Fast and Slow Skeletal Troponin I in Serum from Patients with Various Skeletal Muscle Disorders: A Pilot Study

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Background: Detection of skeletal muscle injury is hampered by a lack of commercially available assays for serum markers specific for skeletal muscle; serum concentrations of skeletal troponin I (sTnI) could meet this need. Moreover, because sTnI exists in 2 isoforms, slow (ssTnI) and fast (fsTnI), corresponding to slow- and fast-twitch muscles, respectively, it could provide insight into differential injury/recovery of specific fiber types. The purpose of this study was to investigate whether the 2 isoforms of sTnI and their modified forms are present in the blood of patients with various skeletal muscle disorders.

Methods: Serial serum samples were obtained from 25 patients with various skeletal muscle injuries. Serum proteins were separated by a modified sodium dodecyl sulfate–polyacrylamide gel electrophoresis protocol followed by Western blotting for sTnI with monoclonal antibodies specific to ssTnI and fsTnI.

Results: We observed (a) intact and, in some cases, degraded sTnI products; (b) evidence of posttranslational modifications in addition to proteolysis; and (c) differential detectability of both skeletal isoforms in the same patient.

Conclusions: It is possible to monitor both sTnI isoforms; this could lead to the development of new diagnostic assays for skeletal muscle damage.

Currently, the diagnosis of skeletal muscle injury (encompassing all types of muscle diseases) in a clinical setting is a challenge. Direct methods are either invasive (biopsy) or difficult to interpret (e.g., magnetic resonance imaging) (1). Indirect indices of skeletal muscle damage include quantifying muscle soreness, measuring the force generated during maximal voluntary contractions, and monitoring the concentrations of serum markers (1). Despite their widespread experimental use, assessment of muscle soreness and measurement of force during maximal voluntary contraction are not ideal in a clinical setting; the former is subjective and the latter requires a baseline measurement for comparison. Their utility is further compromised by patients who cannot cooperate.

The commonly used serum markers, e.g., lactate dehydrogenase, creatine kinase (CK), and myoglobin, lack specificity to skeletal tissue. Recently, several groups (2–5) used immunoassays to investigate use of the skeletal isoform of troponin I (sTnI; a myofilament regulatory protein) as a marker of skeletal muscle injury, just as its cardiac isoform (cTnI) is currently the gold standard for detecting cardiac muscle injury (6).

sTnI exists as 2 different isoforms, slow sTnI (ssTnI) and fast sTnI (fsTnI), produced in slow- (ST) and fast-twitch (FT) fibers, respectively. This makes detection of injury to skeletal muscle problematic because human and animal models of injury indicate that it can be FT- or ST-fiber dominant (7–14). The ability to identify and track injury to specific fiber types is therefore desirable and could provide clinically relevant information.

The usefulness of sTnI assays depends on the ability of

4 Nonstandard abbreviations: CK, creatine kinase; fsTnI and ssTnI, fast and slow skeletal troponin I, respectively; cTnI and cTnT, cardiac troponin I and T, respectively; ST and FT, slow and fast twitch, respectively; mAb, monoclonal antibody; PTM, posttranslational modification; WB-DSA, Western blot–direct serum analysis; and SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.
the monoclonal antibody (mAb) to detect intact ssTnI and fsTnI and any of their modified forms. Enzymatic (e.g., phosphorylation and degradation) or chemical (e.g., oxidation and acetylation) modifications, collectively referred to as posttranslational modifications (PTMs), of an analyte either before its release or once in the blood can affect antibody binding and thus detection (15). We previously reported (16) that sTnI is proteolyzed in the hypoxic canine diaphragm. In addition, proteolytic fragments of fsTnI were present in the serum of a patient with drug-induced rhabdomyolysis (17). The development of a sensitive and specific diagnostic assay for sTnI requires characterization of the exact isoforms and form(s), intact or modified, in the blood after injury. The use of Western blot–direct serum analysis (WB-DSA) allows detection of TnI isoform degradation products because it separates reduced and denatured proteins by size, allowing analysis of degraded products and other modifications that could alter mass.

In this pilot study, we used only isoform-specific mAbs for Western blot analysis, allowing simultaneous and independent detection of isoforms and degradation products, to probe for fsTnI and ssTnI in the sera of patients with various skeletal muscle disorders.

