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RESEARCH ARTICLE

Insignificant role of the N-terminal cobalt-binding site of albumin in the assessment of acute coronary syndrome: discrepancy between the albumin cobalt-binding assay and N-terminal-targeted immunoassay

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Abstract
The aim of this study was to evaluate whether the N-terminus of human serum albumin (HSA) has a role in the cobalt binding detected using albumin cobalt-binding (ACB) assay. We compared the results obtained using an enzyme-linked immunosorbent assay (ELISA) for N-terminal-modified HSA with those of a conventional ACB assay in two groups: acute coronary syndrome ($n = 43$) and non-ischemic chest pain ($n = 39$). ACB and cardiac troponin-I levels were higher in the acute coronary syndrome group. No significant correlation between ACB assay and ELISA results was observed in either of the two patient groups. In acute chest pain patients, the N-terminal site of HSA has a negligible or limited role in cobalt binding in the ACB assay.

Keywords: Ischemia-modified albumin, enzyme-linked immunosorbent assay, myocardial ischemia, cobalt-binding assay

Introduction

Acute coronary syndrome (ACS) refers to a spectrum of clinical presentations from myocardial ischemia to myocardial infarction caused by rupture or erosion of atherosclerotic plaques and partial or complete thrombosis of the infarct-related coronary artery or arteries (Rioufol et al. 2002, Anderson et al. 2007). According to criteria of myocardial infarction updated in 2007 by the Joint European Society of Cardiology/American College of Cardiology Foundation/American Heart Association/ World Heart Federation (ESC/ACCF/AHA/WHF) Committee, the current diagnosis of myocardial infarction is based on clinical evidence of ischemia revealed by history, electrocardiography, non-invasive or invasive tests, and an increased level of cardiac biomarkers (Thygesen et al. 2007). The cardiac troponins, markers of myocardial cell necrosis, are the preferred biomarkers for detecting myocardial necrosis with high sensitivity (Jaffe et al. 2000). Nonetheless, nearly 50% of patients who present early after the onset of chest pain show undetectable levels of troponins; hence, the diagnostic power of troponins is limited by the time dependence of their appearance. In addition to this time dependence, the level of troponins is usually very low in patients who display an acute myocardial ischemia without necrosis (Morrow et al. 2003). Notwithstanding these limitations, the diagnosis of myocardial infarction is much more straightforward than that of unstable angina due to myocardial ischemia, for which there are no well-known uniform diagnostic criteria or biomarkers. Thus, from a clinical point of view, identification of myocardial ischemia in advance or in the absence of myocardial necrosis requires the development of well-defined biochemical markers.

To date, the only clinical test approved by the Food and Drug Administration for myocardial ischemia is the

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albumin–cobalt binding (ACB) assay, which relies on detecting a decrease in the cobalt-binding capacity of ischemia modified albumin (IMA) compared with non-ischemic normal albumin (Bar-Or et al. 2000). It has been thought that the ischemia-driven modification of human serum albumin (HSA) is localized within the NH$_2$-Asp-Ala-His-Lys sequence of HSA. This modification was postulated to be caused by the free radicals produced during ischemia or reperfusion, acidosis, reduced oxygen, and/or cellular electrolyte pump disorders (Bar-Or et al. 2001, Christenson et al. 2001, Bhagavan et al. 2003). IMA is used in conjunction with electrocardiography and troponins to increase diagnostic sensitivity in acute myocardial ischemia or infarction (Peacock et al. 2006, Roy et al. 2004). The high negative predictive value observed in some studies suggested that IMA might be useful as an "elimination" marker in the emergency department (Peacock et al. 2006). Despite the widespread use of the ACB assay, no absolute cutoff value has been established for the diagnosis of myocardial ischemia. In addition, some controversial results have been reported. For example, IMA was shown to yield frequent false positives, resulting in a low specificity that limits the practical significance of the test (Keating 2006, Dominguez-Rodriguez & Abreu-Gonzalez 2010). Moreover, several studies have revealed a weak binding affinity of the N-terminus for cobalt compared to other sites of HSA, strongly implying that the primary binding site for cobalt is not the N-terminal region of HSA (Mothes & Faller 2007, Sokołowska et al. 2009). Thus, an explanation for the low specificity of IMA in the ACB assay requires a greater understanding of the details of cobalt binding to IMA, which remain unclear.

