Non-competitive immunochemiluminometric assay for cardiotrophin-1 detects elevated plasma levels in human heart failure

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ABSTRACT
Cardiotrophin-1 (CT-1) leads to a specific form of ventricular hypertrophy characterized by sarcomeres added in series, and has been reported to be elevated in heart failure. Previous competitive assays for CT-1 necessitate the extraction of plasma and involve prolonged incubations. We describe the development of a non-competitive assay for CT-1 that can measure plasma levels without the need for extraction. Two antibodies specific for the mid-section (amino acids 105–120) and C-terminal (amino acids 186–199) portions of CT-1 were developed in rabbits. One antibody was immobilized and used as the capture antibody. The other antibody was affinity purified and biotinylated. Unextracted plasma was incubated with these antibodies, and detection was with methylacridinium ester-labelled streptavidin. Plasma was obtained from 40 patients with heart failure and 40 normal control subjects. The non-competitive assay demonstrated a linear increase in chemiluminescence (measured as relative light units) with increasing amounts of full-length recombinant CT-1, with no evidence of a hook effect at high concentrations. The lower limit of detection was 2.9 fmol/ml. Intra-assay coefficients of variation ranged from 3.1% to 4.2% in the 10–40 fmol/well concentration range, and interassay coefficients of variation ranged from 3.5% to 4.5% in the 550–950 fmol/ml range. Measurements of CT-1 levels in patients with heart failure (median 166.5 fmol/ml; range 49.5–2788 fmol/ml) revealed very significantly elevated levels compared with those in normal controls (median 43.5 fmol/ml; range 11.2–258.6 fmol/ml; P < 0.0001 by Mann–Whitney test). At a CT-1 concentration of 68 fmol/ml, sensitivity and specificity were 95% and 82.5% respectively. Thus this new non-competitive immunochemiluminometric assay for CT-1 could successfully detect full-length recombinant CT-1 in unextracted plasma, and demonstrated that plasma levels of CT-1 were significantly elevated in patients with heart failure.

INTRODUCTION
Cardiotrophin-1 (CT-1) is a member of a family of cytokines which includes interleukin-6 and leukaemia inhibitory factor (LIF) [1]. The receptors for each of these factors contain a common, expressed cell surface polypeptide known as gp130 (glycoprotein 130) [2]; in addition, CT-1 and LIF share a common second receptor component known as LIF receptor subunit β [2]. CT-1 was originally described as a factor that induces cardiac myocyte hypertrophy, adding sarcomeres in series rather than in parallel [1] and leading to increased cardiac myocyte cell length and thus ventricular dilatation. The hypertrophic response of cardiac myocytes to CT-1 is mediated via the Jak/STAT (Janus kinase/signal transduction and activators of transcription) signalling pathway [3]. In addition, recent evidence suggests that CT-1 exhibits cytoprotective properties in both human [4] and

Key words: cytokines, growth factors, heart failure, hypertrophy, immunology.
Abbreviations: CT-1, cardiotrophin-1; CTα and CTβ, oligopeptides corresponding to amino acids 105–120 and 186–199 respectively of CT-1; LIF, leukaemia inhibitory factor; MAE, methylacridinium ester; RLU, relative light units.
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We have described a competitive immunoluminometrical assay for CT-1 which detects elevated levels in patients with heart failure [8]. More recently, a competitive RIA for CT-1 was described which confirmed these findings [9]. Both of these assays employ plasma that has been extracted previously on C18 cartridges, and involve prolonged incubations. In this present study, we describe the development of a sensitive and specific non-competitive immunoluminometric assay for human CT-1 which detects full-length CT-1 in unextracted plasma. This enabled the rapid measurement of plasma CT-1 levels in human heart failure, confirming the high levels reported previously.