**Materials and Methods**

**PATIENT SAMPLES**

After obtaining approval from the Research Ethics Board of Queen’s University, we analyzed serum samples from 25 patients admitted to Kingston General Hospital with increased CK concentrations and indications of skeletal muscle disorders. Serum aliquots were obtained from blood samples taken for routine care and not by a defined study time course. Blood was collected in serum separator tubes, centrifuged, subjected to routine biochemical tests, including total CK (Modular Analytics E170; Roche Diagnostics GmbH; upper reference limits of 197 U/L for men and 155 U/L for women), and stored at 4 °C. Aliquots were obtained and subjected to WB-DSA (18). Because this was a pilot study, only limited patient information was available (e.g., onset of disorder, whether acute or chronic, reason for presentation to hospital). Chart review by a clinician was used to classify patients according to their skeletal muscle disorder(s) (Table 1).

**SEUM ANALYSIS**

Because signal loss has been observed with samples frozen for longer than 6 months, we analyzed samples stored at 4 °C within 10 days of collection. Serum proteins were separated by a modified sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) protocol (18). In brief, SDS-PAGE was performed under denaturing and reducing conditions with a sample buffer containing 3.3 g/L SDS, 3.3 g/L CHAPS, 3.3 g/L NP-40, 0.1 mol/L dithiothreitol, 1.4 mol/L urea, and 50 mmol/L Tris-HCl (pH 6.8) in 100 mL/L glycerol. Serum was diluted 11-fold in sample buffer to prevent precipitation of serum proteins during boiling. Diluted samples were then boiled for 10 min to assure separation of the troponins from serum proteins and to break up binary and ternary complexes. After boiling, 10 μL of diluted serum (equivalent to 1 μL of undiluted serum) was loaded on 12.5% gels and run at 100 V until the dye front reached the bottom of the gel (~3 h).

Gel-electrophoresed proteins were transferred to nitrocellulose (45 μm; Micton Separation, Inc.) in the presence of 10 mmol/L CAPS (pH 11.0) for 45 min at 100 V and 4 °C in a Trans-Blot Cell apparatus (Bio-Rad). Nitrocellulose blots were transiently stained with Ponceau S (Sigma) to identify albumin, which nonspecifically binds both primary and secondary antibodies. Blots were then cut at ∼50 kDa to remove the albumin band. Membranes were blocked overnight at 4 °C in 100 mL/L blocking reagent (Roche). Primary antibodies of confirmed isoform specificity were used (16, 17). These included FI-32 and FI-23 (Spectral Diagnostics) and SI-1 (Hytest), which are specific for fsTnI, and MYNTS (courtesy of N. Matsumoto), which is specific for ssTnI. At the time of this work, these mAbs were the best available, based on our evaluation of 27 different anti-sTnI mAbs. Blots were incubated with primary antibody (0.5 mg/L) followed by an anti-mouse IgG antibody–alkaline phosphatase conjugate (Jackson Laboratories). All antibodies were diluted in 10 mL/L blocking reagent and incubated for 45 min at room temperature. Signals were visualized with chemiluminescence substrate (Roche) and X-Omat Scientific Imaging Film (Eastman Kodak). Exposure times of Western blots were chosen to maximize visualization of intact sTnI and/or its degradation products; consequently, comparisons of the intensities of bands from different patients were not possible.

A sample was considered positive when ≥2 mAbs detected the isoform and/or its fragments and the intact isoform migrated at the appropriate molecular mass. Serum known to be troponin negative was used as a control. Serial dilution studies of serum supplemented with fsTnI indicated that we can detect down to at least ~1.56 pg per lane (data not shown).

**Results**

fsTnI was detected in the blood of all 25 patients and ssTnI in 16 of the 21 patients tested (Table 1). Representative Western blots of serum samples from 5 different patients, probed independently for fsTnI and ssTnI, are shown in Fig. 1A. The lower band of the doublet of fsTnI migrated with intact fsTnI, as determined from serum samples supplemented with skeletal muscle tissue (data not shown). Both slow and fast isoforms were detected, and both had degradation products, although not in all patients. On the basis of migration on 1-dimensional SDS-PAGE, a maximum of 7 proteolytic fragments were observed for fsTnI and 3 for ssTnI. The extent of proteolysis and number of fragments varied among patients, but...
this was unrelated to CK concentrations (compare the blots of patients A and B with their corresponding CK concentrations; Fig. 1B). Proteolytic fragments of fsTnI and ssTnI were observed in only 13 of 25 (52%) and 7 of 16 (44%) patients, respectively (Table 1).

