Prolonged Stability of Endogenous Cardiotrophin-1 in Whole Blood

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Cardiotrophin-1 (CT-1) is a recently identified cytokine of the interleukin-6 (IL-6) family that signals through the gp130 signalling pathway. CT-1 may be of central importance to the pathogenesis of ventricular remodelling in patients with acute myocardial infarction (AMI) and therefore have clinical value in the identification of patients with impaired ventricular function. Central to the clinical use of CT-1 is in the in vitro stability of the peptide. Twelve subjects were recruited. A total of 25 mL of peripheral venous blood was collected into chilled polypropylene tubes containing EDTA and aprotinin and divided into 5 aliquots. One sample was spun in a prerefrigerated centrifuge (4°C) at 3,000 rpm for 10 minutes and plasma separated and frozen at −70°C immediately. Remaining samples were stored for 24 and 48 hours at room temperature or on ice. CT-1 in extracted plasma specimens was measured with a competitive chemiluminescent assay. The concentration of CT-1 in samples stored optimally was 43.1 ± 6.05 fmol/mL. CT-1 levels for storage at room temperature compared with ice at the remaining time points were as follows: 24 hours, 41.5 ± 5.76 *v* 37.5 ± 8.66; and 48 hours, 42.6 ± 6.28 *v* 41.0 ± 5.42 fmol/mL. There were no significant changes in concentrations of CT-1 stored optimally or kept for up to 48 hours in aliquots of whole blood at room temperature or on ice. We conclude that CT-1 is stable in specimens of whole blood treated with EDTA and aprotinin and stored for up to 48 hours at room temperature or on ice, hence permitting its development in the routine clinical investigation of patients with heart failure.

IN THE EARLY PERIOD following an acute myocardial infarction (AMI), a process of infarct expansion[1] has been recognized to occur and represents a process of lengthening of the noncontractile region of the ventricular myocardium, which provides an important homeostatic response to preserve ventricular stroke volume. Dilatation of the infarcted myocardium causes an increase of ventricular wall stress that triggers the synthesis of myofibrillar proteins added together in series.[2] The subsequent increase in wall thickness reduces ventricular strain by distributing excess stress among an increased number of new sarcomeres. However, when the cumulative loss of myocardium is large, the compensatory hypertrophy described is unable to offset the exaggerated wall stress and more progressive and long-term transformations in the shape and size of the left ventricle develop, which encompass the term ventricular remodelling.[1,3] A deleterious cycle of augmented wall stress and insufficient hypertrophy ensues, which promotes further dilatation and, in turn, further increases in wall stress. The end result is a progressive deterioration of ventricular function.

Cardiotrophin-1 (CT-1) is a recently identified member of the interleukin-6 (IL-6) super family of cytokines that signals through heterodimerization of glycoprotein 130 (gp130) with the leukemia inhibitory factor receptor (LIFR).[4] CT-1 was initially identified as a cardiac myocyte hypertrophy-inducing factor, producing increases in myocyte length through the series addition of myocytes,[5] which bears close similarity to the eccentric hypertrophy of volume overload. Furthermore, augmented expression of gp130 has been demonstrated in a rat model of AMI and may play a central role in the pathogenesis of remodelling in humans.[6] In addition, plasma CT-1 is elevated in patients with left ventricular systolic dysfunction (LVSD),[7] and levels are correlated with measures of left ventricular (LV) function. The measurement of CT-1 may therefore potentially have a clinical role in the identification of patients at risk of remodelling following AMI and of those with left ventricular systolic dysfunction. Before the clinical use of this cytokine as a diagnostic aid, it is important to establish its stability and robustness in clinical situations. Accordingly, we measured the stability of CT-1 in samples collected and stored in a variety of ways.

SUBJECTS AND METHODS

Subjects and Experimental Procedures

A total of 12 subjects (8 men, 4 women), median age, 64.5 (range, 21 to 84 years) were enrolled for participation in the study. All subjects provided verbal informed consent for entry into the study, which was approved by the local hospital ethical committee. There was a confirmed diagnosis of AMI in 7 patients (3 anterior, 3 inferior, and 1 posterior), and 3 patients had a diagnosis of unstable angina. The 2 subjects without a cardiovascular history were symptom free, on no medication, and normotensive with no evidence of heart failure. This enabled assessment of CT-1 stability over a broad range of values. Subjects remained in a relaxed supine position 20 minutes before venesection. A total of 25 mL of peripheral venous blood was drawn from the antecubital fossa of each patient and collected into chilled polypropylene tubes containing EDTA 1 mg/mL and aprotinin (Trasylol; Bayers, Newbury, UK) 500 kIU/mL. Blood was divided into 5 equal aliquots. One sample was spun in a prerefrigerated centrifuge (4°C) at 3,000 rpm for 10 minutes and plasma separated and frozen at −70°C immediately. Other blood samples were stored for 24 and 48 hours at room temperature or on ice and plasma separated and stored until extraction and analysis. Average room temperature measured at 9:00 AM and 5:00 PM on each day was 24°C.