MATERIALS AND METHODS

Materials

Recombinant human CT-1 and streptavidin were obtained from Chemicon International Inc. (Temecula, CA, U.S.A.). All other chemicals and foetal calf serum were from Sigma Chemical Co. (Poole, Dorset, U.K.). Methylacridinium ester (MAE; 4-(2-succinimidyl)oxycarbonyl ethyl)phenyl-10-methylacridinium 9-carboxylate fluorosulphonate) was obtained from Molecular Light Technology (Cardiff, U.K.). Sulphosuccinimidyl-6-(biotinamido)hexanoate was from Calbiochem (Nottingham, U.K.). ECL kits and goat anti-(rabbit IgG)–horseradish peroxidase conjugate were from Amersham Pharmacia Biotech (Little Chalfont, Bucks., U.K.). Aprotinin (Trasylo) was from Bayer (Newbury, Berks., U.K.). Microlite 2 microtitre plates were supplied by Dynex Technologies Inc. Bio-Rad Laboratories (Hemel Hempstead, Herts., U.K.) supplied the Affigel-10 matrix.

Production of anti-CT-1 antibodies

Oligopeptides corresponding to the mid-section (amino acids 105–120; CRQQAELNPRAPLLR; referred to as CTa) and the C-terminus (amino acids 186–199; SRTEGDLQLP GG; referred to as CTb) of the human CT-1 sequence were synthesized in the MRC Toxicology Unit, Leicester University, and then purified by HPLC [4,8]. Both pure peptides were confirmed to have the correct predicted molecular masses using matrix-assisted laser desorption MS. The peptides were conjugated to haemocyanin with e-maleimidocaproic acid N-hydroxysuccinimide ester as described previously [8]. Two rabbits were inoculated with subcutaneous injections of antigen (1 mg) emulsified with complete Freund’s adjuvant, with booster injections (0.5 mg) given subcutaneously every 2 weeks. The IgG fractions of the anti-CT antisera were obtained by Protein A–Sepharose chromatography [8], and bound specifically to the respective peptide sequences against which they were raised. IgG fractions of the antibodies to CTa and CTb were called G187 and G203 respectively.

SDS/PAGE and immunoblotting

Human recombinant CT-1 was resolved by SDS/PAGE (15% gels) and then electroblotted on to reinforced nitrocellulose (0.2 μm). Blots were blocked overnight in TBS/Tween (20 mmol/l Tris/HCl, 135 mmol/l NaCl and 0.1% Tween) containing 1% (w/v) dried milk powder. Detection by G203 and G187 used 1 μg/ml antibody in TBS/Tween for 1 h. After extensive washes in TBS/Tween, blots were incubated in a 1:1000 dilution of anti-(rabbit IgG)–horseradish peroxidase conjugate in TBS/Tween for 1 h, and detection was with ECL* kits with exposure of blots on to pre-flashed X-ray films.

Subjects

Blood was obtained from 40 normal control subjects and 40 patients with moderate to severe heart failure after informed consent was obtained. The studies were approved by the Leicester Health Authority Ethics Committee. Normal control subjects were outpatients who were scanned in the echocardiography department and were found to have no significant cardiovascular abnormality. None were on any treatment. Heart failure was defined as an ejection fraction of less than 40%, as defined by echocardiography. Samples of 20 ml of blood were transferred into chilled tubes containing 500 units/ml aprotinin and 1.5 mg/ml EDTA. Following centrifugation, plasma was stored at −70 °C until the assay was performed. All samples were analysed within 2 months of venesection. Plasma specimens were defrosted and assayed without any further treatment.

Immunoluminometric assay for CT-1

The purified IgG raised against the peptide epitope CTb (G203) was initially affinity purified on Affigel matrices on which the CTb peptide was chemically immobilized (5 μg of peptide to 1 ml of gel) using the manufacturer’s protocol. After binding the IgG on to 1 ml of the peptide–Affigel matrix, unbound antibodies were washed off with PBS, and specific affinity-purified antibodies were eluted using 0.1 mmol/l HCl (pH 3.0) and then 10 mmol/l HCl (pH 2.0). Eluates were neutralized rapidly using 0.1 mmol/l Na2HPO4 buffer (pH 8.0). Biotinylation of the antibody was performed in 1 ml of 0.1 mmol/l Na2HPO4 buffer (pH 8.0), using 50 μg of the biotinylating reagent [sulphosuccinimidyl-6-(biotinamido)hexanoate] for every 1 mg of antibody. Incubations were at room temperature for 2 h. Reactions were quenched with 100 μl of 10 mg/ml lysine, and then the biotinylated antibody was isolated from unbound biotin.
by gel filtration on a Sephadex G-25 column. In order to reduce non-specific binding, the biotinylated antibody was then incubated overnight at 4 °C with 1 ml of Affigel 10 on which were immobilized 5 mg of BSA and 2 mg of rabbit IgG. The supernatant antibody was recovered the next day.