Here, we investigated the role of the N-terminal site of HSA in cobalt binding in an *in vivo* context by comparing the results of the ACB assay with those of an enzyme-linked immunosorbent assay (ELISA) that specifically targets N-terminal-modified HSA. We assessed the correlation and diagnostic characteristics of the two assays in patients with myocardial infarction and unstable angina due to myocardial ischemia.

Methods

Study population
Patients from the Asan Medical Center, Seoul, Korea, were enrolled prospectively in this study. Eighty-two patients presenting to the emergency department with chest pain were divided into the following two groups: (1) the ACS group (*n* = 43; mean age ± standard deviation [SD], 56.8 ± 12.7 years), ultimately diagnosed with acute myocardial infarction or unstable angina (Anderson et al. 2007); and the non-ischemic chest pain (NICP) group (*n* = 39; mean age ± SD, 55.0 ± 16.1 years). The final diagnosis of acute myocardial infarction was based on cardiac troponin-I (cTnI) elevation in conjunction with electrocardiographic and clinical findings, according to the defining criteria of the ESC/ACCF/AHA/WHF Joint Committee (Thygesen et al. 2007). These included the

detection of an increase in the cTnI level above the 99th percentile of the upper reference limit (>1.5 ng mL^{-1}) together with signs indicative of the following: relevant clinical symptoms of ischemia, new ST-segment changes, development of new Q waves in the electrocardiography, and echocardiographic findings of new regional wall motion defects. Unstable angina or non-ST elevation myocardial infarction is defined by ST-segment depression or prominent T-wave inversion, or positive cTnI in the absence of ST-segment elevation in an appropriate clinical setting (chest discomfort or angina equivalent) (Anderson et al. 2007). Patients were classified as NICP if cTnI results were negative on serial sampling, a normal electrocardiography was present, or a reported non-cardiac mechanism was confirmed by invasive coronary study as the cause of pain. We excluded 9 patients with acute or chronic kidney disease, 5 with cerebrovascular diseases, 2 with trauma, 11 with cancer, 6 with chronic heart failure or valvular heart disease, and 3 with evidence of infection upon presentation to the emergency department from the study population. The protocol of this study was approved by the institutional review board of ASAN Medical Center (IRB number: 2010-0410).

Biochemical analysis
All blood samples were obtained with informed consent at the time patients first presented and within 12 hours following the onset of acute symptoms. Serum levels of cTnI were measured using a chemiluminescence immunoassay on an ADVIA (Siemens, Deerfield, IL, USA), and serum albumin levels were measured by the bromocresol green method using a Cobas Mira chemistry analyzer (Roche Diagnostics, Basel, Switzerland). Reference ranges for cTnI and creatinine kinase-MB (CK-MB) were <1.5 and 5 ng mL^{-1}, respectively. C-reactive protein (CRP) was also analyzed at the same time points. For the ACB assay and the ELISA for N-terminal-modified HSA, blood samples were obtained in Vacutainer tubes (BD Diagnostics, Plymouth, UK) without anticoagulants, followed by centrifugation at 1900×g for 15 min at 4°C within 1 hour of collection; aliquots of serum samples were stored at –80°C prior to analysis. Serum IMA was analyzed by a colorimetric ACB assay as described elsewhere (Bar-Or et al. 2000). Briefly, 50 µL of 0.1% cobalt chloride (CoCl$_2$•6H$_2$O; Sigma) in H$_2$O was added to 200 µL of serum, gently mixed, and then incubated for 10 min to allow cobalt–albumin binding. Fifty microliters of dithiothreitol (DTT, 1.5 mg mL^{-1}•H$_2$O; Sigma) was added as a colorizing agent, and the reaction was quenched 2 min later by adding 1.0 mL of a 0.9% NaCl solution. The color development produced by dithiothreitol was measured at 470 nm using a spectrophotometer GeneQuant 1300 (GE Healthcare, England, UK). A serum–cobalt blank without dithiothreitol was used for comparison, and the results were presented in absorbance units (ABSU). The within-series coefficient of variation was 3.1%. A reference level was previously determined by our group from a control reference population of 209 healthy blood donors using a Synchron LX20 system (Beckman Coulter,

High Wycombe, Bucks, UK). Blood samples were handled and analyzed according to the manufacturer's protocols.