Plasma specimens were defrosted at room temperature. A total of 1 mL was acidified with 1 mL of buffer A (1% trifluroacetic acid) and allowed to precipitate for 20 minutes on ice before centrifugation at 3,000 rpm for 30 minutes. C_{18} extraction columns (Peninsula Laboratories, Merseyside, UK) were primed with 1 mL of buffer B (60% acetonitrile and 1% trifluoroacetic acid) followed by 9 mL of buffer A. A total of 1.8 mL of the supernatant was loaded onto the columns. The columns were washed with 6 mL of buffer A before the peptides were eluted with 2 mL of buffer B. Eluates were dried overnight under a

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vacuum and stored at −70°C until assay. Mean recovery of CT-1 on C_{18} columns was 76% ± 3%.

Assay

Details of this assay have been described previously.[7] Briefly, a peptide corresponding to a midsection domain of the CT-1 sequence (CRRQAELNPRAPRLLR) was synthesized in the MRC Toxicology Unit, Leicester University. Peptides dissolved in 0.1 mmol/L phosphate buffer (pH 7) were conjugated to keyhole limpet hemocyanin with the hetero-bifunctional cross-linker EMCS (ε-maleimidocaproic acid N-hydroxysuccinimde ester). Rabbits were injected subcutaneously with 100 μg of the antigens emulsified with Freund's adjuvant and underwent subsequent monthly intravenous injections with 100 μg of the antigens. The antisera produced was serum G187 reactive to amino acids 105-120 of the CT-1 sequence. The IgG fraction of this antiserum was obtained following chromatography using Protein A-sepharose CL4B gel (Pharmacia, Herts, UK).

The CT-1 peptide (105-120) was labelled with the methyl acridinium ester label 4-(2-succinimidyloxycarbonylethyl)phenyl-10-methylacridinium 9-carboxylate fluorosulfonate (kindly donated by Drs Stuart Woodhead and Ian Weeks, Molecular Light Technology Ltd, Cardiff, UK) as previously described.[7]

All extracted samples were analyzed within the same assay. Dried eluates were reconstituted in 0.5 mL of immunoluminometric assay (ILMA) buffer,[7] vortexed and centrifuged at 3,000 rpm for 30 minutes at 4°C, and assayed immediately. A total of 100 μL of assay buffer containing 20 ng of antibody G187 was pipetted into polypropylene tubes and incubated overnight at 4°C with 100 μL of the reconstituted plasma samples or 100-μL peptide standards in the range 0 to 2,000 fmol per tube. Samples and standards were assayed in duplicate. A total of 100 μL of assay buffer containing approximately 10^6 relative light units (RLU) of labelled CT-1 was added to the tubes and incubated in the dark for a further 24 hours at 4°C. Bound and free CT-1 were separated by adding goat antirabbit IgG (Metachem Diagnostics Ltd, Northampton, UK) coated onto paramagnetic particles. Samples were washed with 1 mL of wash buffer,[7] vortexed, and immunoprecipitates recovered using a magnetized tube rack. Solutions were drained and the process repeated 3 times. Immunoprecipitates were resuspended by adding 100 μL of distilled water.

Measurement of chemiluminescence from the immunoprecipitates was obtained on a Berthold Autolumat LB953 luminometer (Stratec Electronic GMBH, Birkenfeld, Germany). The label was stimulated to luminesce[8,9] by injecting 100 μL of 100 mmol/L HNO_3 containing 0.05% hydrogen peroxide with a sequential injection of 100 μL of 250 mmol/L NaOH containing 0.25% cetyl triethylammonium bromide 4 seconds later. Chemiluminescence was measured over the following 2 seconds. Results were expressed as RLU using the mean of the duplicate values. Standard curves were fitted using Rodbard's 4 parameter equation on Fig P (Biosoft, Cambridge, UK) and plotted RLU (Y axis) versus CT-1 peptide (X axis [log scale]). Plasma concentrations of CT-1 were determined from the standard curve and adjusted without correction for loss during the extraction procedure to provide a concentration in fmol/mL. The intra and interassay coefficients of variation for the CT-1 assay (at 30 fmol/mL) are 6.2% and 10.3%, respectively. Cross-reactivity of the assay with atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP), C-type natriuretic peptide (CNP), IL-6, and LIF were all less than 0.1%.