Streptavidin (100 μg) was labelled with MAE (25 μg) in 0.1 mmol/l Na2HPO4 buffer (pH 8.0) for 30 min at room temperature. The reaction was then quenched using 100 μl of 10 mg/ml lysine. Labelled streptavidin was separated from low-molecular-mass products using Sephadex G-25, with 0.1 mmol/l Na2HPO4 (pH 6.0) as the eluting buffer. The low pH ensured that the chemiluminescent streptavidin remained stable for several months when stored in aliquots at −70 °C.

The wells in Microlite 2 plates were coated with 500 ng of purified IgG (G187) dissolved in 100 μl of 0.1 mol/l sodium bicarbonate buffer, pH 9.6. After overnight incubation, wells were washed three times with a wash buffer (1.5 mmol/l NaH2PO4, 8 mmol/l Na2HPO4, 340 mmol/l NaCl, 0.5 g/l Tween and 0.1 g/l sodium azide). Wells were then blocked for 2 h using 10% (v/v) foetal calf serum in bicarbonate buffer. Following three washes with the wash buffer, the plates were ready for performing the assays. The biotinylated affinity-purified G203 antibody was diluted into assay buffer, composed of 1.5 mmol/l NaH2PO4, 8 mmol/l Na2HPO4, 140 mmol/l NaCl, 1 mmol/l EDTA, 1 g/l BSA and 0.1 g/l sodium azide, so that each 1 ml contained 20 ng of antibody (a great excess). Normal rabbit serum was added (1% (v/v)) to reduce non-specific binding. Other additives such as casein, poly(ethylene glycol) or Triton X-100 did not improve performance of the assay. Into each well was placed 20 μl of a subject’s plasma or 20 μl of a standard. Standards were serial dilutions of human recombinant CT-1 (10–80 fmol per well) dissolved in pooled normal human plasma that had been pre-extracted on C18 columns to deplete its peptide content. It was essential to mimic the matrix of the assayed plasma in order to ensure reproducibility. Incubations were performed at room temperature overnight on a microtitre plate shaker set at 300 Hz. Following this, plates were washed six times with wash buffer, and then MAE-labelled streptavidin [3 × 106 relative light units (RLU)/100 μl of assay buffer] was added. Incubations were performed at room temperature for 2 h with plates protected from light. The plates were then washed six times with wash buffer, and chemiluminescence was measured in a Dynex MLX luminometer. Each well was initiated with sequential injections of 100 μl of 100 mmol/l HNO3 containing 0.05% hydrogen peroxide, followed 4 s later by an injection of 100 μl of 250 mmol/l NaOH containing 0.25% cetyl triethyl-ammonium bromide. Chemiluminescence was measured for 2 s following the second injection, and was expressed in RLU.

**Statistical analysis**

All data are presented as mean±S.E.M. or median (range). All values were compared using the Mann–Whitney test for data that are not normally distributed. Statistical significance was assumed at the P < 0.05 level. Receiver-operating characteristic curves were constructed using SPSS software (SPSS Inc., Chicago, IL, U.S.A.).

**RESULTS**

Figure 1 shows Western blots, with recombinant CT-1 resolved at around 21 kDa. Both antibodies (G187 and G203) detected CT-1 specifically, with immunoreactivity being abolished by preincubation of the antibodies with an excess of their respective peptides.