Immunoassay for N-terminal-modified HSA

IMA in human serum was quantitatively measured in 96-well plates using two ELISA kits (both made using the same process) that are specific for the N-terminal-modified HSA (USCNLIFE Science & Technology Co. LTD., Wuhan, China, and USCN Life Science Inc., Wuhan, China) according to manufacturers' instructions. Briefly, wells in the plate were pre-coated with a biotin-conjugated polyclonal antibody that specifically targets N-terminal-modified HSA. Initially, the optimal sample dilution was fixed at 50,000:1 by taking into account the standard curve of the 7.8–500 ng mL^{-1} detection range indicated in the manual. Diluted sera were added to the pre-coated microtiter plate wells, and avidin-horseradish peroxidase (HRP) conjugate was added. Following incubation for a predetermined time, a 3,3′,5,5′-tetramethylbenzidine (TMB) solution was added to each well. Significant changes in color were observed in wells that contained patient serum, biotin-conjugated antibody, and avidin-HRP conjugate. Unless otherwise specified, standard washing steps were applied throughout the assay, and all reagents and human serum samples were loaded at 100 μL each per well in 96-well plates. The enzyme reaction was stopped by addition of a sulfuric acid solution, and the color change was measured at 450 nm using an Infinite M200 microplate reader (Tecan, Männedorf, Switzerland). The levels of N-terminal-modified HSA in sera were expressed as concentration (ng mL^{-1}) by reference to a standard curve prepared using serial dilutions of standards supplied with the kits. Each serum sample was tested repeatedly to evaluate within-series coefficient of variation.

Statistical analysis

Student's t-test was used to evaluate the significance of differences between test groups. Pearson's correlation was used to investigate the correlation between the levels of N-terminal-modified HSA obtained by ELISA and IMA levels measured by the ACB assay. A receiver operator characteristic curve was used to quantify the overall ability of the ELISA and the ACB assay to diagnose ACS patients. The sensitivity and specificity of the two tests were determined by ROC curve analysis. A p value <0.05 was considered statistically significant.

Results

We first investigated the basic characteristics of patients from whom blood samples were obtained and analyzed (Table 1). The ACS group exhibited higher serum levels of cTnI than the NICP group ($p < 0.01$), but serum albumin levels were not significantly different between the two groups. The levels of IMA measured by the ACB assay were distinctly higher in patients with ACS than among those in the NICP group ($p < 0.05$). On the other

Table 1. Baseline characteristics of patients.

	NICP group	ACS group (STEMI, NSTEMI, UA)
n	39	43
Male (%)	69.2	88.4
Age (years)	55.0 ± 16.1	56.8 ± 12.7
Diabetes mellitus	8	6
Hypertension	18	21
Hypercholesterolemia	3	5
Current smoking	4	13*
Chest pain		
Pain within 3 hours	22	22 (16, 3, 3)
Ongoing pain	17	21 (9, 6, 6)
ECG		
ST segment elevation	0	25
Non-diagnostic/normal	39	18
Albumin (g·L^{-1})	4.00 ± 0.52	3.96 ± 0.47
cTnI (ng·mL^{-1})	0.05 ± 0.03	3.11 ± 7.01** (3.34 ± 8.37, 5.46 ± 5.61*, 0.11 ± 0.16)
ACB (ABSU)	0.39 ± 0.18	0.48 ± 0.16*
IMA-ELISA (ng·mL^{-1})	20.42 ± 5.64	20.20 ± 7.77

Data are expressed as means ± SDs.
*$p < 0.05$, **$p < 0.01$.
ACS, acute coronary syndrome; cTnI, cardiac troponin I; ACB, albumin cobalt binding test; ABSU, absorbance units; IMA, ischemia modified albumin; ELISA, enzyme-linked immunosorbent assay; NICP, non-ischemic chest pain; Non-STEMI, non ST segment elevation myocardial infarction; STEMI, ST segment elevation myocardial infarction; UA, unstable angina.

hand, there was no difference in the levels of N-terminal-modified HSA measured by ELISA between the ACS and NICP groups ($p > 0.05$). We used an albumin-adjusted correction to evaluate the influence of albumin levels on the ACB assay, as described elsewhere (Lippi et al. 2007). After correction, differences between ABSU values in the ACS group (0.43 ± 0.17) and NICP group (0.35 ± 0.20) remained significant ($p < 0.05$).