Statistical Analysis

All statistical analyses were performed using the software package Minitab (Minitab Inc, State College, PA). Plasma CT-1 concentrations are expressed as mean ± SEM. Mean and percentage differences in specimens stored for 24 or 48 hours compared with plasma stored optimally were determined with corresponding 95% confidence intervals. Further analysis required logarithmic transformation of the data, as it was not normally distributed. Variation between the samples was assessed by analysis of variance (ANOVA) and the paired *t* test. Pearsons correlation coefficients were determined. Comparisons of $P < .05$ were deemed significant.

RESULTS

Table 1 presents the clinical characteristics of the subjects studied, with the plasma CT-1 levels stored optimally (reference levels), stored at room temperature, or on ice for 48 hours. Plasma CT-1 was detected in all samples, and concentrations ranged from 16 to 87 fmol/mL in plasma stored optimally (reference storage). The concentration of CT-1 in samples stored optimally was 43.1 ± 6.05 fmol/mL. CT-1 levels for storage at room temperature compared with ice at the remaining time points were as follows: 24 hours, 41.5 ± 5.76 versus 37.5 ± 8.66; and 48 hours, 42.6 ± 6.28 versus 41.0 ± 5.42 fmol/mL. Mean plasma concentrations of CT-1 were not significantly different when processed immediately or if kept at room temperature or on ice for 24 hours or 48 hours (Table 2).

Pearson's correlation for reference storage CT-1 compared with CT-1 concentrations at 24 hours at room temperature or on ice were $r = .85$ and $r = .99$, respectively ($P < .0001$) for both. Pearson's correlation for reference storage CT-1 compared with CT-1 concentrations at 48 hours at room temperature or on

Table 1. Characteristics of the Subjects in the Study

Diagnosis	Gender	Age (yr)	Reference CT-1 (fmol/mL)	Room Temperature CT-1 (48 h) (fmol/mL)	Ice CT-1 (48 h) (fmol/mL)
Anterior AMI	M	84	87.4	84	83.5
Anterior AMI	M	79	42.2	39.7	39.7
Anterior AMI	M	83	47.6	47.5	42.2
Inferior AMI	M	69	15.8	13.5	16.5
Inferior AMI	F	54	39.8	35.3	42.9
Inferior AMI	M	60	38.2	33.6	30.6
Posterior AMI	M	45	42.2	43.1	42.7
Unstable angina	M	77	20.7	20.4	19.4
Unstable angina	F	70	23.3	20	22.6
Unstable angina	M	60	38	44.1	38
Normal control	F	45	44.7	49.5	61.4
Normal control	F	21	77	80.3	52.3

Table 2. Mean and Percentage Changes in Plasma Concentrations of CT-1 in Samples of Whole Blood Stored for 24 or 48 Hours at Room Temperature or on Ice Compared With Optimal Collection and Storage

CT-1 Storage Conditions	Mean Change From Reference Storage (fmol/mL) (%)	95% Confidence Intervals (fmol/mL)	95% Confidence Intervals (% change)
24 hours at room temperature	+0.22 (+0.51)	+9.62 to −9.17	+22.3 to −21.3
24 hours on ice	+3.49 (+8.1)	+16.05 to −9.06	+37.3 to −21.0
48 hours at room temperature	−0.49 (−1.14)	+1.81 to −2.79	+4.2 to −6.47
48 hours on ice	−2.09 (−4.9)	+3.84 to −8.02	+8.9 to −18.6

ice were $r = .89$ and $r = .94$, respectively ($P < .0001$) for both (Figs 1 and 2).

DISCUSSION

AMI represents a severe threat to circulatory homeostasis and following such events, a number of cytokines are released, particularly the interleukins and tumour necrosis factor alpha, which have pronounced effects, both systemically and locally on the cardiovascular system.[10] Very recently, augmented secretion of CT-1 has been identified in patients with AMI.[11] Ligands that activate gp130 signalling pathways have been shown to be of physiologic importance in regulating survival of terminally differentiated cells in vivo and, as such, augmented secretion of CT-1 may have an important role in cardiomyocyte salvage in the acute stages of myocardial infarction.

In addition, cardiac muscle hypertrophy represents an important adaptive response of the heart to injury for the maintenance of cardiac homeostasis. CT-1 can activate a distinct form of myocardial cell hypertrophy (the promotion of sarcomere assembly in series),[4] which may augment cardiac function and attenuate increased wall stress in the initial stages of AMI. However, long-standing exposure to hemodynamic stress results in cardiac dilatation producing a thin-walled ventricle, the structural basis of which reflects the long-term series addition of sarcomeres. CT-1 promotes left ventricular remodelling experimentally[5] and can generate a form of hypertrophy with similarity to the eccentric hypertrophy of volume overload. Furthermore, elevated levels of plasma CT-1 have been identified in a population of patients with LVSD.[7,12] These data strongly suggests a putative role for CT-1 in the left ventricular remodelling of heart failure.

