The Journal of Emergency Medicine, Vol. 19, No. 4, pp. 311–315, 2000

0736-4679/00 $–see front matter

PII S0736-4679(00)00255-9

Original Contributions

A NOVEL ASSAY FOR COBALT-ALBUMIN BINDING AND ITS POTENTIAL AS A MARKER FOR MYOCARDIAL ISCHEMIA—A PRELIMINARY REPORT

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□ **Abstract—We initially observed a phenomenon of reduced in vitro binding of exogenous cobalt [Co(II)] to the N-terminus of human serum albumin (HSA) in emergency chest pain patients with early onset unstable angina and myocardial infarction. We then developed a colorimetric assay to measure cobalt-HSA binding and record the results in absorbance units (ABSU). In a preliminary clinical study of 139 emergency patients with acute chest pain, 99 patients with evidence of myocardial ischemia (Group 1) had elevated assay levels (mean ABSU ± SD; 0.519 ± 0.086) compared to 40 patients (Group 2) with no evidence of ischemia (0.316 ± 0.092) ($p < 0.00001$). In Group 1, 95 of 99 (96.0%) patients had levels higher than a decision threshold of 0.400 ABSU and in Group 2, 37 of 40 (92.5%) samples had higher cobalt binding capacity (ABSU ≤ 0.400). Further studies are warranted to determine if an assay measuring altered cobalt-HSA binding is a clinically useful diagnostic test to rule out myocardial ischemia.**

□ **Keywords—myocardial ischemia; chest pain; cobalt; human serum albumin; diagnostic assay**

INTRODUCTION

The diagnosis of acute myocardial ischemia in emergency patients with acute coronary symptoms is often difficult due to an unclear clinical presentation and lack of a rapid, reliable diagnostic test. Resting electrocardiogram (EKG), creatine phosphokinase isoenzymes (CK-MB), myoglobin, and troponin, all of which assist in making the diagnosis of myocardial infarction, have proven unreliable for detecting acute myocardial ischemia (1–3). To be of any clinical value, currently available myocardial infarction markers must be repeated serially, at least 2 to 6 h after the onset of symptoms, to increase the likelihood of signaling an earlier ischemic event (4,5). Consequently, the minimum hospital observation time required to rule out myocardial ischemia is usually 12 to 24 h and occasionally takes up to 3 days. Other tests may include serial EKGs, continuous cardiac monitoring (sometimes including real-time ST segment evaluations), and more definitive tests such as exercise treadmill, stress echocardiogram, radionucleotide stress imaging, or coronary angiography (6,7). Numerous reports indicate that half or more of chest pain patients hospitalized for cardiac testing do not have any evidence of acute myocardial ischemia or acute coronary syndromes (1,4). A rapid blood test that could rule out the presence of acute myocardial ischemia would dramatically improve the triage process of patients with acute coronary symptoms, eliminate many prolonged patient observation times, and reduce health care costs.

Although transitional metal binding to albumin is well known, alterations of human serum albumin (HSA) metal binding sites by ischemic events have not been extensively reported. HSA is a peptide consisting of 585 amino acids (66,500 Da) with a unique amino acid se-

Original Contributions is coordinated by *John A. Marx*, MD, of Carolinas Medical Center, Charlotte, North Carolina

RECEIVED: 4 April 2000; FINAL SUBMISSION RECEIVED: 9 June 2000;
ACCEPTED: 21 June 2000

quence, which may be specific to humans, at its amino terminus ("N-terminus") (8). Previous studies have shown the N-terminus of HSA to be the primary binding site for the transitional metals Co(II), Cu(II), and Ni(II) (9). The HSA metal binding site is also particularly susceptible to biochemical degradation compared with albumin from other species (10).

While specifically looking for biochemical diagnostic markers from emergency patients' serum, one author (D.B–O.) initially observed reduced in vitro binding of exogenous cobalt [Co(II)] to human serum albumin (HSA) in serum from several patients with acute coronary syndrome (unstable angina or very early myocardial infarction) (11). Based on that early observation, we postulated that significant molecular changes to or loss of portions of the N-terminus of HSA could reduce the in vivo transitional metal binding capacity of HSA either during myocardial ischemia or during reperfusion immediately after the ischemic event.

Prior reports indicate that the mechanisms involved in ischemia/reperfusion-induced in vivo changes to albumin may include exposure to endothelial and extracellular hypoxia, acidosis, free radical damage, membrane energy-dependent sodium and calcium pump disruptions, and free iron and copper ion exposure (12–14). Conditions necessary for altering the metal binding site of HSA are known to occur in vivo and probably occur within minutes after the onset of myocardial ischemia (13,15). An assay based on changes in circulating HSA might demonstrate abnormal results minutes or hours before abnormal serum levels of myocardial infarction markers can be detected.