In an effort to gain insight into whether the N-terminal region of HSA is closely linked with cobalt binding, we examined the correlation between the levels of IMA in individual blood samples in the two groups measured by the ACB assay and ELISA. For this purpose, we used commercially available ELISA kits that are specific for the N-terminal-modified HSA. As shown in Figure 1a, there was a negative correlation between the levels of IMA determined by the ACB assay and the levels measured by the ELISA for the ACS group ($r = -0.429$; $p < 0.01$). In the case of the NICP group, no correlation was observed between the levels of IMA measured by the two methods ($r = -0.219$; $p > 0.05$). In all patients, the levels of the N-terminal-modified HSA measured by ELISA were not significantly correlated with the ABSU values of the ACB assay. Intra-assay and inter-assay coefficients of variation were 3.1% and 3.0%, respectively, for the ACB assay; the corresponding values for the ELISA were 3.7% and 5.6%.
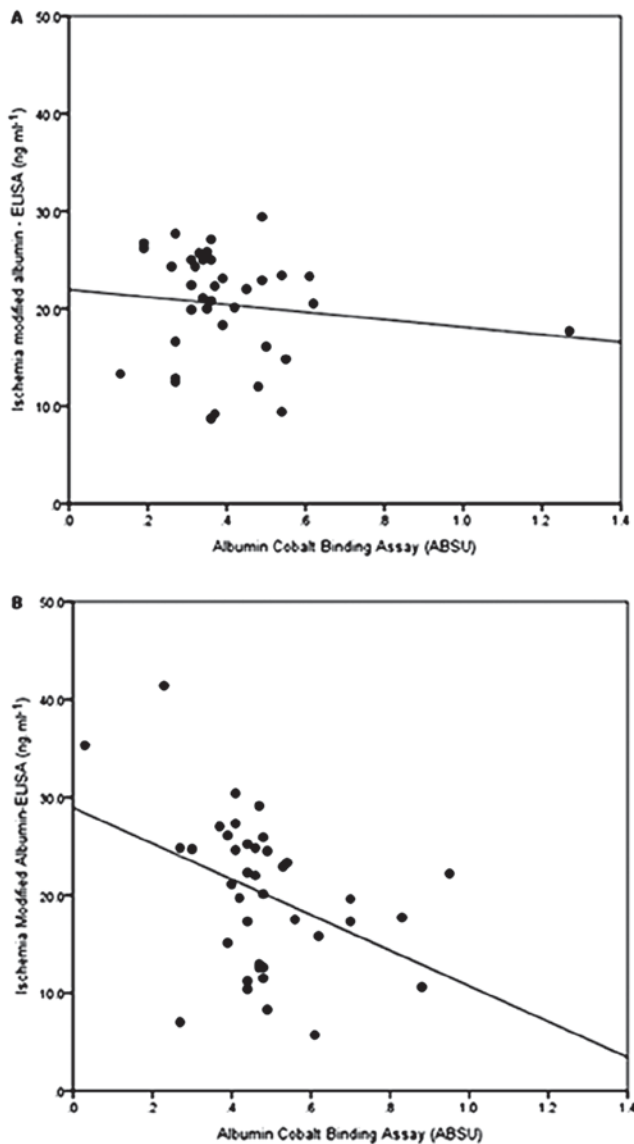
Figure 1. Correlations between IMA measured by ELISA (N-terminal-modified HSA levels) and ACB assay (ABSU values). (A) NICP group ($r=-0.219$; $p > 0.05$); (B) ACS group ($r=-0.429$; $p<0.01$). The levels of N-terminal-modified HSA measured by ELISA are expressed as nanograms per milliliter, and ACB assay results are expressed as absorbance units (ABSU).
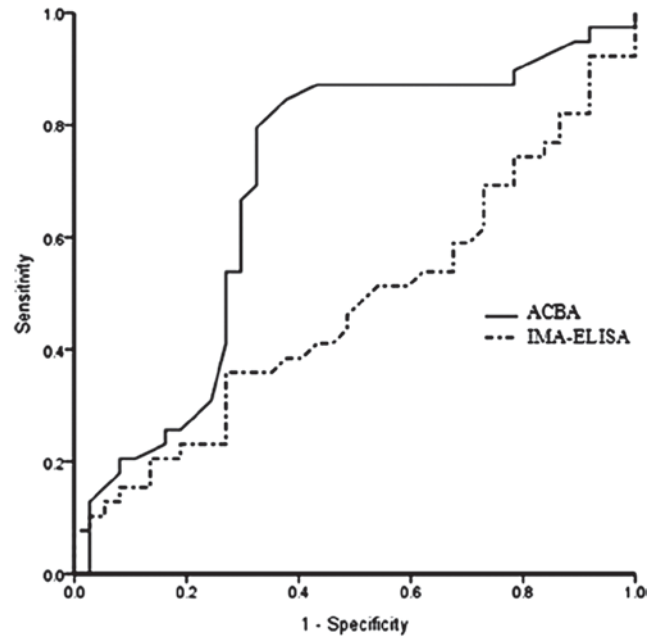

