Cardiac Troponin T Circulates in the Free, Intact Form in Patients with Kidney Failure

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Background: The clinical significance of the increased concentrations of cardiac troponins observed in patients with end stage renal disease (ESRD) in the absence of an acute coronary syndrome (ACS) is controversial. One proposed explanation is that immunoreactive fragments of cardiac troponin T (cTnT) accumulate in ESRD. We used gel-filtration chromatography (GFC) to ascertain whether fragments of cTnT, which could cross-react in the commercial diagnostic immunoassay (Roche Diagnostics), were the cause of the increased cTnT in the serum of patients with ESRD.

Methods: We subjected sera from ESRD patients (n = 21) receiving dialysis and having increased cTnT concentrations to size-separation GFC. We detected cTnT in the chromatography fractions by use of the same antibodies used in the commercial assay for serum cTnT.

Results: In all patients, cTnT immunoreactivity eluted as a major, homogeneous peak in an identical position between the peaks of serum prolactin [relative molecular mass (Mr) 23 000] and albumin (Mr 67 000): the elution pattern of cTnT in samples obtained from ACS patients was identical to that of the ESRD patients. There was no evidence that low–molecular-mass (Mr <23 000) cTnT fragments were the cause of the increased cTnT in the patients studied.

Conclusions: The form of cTnT observed in the serum of patients with kidney failure and immunoreactive in the diagnostic assay is predominantly the free intact form, as in patients with ACS. Our data are consistent with the view that circulating cTnT in renal failure reflects cardiac pathology.

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Troponins T, I, and C [relative molecular masses (Mr) 37 000, 22 000, and 18 000, respectively] are components of the contractile apparatus of muscle. Specific forms of troponin T and I occur in heart muscle: cardiac troponin T (cTnT) and troponin I (cTnI). After injury to heart muscle cells, e.g., after myocardial infarction, cardiac troponins are released into the circulation, and measurement of cTnT and cTnI in blood has become the standard approach to diagnosis and risk assessment of patients with acute coronary syndrome (ACS) (1). Within the myocyte, cTnT exists predominantly in a bound form with troponins C and I (the ternary T-I-C troponin complex), but also as a free intact form in the cytoplasm. Approximately 6% to 8% of cTnT is thought to exist in this latter form.

Increased serum concentrations of troponins, especially cTnT, have been detected in the presence of renal failure (2–4) and also in the predialysis population (5, 6). Although there is general agreement that this finding is a strong indicator of poor prognosis (7–9), its underlying cause remains the subject of controversy. Normal coronary angiograms have been demonstrated in a substantial proportion of kidney disease patients with increased (>0.1 µg/L) cTnT (10), and increased cTnT does not predict coronary artery disease in patients with end stage renal disease (ESRD) (11). It also seems unlikely that left ventricular hypertrophy explains the increased cTnT concentrations observed in patients with kidney disease (5, 6, 12).

Uncertainty over the mechanism causing increased troponin concentrations in kidney disease has raised doubts about their clinical significance. Diris et al. (13) and Michielsen et al. (14) observed fragments of cTnT...
ranging in size from \( M_r 8000 \) to 25 000 in all of 63 ESRD patients receiving hemodialysis. The relative amounts of the different fragments varied, and intact cTnT was not detected. Furthermore, fragments were generated in vitro from intact cTnT added to serum after incubation at 37 °C (14). Diris et al. (13) proposed that the fragments remain reactive in immunoassays and accumulate in blood because of decreased renal clearance in patients with impaired renal function and cause, or contribute to, the increased cTnT concentrations observed in these patients.

cTnT is measured with assays from a single reagent manufacturer (Roche Diagnostics), which has patent rights to the antibodies used in these analytical systems. Critically, Diris et al. (13) did not demonstrate that the cTnT fragments they observed could cross-react in the Roche commercial cTnT assay.