In this preliminary report, we describe a rapid colorimetric assay method measuring ischemia-induced alterations of the binding capacity of HSA to exogenous cobalt. Additionally, we report the results of a preliminary study testing the new assay on serum samples from emergency patients complaining of chest pain, including patients experiencing myocardial ischemia and acute coronary syndromes.

MATERIALS AND METHODS

Patients were enrolled prospectively in this study from Swedish Medical Center, Englewood, Colorado, and Denver Health Medical Center, Denver, Colorado. Institutional Review Board approval and informed consent were obtained for all patients. All blood samples were collected at the time the patient first presented and within 4 h following the onset of acute symptoms. After obtaining blood samples in plain tubes containing no preservatives or separation gels, the sample was allowed to clot for 30 to 90 min and centrifuged before separating the serum. Serum samples were refrigerated at 4°C and analyzed in the same laboratory within 24 h.

A rapid, colorimetric assay was developed to screen human serum samples for decreased cobalt binding to albumin. The assay method involved adding 50 μL of 0.1% cobalt chloride (Sigma, $CoCl_2.6H_2O$) in H_2O to 200 μL of serum, gently mixing, and waiting 10 min for adequate cobalt-albumin binding. Fifty microliters of dithiothreitol (DTT) (Sigma, 1.5 mg/ml H_2O) was added as a colorizing agent and the reaction was quenched 2 min later by adding 1.0 mL of 0.9% NaCl. Using a spectrophotometer at 470 nm (Shimadzu, model UV160U), color development with DTT was compared to a serum-cobalt blank without DTT and reported in absorbance units (ABSU).

The colorimetric assay format quantitatively measures unbound cobalt remaining after cobalt-albumin binding has occurred. Thus, with reduced cobalt-albumin binding, there is more free, unbound cobalt detected, resulting in elevated assay levels. We verified the colorimetric assay method and binding of cobalt to albumin in experiments using radioactive ^{57}Co (unpublished observations). For purposes of this initial study, values greater than 0.400 absorbance units (ABSU) were considered positive for ischemia (lower cobalt binding) and values equal to or lower than 0.400 ABSU were considered negative for ischemia (higher cobalt binding). Two investigators (D.B-O, E.L.) performed all of the colorimetric cobalt-HSA assays.

A preliminary, prospective study was designed to select serum samples from two distinct human populations: Emergency Department (ED) patients with acute chest pain and evidence of early myocardial ischemia (Group 1), including unstable angina and acute myocardial infarction (AMI), and a control group (Group 2) consisting of ED patients with acute chest pain but no subsequent evidence of myocardial ischemia.

Emergency patients were included in the preliminary clinical study if the history of non-traumatic acute chest pain, starting within the immediate 4 h before ED admission, suggested myocardial ischemia or an acute coronary syndrome in the opinion of the Emergency Physician (EP). Patient selection was based on the clinical impression of the attending EP and included varying descriptions of chest pain characteristics. Diagnosis and treatment of all patients with chest pain was determined by attending physicians unaware of the results of the cobalt-albumin assay.

Group 1 included patients with objective evidence of myocardial ischemia including acute coronary syndromes such as unstable angina and AMI. Medical records were reviewed for at least one of the following: 1) a discharge diagnosis consistent with acute myocardial ischemia; 2) ischemia related complications (ven-

tricular fibrillation, sustained ventricular tachycardia [>20 beats], conduction disturbances, symptomatic bradycardia, heart failure, recurrent chest pain requiring treatment with nitroglycerin, pulseless electrical activity, or cardiac arrest); 3) ischemia related interventions (including use of i.v. anti-dysrhythmic medications or pressor support, pacemaker, defibrillator, ventilator support, angioplasty, or coronary artery bypass grafting); or 4) cardiac test reports (exercise treadmill, resting or stress radionucleotide cardiac imaging, stress echocardiogram, or coronary angiography suggesting the presence of acute myocardial ischemia).