Figure 2. ROC curves of the ACB assay and ELISA for the diagnosis of myocardial ischemia. Areas under the curve for ACB assay and ELISA were 0.713 (95% CI, 0.595–0.832; $p=0.001$) and 0.476 (95% CI, 0.345–0.608, $p=0.724$), respectively.

A ROC curve analysis was employed to quantify the overall ability of IMA, measured as N-terminal-modified HSA (ELISA) and ABSU (ACB assay) levels, to discriminate individuals with ACS from those without myocardial ischemia. Areas under the curves for the ELISA and the ACB assay were 0.476 (95% confidence interval [CI], 0.345–0.608; $p > 0.05$) and 0.713 (95% CI, 0.595–0.832; $p < 0.01$), respectively (Figure 2). Thus, these results indicate that only the ACB assay has the ability to discriminate myocardial ischemia. Over the range of ABSU levels >0.4 (limit of detection), the sensitivity and specificity of the ACB assay for assessing myocardial ischemia were estimated to be 81.4% (95% CI, 48.7–85.6%) and 69.2% (95% CI, 34.7–75.9%), respectively. The negative and positive predictive values were 77.1% and 74.5%, respectively.

To address the time factor aspect in IMA, measured as N-terminal-modified HSA levels (ELISA) and ABSU values (ACB assay), we selected patients with non-diagnostic electrocardiography and compared the two groups according to chest pain duration, except for 16 patients with STEMI, and 57 patients who showed non-diagnostic or normal electrocardiography: Among 28 patients who arrived within 3 hours after chest pain onset, the median ABSU values in the ACB assay were not different between ACS (ABSU, 0.565; 95% CI, 0.251–0.729) and NICP (ABSU, 0.360; 95% CI, 0.313–0.502; $p=0.414$) groups (Figure 3a). Among 29 patients who had ongoing chest pain for more than 3 hours, the median ABSU value in the ACB assay was significantly higher in the ACS group (ABSU, 0.465; 95% CI, 0.410–0.608) than in the NICP group (ABSU, 0.360; 95% CI, 0.309–0.448; $p=0.023$; Figure 3b). Similar results were obtained for N-terminal-modified HSA levels measured by ELISA. Among patients who arrived within 3 hours after chest pain onset, median N-terminal-modified HSA levels were not significantly different between the NICP (20.650 ng mL^{-1}; 95% CI, 16.430–22.663) and ACS (20.400 ng mL^{-1}; 95% CI, 8.938–37.012; $p=0.380$) groups (Figure 3c), but were different between patients of the NICP and ACS groups who suffered from ongoing chest pain for more than 3 hours (Figure 3d). In this latter group of patients, the median serum level of N-terminal-modified was measured by ELISA was 22.300 ng mL^{-1} (95% CI, 19.327–23.556) in the NICP and 15.100 ng mL^{-1} (95% CI, 11.659–20.777) in the ACS group ($p=0.037$). Thus, among patients with chest pain for less than 3 hours from last onset, the N-terminal site of HSA did not appear to play any role in differentiating acute coronary syndrome from non-ischemic chest