In the present study, we used a simple and direct technique to ascertain whether cTnT fragments that could cross-react in the commercial diagnostic immunoassay were present in the serum of patients with kidney failure. We identified samples from patients with ESRD being treated by dialysis who had increased serum cTnT. Samples were subjected to size-separation gel-filtration chromatography (GFC) with detection of cTnT in the chromatographic fractions by the Roche commercial cTnT assay.

### Materials and Methods

We performed chromatography with Sephacryl S-100 gel [Amersham Biosciences; separation range \( M_r 1000 \) to 100 000]. Preliminary experiments, with a 40 × 1.6 cm column (bed volume, 75 mL) and maximum sample volume of 3 mL (~5% of the column bed volume), demonstrated that a peak of cTnT immunoreactivity could be detected with the standard Roche assay applied directly to the chromatography fractions when the cTnT concentration in the original serum sample was ≥0.08 \( \mu \text{g/mL} \). In subsequent experiments, we adjusted the volume of serum applied to the column (between 1.70 and 3.00 mL), depending on the cTnT concentration in the sample. We applied serum to the column and eluted the serum with 0.02 mol/L Tris buffer (pH 7.4) containing 0.26 mol/L sodium chloride, 0.006 mol/L sodium azide, and 1 g/L bovine serum albumin. The flow rate was 0.3 mL/min, and 0.6-mL fractions were collected with direct detection of cTnT in the fractions by the Roche assay. Dilution (1 part/4 parts) of 5 serum samples with known cTnT concentrations (range, 0.98–4.25 \( \mu \text{g/mL} \)) in the Tris buffer confirmed that the Roche assay detected all of the cTnT (mean measured concentration, 103% of expected; range, 99%–108%) in this medium.

We calibrated the column with ovalbumin (\( M_r 43 000 \)), chymotrypsinogen A (\( M_r 25 000 \)), and ribonuclease A (\( M_r 14 000 \)), all from Amersham Biosciences, using low sample volumes (<1% of column bed volume) for accurate determination of peak elution volumes. However, with the large sample volumes required to provide sufficient sensitivity when serum cTnT was ~0.1 \( \mu \text{g/mL} \), the relationship between elution volume and \( M_r \) was invalid. We therefore also checked the elution volume of endogenous human serum albumin (\( M_r 67 000 \)) and prolactin (\( M_r 23 000 \)), using low sample volumes of serum (Fig. 1). Serum prolactin and albumin behaved in a manner consistent with their \( M_r \) values of 23 000 and 67 000, respectively, enabling their use as internal standards in patient samples, such that endogenous serum prolactin could serve as a marker of relative molecular mass appropriate to the identification of the cTnT fragments (\( M_r 8000–25 000 \)) detected by Diris et al. (13) when using large sample volumes. We calculated that, using the chromatographic system described above, a change in relative molecular mass from 37 000 to <25 000 for immunoreactive cTnT would produce an increase in elution volume and peaks at least 5 fractions apart.

Patients with ESRD receiving either peritoneal dialysis (Canterbury; \( n = 33 \)) or hemodialysis (Southend; \( n = 62 \)) as outpatients were recruited to the study. All patients gave informed consent, and the study had the full approval of the West Essex and East Kent Local Research Ethics Committees (REC reference numbers 04/Q0301/34 and K214/11/02, respectively). We collected blood samples (15 mL) in the nonfasting state into SST Vacutainers (Becton Dickinson) and allowed the samples to clot before centrifugation. Samples from hemodialysis patients were taken before dialysis. We performed initial measurements of cTnT either within 4 h of sample draw or after storage at 4 °C for up to 48 h. Serum for subsequent analysis was stored frozen at −80 °C for <6 months before chromatography. We selected patients for chromatography if their serum cTnT concentration was ≥0.08 \( \mu \text{g/mL} \); samples from 11 peritoneal dialysis patients (cTnT range, 0.08–0.33 \( \mu \text{g/mL} \)) and 10 hemodialysis patients (cTnT range, 0.11–0.21 \( \mu \text{g/mL} \)) were suitable for chromatographic analysis (Table 1). No patients had recently (within 3 months) suffered an ACS.