Group 1 also included patients with AMI with at least one of the following criteria documented by the medical record or the attending physician: 1) characteristic evolution of elevated blood CK-MB or troponin levels; 2) development of new EKG Q waves (>0.04 s) with ≥ 25% decreased amplitude of the following R wave; 3) sudden death within 72 h of the initial visit with symptoms suggestive of myocardial ischemia. Patients with unstable angina were also included in Group 1 if they had all of the following criteria: 1) chest pain occurring at rest or with minimal exercise; or chest pain lasting longer than 20 min; 2) CK-MB or troponin level less than twice the upper limit of normal during the first 24 h after admission; 3) EKG changes compatible with ischemia. Additionally, patients with "probable unstable angina" were included if they met the first two criteria for unstable angina but did not have characteristic EKG changes of ischemia, yet had relief of chest pain with nitroglycerin within 30 min, or were found to have abnormalities suggestive of myocardial ischemia on subsequent cardiac testing (exercise treadmill, resting or stress radionucleotide cardiac imaging, stress echocardiogram, or coronary angiography).

Group 2 consisted of patients presenting with acute chest pain and a subsequent clinical diagnosis by the attending physician of (or comparable to) "non-cardiac chest pain" with no objective evidence of myocardial ischemia, unstable angina, or myocardial infarction.

All serum samples from patients and volunteers enrolled in the preliminary clinical study were screened using the colorimetric cobalt-HSA binding assay. Diagnostic assay data were reported as mean ± standard deviation (SD). Differences between study patient groups were compared with Student's *t* test. Statistical significance was established at the .05 confidence level.

RESULTS

There were 139 ED patients enrolled in the preliminary study. Group 1 comprised a total of 99 patients meeting diagnostic criteria for evidence of myocardial ischemia and included 58 males (mean age 54.8 ± 13.1) and 41 females (mean age 58.7 ± 15.7). Group 2 comprised a total of 40 patients without evidence of myocardial ischemia and included 30 males (mean age 49.0 ± 13.5) and 10 females (mean age 39.6 ± 8.6).

Serum samples from Group 1 ($n = 99$) had elevated cobalt-HSA assay levels (0.519 ABSU ± 0.086 SD) compared to Group 2 ($n = 40$) samples (0.316 ABSU ± 0.092 SD) ($p < 0.00001$). In Group 1, 95 out of 99 (96.0%) had assay levels > 0.400 ABSU, indicating reduced cobalt binding. In Group 2, 37 out of 40 samples (92.5%) had higher cobalt binding capacity (ABSU ≤ 0.400) (Figure 1).

DISCUSSION

The absence of a dependable blood test for myocardial ischemia makes accurate and safe diagnosis of ED patients with chest pain difficult and expensive. The EKG is rarely abnormal following transient myocardial ischemia, and the ED EKG is normal in approximately half of the patients with acute coronary syndromes (1). Currently available myocardial infarction markers, such as CK-MB, myoglobin, and troponin, appear to be released from intracellular myocyte sources only after irreversible cellular damage and disruption of cell membrane integrity. Shorter and less significant episodes of ischemia do not consistently result in elevated blood levels of these myocardial infarction markers (2,3). In those cases when abnormal myocardial infarction marker results are reported following myocardial ischemia, there is usually a delay of several hours after the onset of symptoms before abnormal levels can be detected. Although several alternatives to prolonged hospital observation or expensive cardiac testing procedures have been proposed, the search for a rapid, reliable blood test for myocardial ischemia continues (4,5,6). This brief report is among the first to suggest that myocardial ischemia may alter the metal binding capacity of circulating serum albumin and to propose a new blood assay based on that mechanism.

Strong binding of transitional metals such as cobalt, copper, and nickel to the N-terminus of human albumin has been previously reported (10,11). Conditions such as acidosis and heat have been reported to affect metal binding to HSA, but there have been few reports regarding ischemic effects on albumin-metal binding sites (11). Reperfusion after an ischemic event may cause as much or more damage to serum albumin and the surrounding tissue as ischemia itself. Biochemical mechanisms involved in the in vivo alterations to metal-albumin binding during either ischemia or reperfusion may include hypoxia, acidosis, free radical damage, membrane energy-dependent sodium and calcium pump disruptions, and