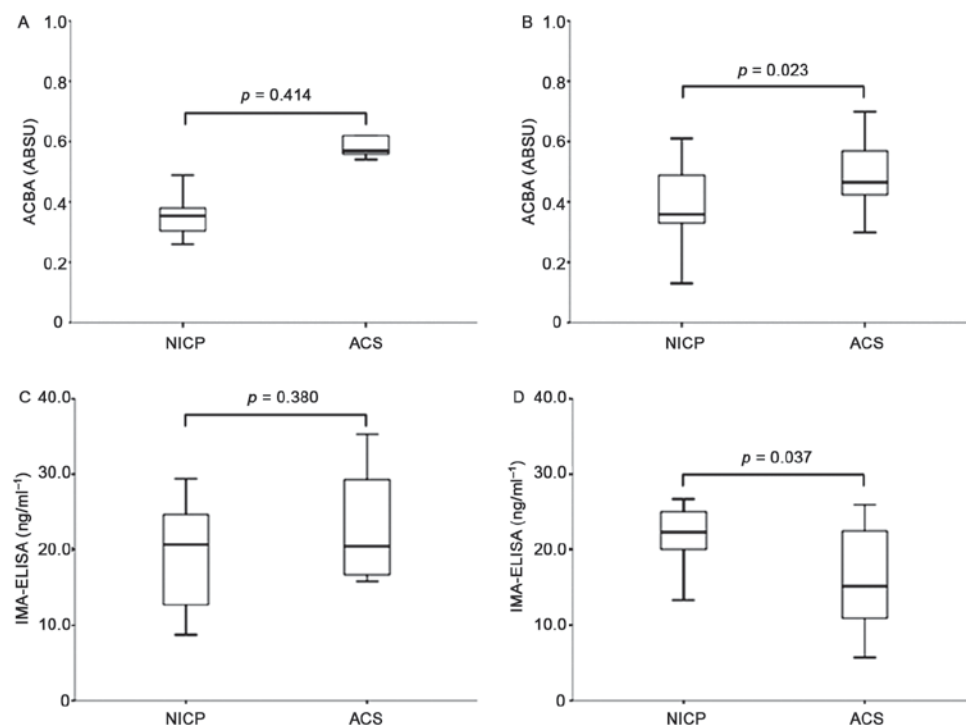
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Figure 3. Comparisons of serum N-terminal-modified HSA levels (ELISA) and ABSU values (ACB assay) between the NICP and ACS groups in patients with non-diagnostic electrocardiography. (A) Comparison of ABSU levels measured by ACB assay in patients who arrived within 3 hours after chest pain onset ($n=28$). (B) Comparison of ABSU levels measured by ACB assay in patients with on-going chest pain ($n=29$). (C) Comparison of the levels of N-terminal-modified HSA measured by ELISA in patients who arrived within 3 hours after chest pain onset ($n=28$). (D) Comparison of the levels of N-terminal-modified HSA measured by ELISA in patients with on-going chest pain ($n=29$). Box plot shows numerical data through their five-number summaries: the smallest observation, lower quartile, median, upper quartile, and largest observation.

pain. There was no significant difference in the levels of serum albumin between the groups ($p > 0.05$).

Discussion

ACS represents a range of clinical symptoms from myocardial ischemia to myocardial infarction due to partial or complete thrombosis of the infarct-related coronary artery or arteries. Accordingly, all patients presenting to the emergency department with ACS should be considered high-priority and diagnosed as early as possible in order to achieve better myocardial perfusion and myocardial salvage by shortening the duration of ischemia (Anderson et al. 2007). The ACB assay is currently used as the primary method for diagnosing myocardial ischemia. The ACB assay is based on a decrease in the cobalt-binding capacity of IMA compared to normal albumin, a decrease thought to reflect deletion of the N-terminal site by reactive oxygen under ischemic conditions species (Bar-Or et al. 2000). Experimentally, HSA has been shown to have a variety of metal-binding sites with different specificities (Sbarouni et al. 2006, Cho et al. 2007, Duarte et al. 2009). Four different sites for the binding of transition metals have been proposed, including A and B sites, the N-terminal site, and Cys34. Of these, the N-terminal site consisting of Asp1-Ala2-His3 has been linked to the binding of transition metals such as copper, and was also