As such, routine measurement of CT-1 may represent an objective and cost-effective means of identifying patients following AMI who are at risk of remodelling and ensuing ventricular failure. However, of critical value to such a biochemical test is the in vitro stability of the peptide under practical working conditions. This study is the first to directly assess the stability of CT-1 and demonstrates that CT-1 is stable in whole blood treated with EDTA and aprotinin up to 48 hours at both room temperature and when stored on ice. However, a limitation of this study is the small number of subjects, making it underpowered to detect any potential gender differences in CT-1 levels. One unexplained finding of this study was the apparently superior in vitro stability of CT-1 kept in blood samples for up to 48 hours, the reasons for which are unclear. The prolonged in vitro stability of CT-1 is a critical determinant in the development of a widespread biochemical test for identifying patients with raised levels of CT-1. Although further work is clearly essential to determine if CT-1 is of potential clinical value in the routine clinical investigation of patients with heart failure, the robustness of this peptide under different storage conditions would enable further studies on its physiology and pathophysiology.

The potential of a biochemical test for the routine investigation of patients following AMI is an attractive and potentially beneficial prospect. Left ventricular function represents the most essential determinant of prognosis subsequent to AMI.[13] However, pre-existing methods for the assessment of patients

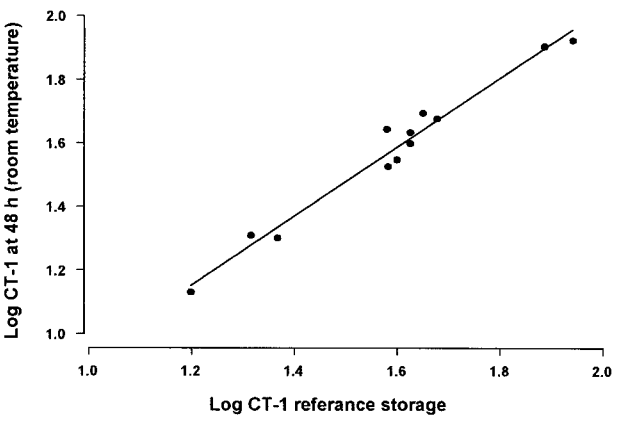

Fig 1. Correlation of plasma specimens of CT-1 stored optimally or kept for 48 hours in whole blood at room temperature.

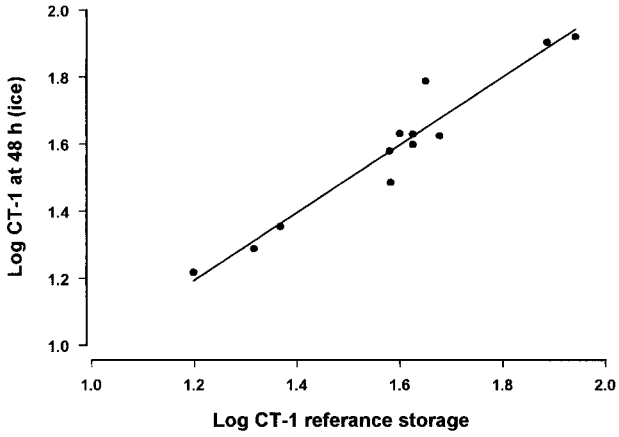

Fig 2. Correlation of plasma specimens of CT-1 stored optimally or kept for 48 hours in whole blood on ice.

with impaired left ventricular function following AMI are subject to certain limitations. Radionuclide ventriculography is a validated method, but is limited as a screening procedure in terms of cost and limited patient access. Echocardiography is the current gold standard of assessing ventricular function following AMI, but requires the availability and expense of trained personnel and is suspect to subjective errors of interpretation. The use of a biochemical marker is clearly unlikely to replace more accurate and established methods of assessing ventricular dysfunction. Increased levels of CT-1 following AMI may identify individuals at risk of remodelling and, as such, result in a streamlined and cost-effective approach in identifying patients who require more formal ventricular assessment. However, it is not known whether changes in this peptide marker occur earlier than changes documented on echocardiography. Nevertheless, the identification of patients with increased levels of CT-1 could form the basis of a new pharmacologic approach using anticytokine intervention. Such an approach could potentially decrease the risk of infarct expansion and positively influence the negative effects of left ventricular remodelling on ventricular performance.

This work represents an attractive start for more formal assessment of the pathophysiologic actions of CT-1 and its ability to identify patients at risk of remodelling.

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