Using G187 as the immobilizing antibody and biotinylated affinity-purified G203 as the detecting antibody, it was possible to measure concentrations of human recombinant CT-1 in the range 10–80 fmol per well (Figure 2). There was no evidence of a paradoxical ‘hook’ effect at high concentrations of CT-1. The lower limit of detection, as defined by three S.D.s of the RLU measurement at zero concentration CT-1, was 2.9 fmol/ml of plasma. Addition of recombinant CT-1 to different plasma specimens resulted in the recovery of 103.0±2.4% of the added CT-1 (mean±S.E.M. of 12 experiments). Figure 2 also shows the serial 2-fold dilutions of patients’ plasma specimens, as compared with the standard curve obtained using recombinant human CT-1. The dilution curves and standard curve are very similar, suggesting that the measured chemiluminescence output was highly likely to be due to CT-1.

A number of different cytokines and peptides that could be elevated in heart failure were tested in the immunochemiluminometric assay. The natriuretic peptides (atrial, brain and C-type) showed no significant reactivity, and this was also true for the cytokines interleukin-6 and LIF (all were below the lower limit of detection of the assay). The intra-assay coefficients of

**Figure 1** Western blot of an SDS/15% PAGE gel, with human recombinant CT-1 (20–40 fmol per lane) resolved and detected with G187 (right panel) or G203 (left panel)

Peptide markers for 17 and 26 kDa are indicated. CT-1 is revealed by both antibodies at a molecular mass of approx. 21 kDa.

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Figure 2 Measurement of CT-1 in human plasma using the immunochemiluminometric assay
Known amounts of recombinant CT-1 were added to peptide-depleted human plasma. The y axis represents chemiluminescence (RLU) detected from the streptavidin bound on to the plate. Means ± S.D. are plotted. The line is a fitted polynomial using a least-squares technique. Plasma from patients with heart failure (broken lines) were also taken through 2-fold serial dilutions, and the readings were compared with the standard curve.

Table 1 Characteristics of normal subjects and heart failure patients
Values are means ± S.E.M. Significance of differences: *P < 0.0001 compared with normal subjects.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Normal subjects</th>
<th>Heart failure patients</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number</td>
<td>40</td>
<td>40</td>
</tr>
<tr>
<td>Age</td>
<td>56.2 ± 2.7</td>
<td>56.3 ± 2.1</td>
</tr>
<tr>
<td>Male gender</td>
<td>31</td>
<td>34</td>
</tr>
<tr>
<td>Left ventricular ejection fraction (%)</td>
<td>70.0 ± 2.6</td>
<td>33.0 ± 2.0*</td>
</tr>
<tr>
<td>NYHA class</td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>–</td>
<td>8</td>
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<tr>
<td>II</td>
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<tr>
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<td>28</td>
</tr>
<tr>
<td>Spironolactone</td>
<td>–</td>
<td>6</td>
</tr>
<tr>
<td>Plasma CT-1 (fmol/ml)</td>
<td>54.4 ± 7.5</td>
<td>409.6 ± 97.5*</td>
</tr>
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</table>

variation at 10, 20 and 40 fmol of CT-1/well were 3.8%, 4.2% and 3.1% respectively. The inter-assay coefficients of variation were 4.5% at 550 fmol/ml and 3.5% at 950 fmol/ml.

The newly developed assay was then used to assess levels of CT-1 in human plasma samples from normal control subjects and from patients with heart failure. The clinical characteristics of the subjects are shown in Table 1. There were no significant differences in age or gender between the groups. Left ventricular ejection fractions were significantly different, by definition. All plasma levels were measured in duplicate; when levels were outside the range of the calibrators, appropriate dilutions were made and measurements were repeated in duplicate. Plasma CT-1 levels were very significantly elevated in the heart failure group compared with the controls [median (range); controls, 43.5 (11.2–258.6) fmol/ml; heart failure patients, 166.5 (49.5–2788) fmol/ml; P < 0.0001 by Mann–Whitney test] (Figure 3). Means ± S.E.M. for the two groups are shown in Table 1. Receiver-operating characteristic curves, plotting sensitivity against 1 specificity, yielded an area under the curve of 0.94. At a plasma level of 68 fmol/ml CT-1, heart failure was detected with a sensitivity of 95% and a specificity of 82.5%. The positive and negative predictive values were 84.4 and 94.2% respectively.