Sera from ACS patients (\( n = 5 \)) with increased cTnT concentrations and normal kidney function were used as controls. Additionally, we performed studies using cTnT extracted from human heart tissue (SCIPAC Ltd.; product code 184-4). That tissue had been homogenized and clarified before purification by ion-exchange chromatography. The troponin extract contained ~32 mg/L cTnT in 0.025 mol/L Tris buffer (pH 7.5) containing 0.15 mol/L sodium chloride and 0.9 g/L sodium azide. This was diluted 1 part/1600 parts in serum with a cTnT concentration <0.01 \( \mu \text{g/mL} \), and 0.25 mL of the diluted material was subjected to chromatography both before and after incubation at 37 °C for 48 h.

We measured serum cTnT with a 3rd-generation electrochemiluminescent immunoassay on Elecsys 1010 or E-170 analyzers (Roche Diagnostics). The 99th percentile upper reference limit of this assay and the limit of
detection are 0.01 μg/L (15, 16). The between-day imprecision (CV) was 5.7% at a concentration of 0.11 μg/L. We confirmed acceptable recovery of cTnT from the GFC fractions by calculating recovery from the columns.

Results

In all ESRD patients studied, both hemodialysis and peritoneal dialysis, the cTnT immunoreactivity eluted as a major, homogeneous peak in an identical position be-

Table 1. Clinical characteristics of the dialysis patients whose samples were subjected to GFC.

<table>
<thead>
<tr>
<th>Patient no.</th>
<th>Age, years</th>
<th>Sex</th>
<th>Primary cause of renal failure</th>
<th>Dialysis modality</th>
<th>Time on dialysis, months</th>
<th>Known diabetes mellitus</th>
<th>Known cardiovascular disease</th>
<th>Total serum cTnT, μg/L</th>
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<tr>
<td>1</td>
<td>77</td>
<td>M</td>
<td>Diabetic nephropathy/IgA nephropathy</td>
<td>PD&lt;sup&gt;c&lt;/sup&gt;</td>
<td>14</td>
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<td>No</td>
<td>0.10</td>
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<tr>
<td>2</td>
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<td>PD</td>
<td>53</td>
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<tr>
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<td>PD</td>
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<td>Yes</td>
<td>0.11</td>
</tr>
<tr>
<td>4</td>
<td>78</td>
<td>M</td>
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<td>PD</td>
<td>65</td>
<td>No</td>
<td>Yes</td>
<td>0.15</td>
</tr>
<tr>
<td>5</td>
<td>69</td>
<td>M</td>
<td>Myeloma</td>
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<td>62</td>
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<td>No</td>
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</tr>
<tr>
<td>6</td>
<td>73</td>
<td>F</td>
<td>Focal and segmental glomerulosclerosis</td>
<td>PD</td>
<td>35</td>
<td>Yes</td>
<td>No</td>
<td>0.11</td>
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<tr>
<td>7</td>
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<td>M</td>
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<td>8</td>
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<td>F</td>
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<tr>
<td>14</td>
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<td>0.11</td>
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<td>45</td>
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<td>No</td>
<td>0.21</td>
</tr>
</tbody>
</table>

<sup>a</sup> All patients were Caucasian, except patient 18, who was South Asian.

<sup>b</sup> Yes indicates patients with known cardiovascular disease, including a previous medical history of any of the following: ACS, cardiac arrhythmia, valvular heart disease, angina, coronary artery bypass surgery, angioplasty, or congestive cardiac failure.