suggested to be a primary binding site for cobalt in the ACB assay (Bar-Or et al. 2001). In the ACB assay, cobalt is exogenously added at a ratio of 1.5 equivalents per HSA; the first two equivalents bind to sites A and B, and only the third may bind to the N-terminal site (Mothes & Faller 2007). This suggests that the N-terminal site of HSA is not a primary binding site for cobalt in the ACB assay. An N-terminal amino acid sequence analysis showed a wild-type N-terminal sequence in six of seven high-level ACB assay patients (Bhagavan et al. 2003). Furthermore, many factors have been implicated in cobalt binding to HSA, and direct evidence for the cobalt-binding site has yet to be provided.

In an effort to gain some insight into the role of the N-terminal site of HSA in the cobalt-binding assay, we analyzed IMA in the sera of patients by two different methods: namely, the cobalt-binding-based ACB assay, described above, and a commercially available ELISA specific for N-terminal-modified HSA. If the N-terminal site of HSA is responsible for the cobalt binding measured in the ACB assay, as presumed, a close relationship between the two assays would be predicted. Unexpectedly, however, we found no positive correlation between the ACB assay and the ELISA for either ACS or NICP group. The statistical power was estimated to be 5% at given alpha level of 0.05. This low value could be an influencing factor in statistically non-significance between two groups.

In fact, a negative correlation between the two methods was observed for the ACS group. These results strongly suggest that the N-terminal site of HSA has a limited or insignificant role in the ACB assay, supporting the recent observations that exogenous cobalt may not bind to the N-terminal site of HSA (Mothes & Faller 2007, Bar-Or et al. 2008).

Notably, even though its correlation with the ELISA was negligible, the ACB assay, which is used for the diagnosis of ACS in the emergency department, displayed distinct positive signals in patients with ACS, confirming its clinical significance in the diagnosis of ACS. Studies have shown that the level of IMA increases within minutes after the onset of ischemia, remains elevated for 6–12 hours, and returns to a normal level within 24 hours (Sinha et al. 2004, Cho et al. 2007). The ACB assay can reduce the delay for a reliable troponin-negative classification by approximately 6–24 hours when used alone or in combination with markers of necrosis, reducing the inappropriate admission of low-risk patients (Christenson et al. 2001). Thus, the ACB assay offers a valuable method for early detection of ischemia prior to the development of myocardial necrosis. Nonetheless, several studies have reported that IMA has a high negative-predictive value in excluding ACS for patients with symptoms in the emergency department. The ACB assay can predict the consequences of cardiac troponins, but it has a low diagnostic sensitivity during the first few hours following presentation (Antman et al. 1995, Bar-Or et al. 2000). The ACB assay was shown to be affected by many factors, including the proportion of HSA with an intact N-terminus, the level of albumin in serum, the ratio of cysteine to cystine, plasma pH, and the oxidation status of HSA Cys34 reflecting interactions with added cobalt and dithiothreitol (Bhagavan et al. 2003, Bar-Or et al. 2008). Of particular note, the sensitivity of cardiac markers is low in the first 6 hours after the onset of symptoms. Approximately 2–10% of patients with acute myocardial infarction may be inadvertently discharged from emergency departments without proper treatment, resulting in serious health and legal consequences. On the other hand, inappropriate admission of a large number of patients without ACS will substantially increase medical expenses (Puleo et al. 1994). Initial IMA levels were reported as a powerful indicator of short-term mortality in ST segment elevated acute myocardial infarction patients (Dominguez-Rodriguez et al. 2009).