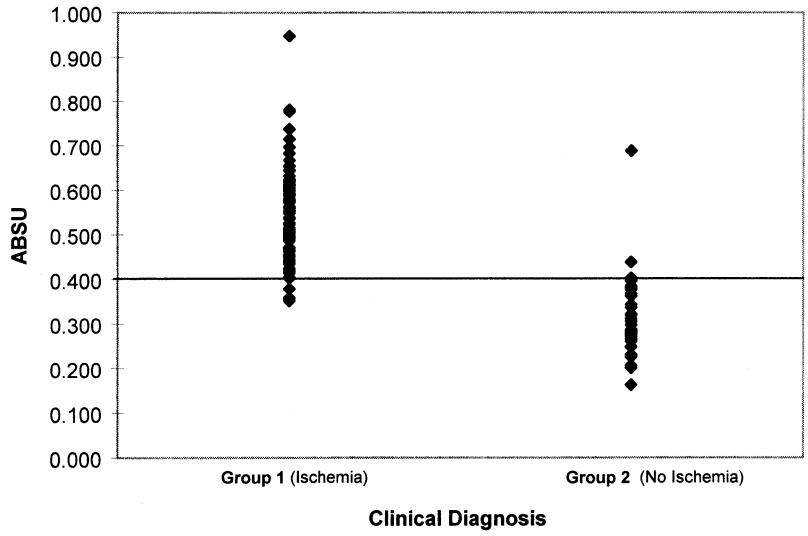

Figure 1. Scatter plot showing cobalt-HSA binding absorbance (ABSU) levels for emergency patients with ischemia (Group 1) and no evidence of ischemia (Group 2). The 0.400 ABSU diagnostic threshold is shown by the horizontal line.

free iron and copper ion exposure (12–14). Most of these conditions occur in vivo within minutes after the onset of acute myocardial ischemia (13,15).

After the initial observation of reduced cobalt binding in patients with acute coronary syndrome, our goal was to develop a rapid assay method that eventually could be used in clinical laboratories. The colorimetric assay method reported here involves adding a predetermined amount of exogenous cobalt to patient sera and indirectly detecting cobalt-HSA binding capacity by measuring the remaining unbound cobalt. In this current format, the assay does not directly measure the ischemia-altered HSA. Future assay formats, including immunoassay, may enhance the test by directly measuring the amount of HSA having an ischemia-altered N-terminus.

Results of the preliminary clinical study in this report indicate that selected emergency patients with evidence of myocardial ischemia and acute coronary syndrome have reduced cobalt binding to HSA when measured by the colorimetric assay method. These initial results are promising but the study has several important limitations. First, patients in the study group were somewhat older than patients in the control group. Second, patients in this preliminary study had a variety of cardiac diagnostic procedures with no consistent "gold standard" test for myocardial ischemia and final results relied primarily upon the attending physician's clinical diagnosis. More consistent testing of the group of patients discharged without evidence of ischemia might have identified previously undetected disease. Additionally, including both unstable angina and AMI patients into the myocardial ischemia group may have affected the overall results and precluded any final distinction between transient ischemia and myocyte necrosis. Sampling patient serum for the cobalt-albumin assay only upon arrival in the ED does not take into account the specific elapsed time from the onset of symptoms or kinetics of quantitative assay changes over time. This study did not determine how long after the ischemic event any cobalt-binding changes are detected by the assay. Although the study included only ED patients with chest pain, other non-cardiac sources of ischemia could not be conclusively excluded and, theoretically, the assay may not be specific for cardiac ischemia alone.

This preliminary clinical study was intended as an initial evaluation of the cobalt-HSA assay's ability to rule out myocardial ischemia in an ED setting. The study's inherent design limitations preclude an accurate estimation of sensitivity, specificity, and positive or negative predictive value. There was no attempt to test the assay's ability to distinguish acute myocardial ischemia from acute coronary syndromes that include unstable angina and AMI. Most acute coronary syndromes probably involve varying degrees of intermittent regional ischemia and thereby distort distinctions between these diagnoses. Larger clinical studies with strict inclusion criteria for myocardial ischemia alone will be needed to compare the assay against specific diagnostic parameters for myocardial ischemia and to measure quantitative assay changes as a function of time of onset, duration and magnitude of the ischemic event, and specific cardiac conditions such as vasospasm and drug toxicity (e.g., cocaine).

Despite the limitations of this preliminary study, we

conclude that the phenomenon of altered cobalt binding by the N-terminus of HSA may prove to be an early biochemical marker of ischemia. Further confirmatory investigations will be necessary to determine if an assay based on these observations has clinical utility as a diagnostic test to rule out myocardial ischemia.

Acknowledgments—Funding was provided by DMI BioSciences, Inc. (Englewood, CO, USA) and Ischemia Technologies, Inc. (Denver, CO, USA). Each of the authors, D.B-O., E.L., J.V.W., is employed by DMI BioSciences, Inc. and owns stock in DMI BioSciences, Inc. which in turn owns stock in Ischemia Technologies, Inc. D.B-O. is a director of Ischemia Technologies, Inc. and DMI BioSciences, Inc. and is named in two international patents for the cobalt-albumin ischemia test currently assigned to Ischemia Technologies, Inc.

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