet the appropriate inclusion and exclusion criteria will be invited to participate in this study. The study rationale and risk/benefits will be explained to the patient in the appropriate language. Study participation will be entirely voluntary and no monetary compensation will be given.

I. Confidentiality of Study Data

Study data will be coded and stored in a secure location in accordance with IRB regulations.

J. Potential Conflict of Interest

None known

K. Location of the Study

This study will take place in outpatient clinical areas (AIM clinic and Naomi Berrie Diabetes Center) at CPMC.

L. Potential Risks

There will be minimal risk from single collection of 10cc of blood samples by venipuncture. There may be pain and bruising at the site of venipuncture for a couple of days

M. Potential Benefits

There are no likely benefits to the subjects in this study.

a. Scientific benefits

There is a possibility that this study would enhance our understanding of in-vitro RAGE expression in human diabetics and may contribute to further characterization of RAGE in human diseases.

N. Alternative Therapies

This is not a therapeutic trial, and alternative therapies for diabetes and atherosclerosis will not be discussed with subjects by study investigators. There are no alternate diagnostic tests in clinical practice and will not be offered to study subjects.

O. Compensation to Subjects

No compensation will be offered to patients.

P. Costs to Subjects

No additional costs are foreseen for subjects in this study.

Q. Minors as Research Subjects

No minors will participate in this study.

R. Radiation or Radioactive Substances

This study will not expose any subject to radiation or radioactive substances.

S. References

- 1) J Beckman, M Creager, P Libby. Diabetes and atherosclerosis. *Journal of the American Medical Association* 2002; 287: 2570-2581.
- 2) A Schmidt et al. The multiligand receptor RAGE as a progression factor amplifying immune and inflammatory responses. *Journal of Clinical Investigation* 2001; 108: 949-955.
- 3) B Hudson et al. Blockade of receptor for advanced glycation endproducts: a new target for therapeutic intervention in diabetic complications and inflammatory disorders. *Archives of Biochemistry and Biophysics* 2003; 419: 80-88.
- 4) P Libby, P Ridker, A Maseri. Inflammation and atherosclerosis. *Circulation* 2002; 105: 1135-1143.
- 5) G Blake, P Ridker. C-reactive protein and other inflammatory risk markers in acute coronary syndrome. *Journal of the American College of Cardiology* 2003; 41: 37S-42S.
- 6) B Hudson et al. Characterization of allelic and nucleotide variation between the RAGE gene on chromosome 6 and a homologous pseudogene sequence to its 5' regulatory region on chromosome 3. *Diabetes* 2001; 50: 2646-2651.