Our results suggest that cobalt binding to the N-terminal site of HSA may be significant in the ACB assay for patients with chest pain for more than 3 hours due to myocardial ischemia or infarction. But, the binding affinity of the N-terminal site of HSA to cobalt may decrease drastically in patients with ACS after 3 hours from the onset of chest pain. It was previously shown that generation of IMA occurs in the initial stage of the development of atheromatous plaques, and may provide an early assessment of overall patient risk (Piwowar et al. 2008, Duarte et al. 2009, Kazanis et al. 2009). The

rapid clearance of N-terminal-truncated albumin from the human body could provide a possible explanation (Bar-Or et al. 2006). In the current study, we found that the levels of the N-terminal site of HSA measured by ELISA increased in patients with chest pain less than 3 hours from last onset, but were not significantly different in the ACS group. Both N-terminal-modified HSA levels (ELISA) and IMA levels measured by ACB assay (ABSU values) trended higher in the ACS group, but did not reach statistical significance. In the late phase of chest pain, HSA levels measured by ELISA were significantly lower in the ACS group. These time-dependent differences in N-terminal-modified HSA levels imply that N-terminal-modified HSA has a confounding effect on the ACB assay in the late phase of chest pain, which could explain the low specificity of the ACB assay. However, to date, there have been no reports on the effects of serial clearance of N-terminal-modified albumin on the ACB assay.

In diabetes mellitus, hyperglycemia, oxidative stress, and increased lactate may induce conditions of chronic hypoxia, stimulating the generation of modified albumin (Piwowar et al. 2008). The levels of IMA have been shown to be high in patients with hypercholesterolemia (Duarte et al. 2009). However, our study revealed no difference in the number of hypercholesterolemia patients or the levels of C-reactive protein between the ACS and NICP groups (0.69 ± 2.29 vs. $1.58 \pm 4.62\,\mathrm{mg\,L^{-1}}$; $p = 0.32$). In patients with documented stable coronary artery disease, IMA was found to be higher than in controls (Kazanis et al. 2009). Our understanding of the significance of IMA as a marker of total atherosclerotic burden is continuing to evolve, and further investigation is required to refine this linkage.

It was recently shown that the diagnostic sensitivity of cardiac troponins for the detection of unstable angina is low or moderate, suggesting that these assays may be of limited use in the diagnosis of unstable angina (Reiter et al. 2011, Hjortshoj S. et al. 2010, Kim et al. 2010). We think that controversial results about the significance of IMA as a reliable standard marker come from the low specificity and sensitivity of the current assay method for IMA in the diagnosis of the ACS. IMA is currently the most reliable biomarker for the detection of myocardial ischemia without necrosis and is not influenced by gender (Sbarouni et al. 2011). The clinical significance of IMA lies in its negative predictive value, namely excluding the presence of ischemia in a population with a low prevalence of coronary artery disease. However, low specificity together with a high false-positive rate could also be a problem in an overcrowded emergency department. Thus, it is important to elucidate the mechanism underlying the high false-positive IMA results. Moreover, the prognostic performance of IMA has not yet been studied in patients with unstable angina and a normal level of troponins. Little is known about the use of IMA in primary prevention or its ability to detect asymptomatic ischemia or hypoxic stress in high-risk cardiovascular patients. Many questions

remain unanswered regarding IMA mechanism of action, cardio-specificity, and kinetics during the first hours after ACS, and the optimal cutoff point of IMA to use in clinical validation. A coronary angiogram was not performed in all enrolled patients, and identification of the exact cause of chest pain is often difficult in clinical practice. A coronary angiogram is not indicated in patients with a low risk of cardiac events. We believe that the selection of diagnostic tools selected in our study, while not controlled, is representative of what used in clinical practice.

Conclusions

In summary, the present study investigated the role of the N-terminal site of HSA in cobalt binding in an *in vivo* context using an ELISA that specifically targets N-terminal-modified HSA. We conclude that the N-terminal site of HSA is likely to play a negligible or limited role in cobalt binding in the ACB assay. However, the time-dependent variation in the levels of N-terminal-modified HSA measured by ELISA indicates that the N-terminal site could exert a confounding effect on the ACB assay in differentiating chest pain patients. Thus, further studies are required to assess time-dependent changes in the specificity of IMA in differentiating acute coronary syndrome and non-ischemic chest pain.

Declaration of interest

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