<sup>c</sup> PD, peritoneal dialysis; HD, hemodialysis.
Fig. 2. GFC on Sephacryl S-100.

cTnT is shown as a solid line. ○ and ■ indicate that cTnT was measured in this fraction but was below the limit of detection.Albumin and prolactin are shown as short- and long-dashed lines, respectively: (A), serum from patient 9, who was receiving peritoneal dialysis (serum cTnT, 0.28 μg/L; sample volume, 3.0 mL); (B), serum from patient 14, who was receiving hemodialysis (serum cTnT, 0.19 μg/L; sample volume, 2.3 mL); (C), serum from a patient who had suffered an ACS (serum cTnT, 1.60 μg/L; sample volume, 0.50 mL); (D), serum containing troponin extract (SCIPAC Ltd.) both before (●) and after [■] incubation for 48 h at 37 °C.
between the peaks of serum prolactin (M_r 23 000) and albumin (M_r 67 000; Fig. 2, A and B). The elution pattern of cTnT in serum samples obtained from patients who had experienced an ACS was identical to that of the ESRD patients (Fig. 2C). Chromatography of cTnT extract that had been added to serum yielded a major peak coinciding with that of albumin and a second, lower–molecular-mass peak in an position identical to that observed in samples from our patients (Fig. 2D). It seems likely that the former peak represents the T-I-C complex and the latter free cTnT. After incubation at 37 °C for 48 h, the pattern changed, showing evidence of disintegration of the T-I-C complex and augmentation of the free cTnT peak. In all cases, there was no evidence of peaks or shoulder peaks with M_r <23 000, which would indicate the presence of low–molecular-mass fragments of cTnT reactive in the Roche assay. Median (range) recovery of cTnT from the columns was 76 (60–102)% in peritoneal dialysis patients and 100 (86–154)% in hemodialysis patients.

Discussion

After ischemic myocardial injury and reperfusion, troponins T and I are present in blood in a variety of forms, including complexes of cTnT, cTnI, and troponin C; degradation products; and free cTnT. These forms react differently in immunoassays (17). The pattern of release of troponins after myocardial injury has not been fully characterized. In particular, there has been relatively little work addressing the release pattern of cTnT. Wu et al. (17) used GFC on Sephacryl S-200 to examine the nature of immunoreactive cTnI and cTnT in 3 patients after acute myocardial infarction. The approach had limited sensitivity; therefore, all patients studied were selected on the basis of markedly increased troponin concentrations. They observed a variable pattern of proteins; an I-C complex, free cTnT, and cTnT fragments, but no free cTnI, were observed in all patients, and in addition, a ternary T-I-C complex was detected in 2 individuals and, possibly, cTnT fragments in 1 individual. In all 3 cases, the major cTnT peak observed corresponded to intact free cTnT.

We modified the approach of Wu et al. (17), using Sephacryl S-100 gel to optimize resolution of low–molecular-mass components. In all patients studied, cTnT eluted in an identical position consistent with the molecular mass of the free intact protein. We did not observe any lower–molecular-mass immunoreactive fragments of cTnT. All of the immunoreactive troponin we observed eluted earlier (i.e., in a higher molecular mass position) than the M_r 23 000 endogenous marker protein, prolactin. Our observations conflict with those of Diris et al. (13) and support the hypothesis that the increased cTnT concentrations observed in patients with kidney failure are caused by the presence of intact cTnT.

After release, the further metabolism and clearance of cTnT is not well understood. Given the size of both free cTnT and its ternary complex (M_r 37 000 and 77 000, respectively), it is unlikely that the kidney will represent the main clearance mechanism, and it is probable that clearance occurs via the reticuloendothelial system. Ellis et al. (18) calculated plasma half-lives and elimination rate constants of cTnI in post-myocardial infarction patients with and without renal failure and concluded that they were not statistically different, arguing against a significant effect of renal function on clearance. Furthermore, renal transplantation did not have a large influence on circulating cTnT concentrations in patients with ESRD (19). Nevertheless, cTnT has been detected in the urine of patients with renal failure (both in the presence and absence of a concomitant ACS) but not in the urine of nonuremic individuals after myocardial injury (20).