DISCUSSION

We and others have previously measured CT-1 in human plasma specimens using competitive assays (immuno- luminometric assays or RIAs) [8,9]. In normal subjects we obtained a range of 6.9–48.3 fmol/ml with a median value of 29.6 fmol/ml using the CTa peptide as a standard [8]. Another group [9] used recombinant human CT-1 as a standard and reported a mean plasma CT-1 level of approx. 570 fmol/ml. Both of these competitive immunoassays required prior extraction of plasma on C18 columns and then time-consuming drying of the extracts on centrifugal evaporators; these pre-assay steps taking up to 24 h. In the present non-competitive immuno-
chemiluminometric assay, it was possible to measure CT-1 in specimens without prior extraction on C_{18} columns. Moreover, incubations were performed overnight, producing a result within 24 h. This contrasts with the previous competitive assays, in which the sequential addition of the CT-1 tracers to pre-reacted samples and antibodies led to assay durations of up to 48–72 h. The problem of the recovery of extracted samples was also circumvented, with recovery of all of the added recombinant CT-1 in the present assay. The present assay also has the advantage of using non-radioactive detection of the ligand, with the highly sensitive chemiluminescence emitted from MAE-labelled antibodies or streptavidin [10]. These reagents have been demonstrated to be stable for more than 6 months when stored at pH 6.0 at −70 °C.

With the present assay, using recombinant CT-1 as a standard, we found the concentration of CT-1 in plasma from control subjects to range from 11.2 to 258.6 fmol/ml, with a median value of 43.5 fmol/ml. These results differed from our previously reported normal range for CT-1 (median 29.6 fmol/ml; range 6.9–48.3 fmol/ml) [8], where standardization was to the CTA peptide and not to full-length recombinant CT-1. Standardization of immunoassays using short peptide sequences can lead to variations in results compared to the use of full-length standards due to differences in immunoreactivity between the antibody and the peptide or the full-length standard. The present results are slightly lower than those reported by Asai et al. [9], who reported levels in normal humans of approx. 570 fmol/ml using recombinant CT-1 as standard, but competitive assays are known to be less specific and more prone to errors due to the extra extraction steps.

It was necessary to affinity purify the detector antibody before labelling it with biotin. Following this, binding at zero levels of CT-1 was reduced by incubating the biotinylated antibody with Affigel to which was coupled BSA and rabbit IgG. Other additives, such as casein, poly(ethylene glycol) or detergents (e.g. Triton X-100), were not useful in reducing the binding of the biotinylated antibodies at zero levels of CT-1. These measures resulted in the low detection limit of 2.9 fmol/ml of plasma for the assay.

Using the present assay, we could measure CT-1 levels in patients’ plasma successfully, confirming the 3-fold elevated levels seen in heart failure. The role of elevated CT-1 in the pathophysiology of heart failure remains to be further investigated, but its effect of inducing myocyte hypertrophy by the addition of sarcomeres in series is suggestive of a role in ventricular dilatation. Moreover, cardiac CT-1 mRNA is induced in various animal heart failure models [11,12], although plasma levels were not reported in those studies.

Other cardiac-derived peptides have been suggested as indicators of left ventricular function, including brain natriuretic peptide ([13,14]; reviewed in [15]) and atrial natriuretic peptide [13,14]. A direct comparison between the utility of these peptides in the detection of heart failure in the community is currently in progress.

This is the first report of the use of a non-competitive assay for measurement of plasma CT-1 concentrations in humans, and defines the levels expected in both normal subjects and heart failure patients. The assay of CT-1 should facilitate the investigation of the physiology of the secretion of this cardioprotective, hypertrophy-inducing peptide in a variety of cardiovascular conditions, and the effects of therapy upon it. The assay will also facilitate the measurement of CT-1 levels achieved in plasma following exogenous administration in any future investigation of the cardioprotective effects of this peptide. Furthermore, the assay will also prove useful in investigations of the secretion of CT-1 into culture media from cardiac tissue incubated in vitro.

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