Although the majority of cTnT released from myocytes is thought to be intact (17), there is some evidence of limited proteolysis, possibly before release into the circulation (21). Certainly proteolytic degradation of cTnI has been shown to occur (22). It is possible, therefore, that the cTnT molecule could undergo proteolytic cleavage with formation of lower–molecular-mass fragments that remain reactive in the Roche assay. The Roche assay uses 2 antibodies directed to sequences located in the N-terminal tail region of the molecule: amino acid residues 125–131 and 136–147. This region of the molecule has a high concentration of α-helices that render it resistant to proteolysis, whereas the area of the molecule encompassed by residues 183–200 is highly susceptible to proteolysis (23). Cleavage of the free cTnT molecule in the 183–200 region, for example, could potentially lead to the formation of 2 fragments with approximate M_r of 25 000 and 12 000—the former remaining immunoreactive in the Roche assay, whereas the latter would not be recognized. Hence, it might be reasonable to suggest that the increased concentrations of cTnT observed in dialysis patients are the consequence of reduced renal clearance of fragments. However, in our system, we were unable to observe such fragments, and we were also unable to confirm the findings of Diris et al. (13), that fragments of cTnT could be generated in vitro on prolonged incubation of serum enhanced with intact cTnT (14).

Diris et al. (13) used a complex analytical approach to demonstrate the presence of such fragments in patients on hemodialysis, including immunoprecipitation, separation by gel electrophoresis, and visualization by Western blotting. The validity of their findings has been questioned by Giannitsis and Katus (24), who have suggested that the bands on electrophoresis interpreted by Diris et al. (13) as fragments of cTnT were, in fact, the result of accumulation and nonspecific binding of antibodies on the electrophoresis gel, an artifact to which the Western blot technique is prone. Certainly, they did not perform amino acid sequencing of the bands they identified. In particular, Diris et al. (13) did not demonstrate directly that the cTnT fragments they detected reacted in the commercial assay for serum cTnT. It is possible that the differences observed between our studies are the result of fragments that do
not react in this assay. However, whereas the presence of such fragments would be of scientific interest, that presence would not explain the clinical observation in patients with kidney disease. The global use of cTnT as both a diagnostic marker and a prognostic indicator is based solely on experience with the Roche assay.

Our application of the Roche assay to chromatographic fractions was at the limit of sensitivity of the analytical system. As a consequence, calculated recovery from the column was subject to inaccuracy. It is possible that smaller molecular-mass fragments may have been present in our samples and remained undetected in the fractions. It is also possible that cTnT fragments may have been present at low concentrations in the sera of patients whose total cTnT concentrations were too low to be subjected to chromatography. However, it is clear from our data that such fragments, if present, do not represent the major form of cTnT seen in patients with ESRD. Using immunoprecipitation, Diris’s group (13, 14) were able to study cTnT fragments even in samples with total cTnT below the detection limit of the commercial assay. However, the study by Diris et al. (13) was remarkable because no intact free cTnT was detected in any of the serum samples from hemodialysis patients, whereas this component appeared to be ubiquitous in our study.

In conclusion, our data suggest that the form of cTnT observed in the serum of patients with kidney failure and immunoreactive in the diagnostic assay is predominantly the free intact form. We found no evidence of immunoreactive cTnT fragments or of in vitro formation of cTnT fragments that react in the Roche assay. This does not exclude the possibility that there may be fragments of cTnT present or that the circulating pattern of cTnT forms differs between patients who have suffered an acute coronary event and patients with kidney disease; further evaluation of this possibility awaits the availability of more sensitive assays for cardiac troponins. Although the underlying pathology of the increased cTnT concentrations observed in kidney disease remains elusive, it is not attributable to the accumulation of cTnT fragments.

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References