The temporal immunoreactivity profiles obtained with 2 fsTnI-specific mAbs in 8 samples from 2 different patients are shown in Fig. 2. In the first patient (Fig. 2A), mAb SI-1 (bottom panel) displayed less immunoreactivity in the first 4 samples than in the last 4, whereas mAb FI-32 (top panel) displayed relatively equal immunoreactivities in all 8 samples. The differential detection of fsTnI and ssTnI over time in another patient is shown in Fig. 2B. The initial increase in ssTnI was followed by a rapid decrease to lower concentrations (prolonged exposures were required for visualization). In contrast, fsTnI increased, peaking after ssTnI peaked, and remained increased.

Although both isoforms were detected in most patients (Table 1, patients 1–4, 6–11, and 13–18), only the fast isoform was detected in the sera of others (patients 20–23 and 25) with activity-induced (exercise or exertion) injury. The findings in 2 such patients are shown in Fig. 3. One patient had both cardiac [confirmed by cardiac troponin T (cTnT)] and respiratory complications (Fig. 3A), whereas the other had only respiratory complications (Fig. 3B; no increased cTnT).

**Discussion**

Using WB-DSA, we have detected both slow and fast isoforms of sTnI in patients with skeletal muscle disorders, raising the possibility that their detection may eventually provide useful diagnostic information. A limitation of our technique, WB-DSA, is that it cannot detect PTMs (e.g., oxidation and glycosylation) that do not alter the electrophoretic migration of the protein on 1-dimensional SDS-PAGE. However, differential detection of the same isoform by different mAbs indicates the presence of a PTM (Fig. 2).

The doublet of the intact fsTnI isoform (Fig. 1A) is not the result of proteolysis but a different modification. Phosphorylation is one type of modification that can cause a shift in electrophoretic mobility on 1-dimensional SDS-PAGE, creating a doublet, the parent protein with the higher molecular mass band (≈2–4 kDa) being the phosphorylated form (19, 20). Although we found no difference in the ratio of the 2 intact fsTnI bands after dephos-

<table>
<thead>
<tr>
<th>Patient</th>
<th>fsTnI</th>
<th>ssTnI</th>
<th>Skeletal muscle disorder</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>+</td>
<td>-</td>
<td>Sepsis/Cellulitis</td>
</tr>
<tr>
<td>2</td>
<td>+</td>
<td>+</td>
<td>Drug-induced rhabdomyolysis/sepsis</td>
</tr>
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<td>+</td>
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<td>-</td>
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</table>

Total positive, n 25 13 16 7

* +, detected; −, not detected; NA, not available.

**Table 1. Detection of fast and slow sTnI (intact form and degradation fragments) in patients with skeletal muscle disorders.**

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phorylation with alkaline phosphatase (18), this result does not exclude the possibility that the upper band was a phosphorylated form (or some other PTM, e.g., oxidation, glycosylation, or nitration) of fsTnI. Of interest, the ratios of the 2 bands varied among patients (Fig. 1B) independent of proteolysis and/or CK concentrations. Both of these results, the doublet and variations in its ratio, are similar to those observed for cTnI in the sera of patients after acute myocardial infarction (18).

The nature of modifications changed during the course of the disease (Fig. 2). The altered immunoreactivity of mAb SI-1 does not reflect changes in analyte concentration because mAb FI-32 revealed relatively constant amounts throughout. Thus, differential detection of fsTnI by mAbs FI-32 and SI-1 indicates the presence of a modified form that affects the binding of mAb SI-1 but not FI-32. The clinical relevance of these modifications remains to be determined.

Did proteolysis occur in the tissue or after its release into blood? We previously showed, in severely hypox-
emeric dogs, that proteolysis of sTnI occurs in the dia-
phragm, demonstrating that proteolytic fragments form
in the muscle cell before release (16). This is similar to
what happens in the myocardium, where the cTnI under-
goes specific and progressive proteolysis (21, 22); recom-
brinant human cTnI added to serum undergoes little, if
any, proteolysis over 24 h at 37 °C (18). Considering the
biochemical similarities of cardiac and skeletal TnI, these
results suggest that proteolysis of sTnI occurs in the tissue
rather than after release.

The ability to monitor independently both sTnI iso-
forms permits tracking of injury to each fiber type. Skele-
tal muscles are generally classified as either ST (also
referred to as type I) or FT (also referred to as type IIA and
IIB) based on histochemical staining for myosin adenosine
triphosphatase [for a review, see Ref. (23)]. ST fibers are
equipped to work aerobically, whereas FT fibers are
equipped to work anaerobically. Most adult human
muscles are composed of various proportions of ST and
FT fibers, allowing them to function over a wide range of
contractile demands.

Several studies in animals and humans have suggested
that activity-induced injury can be FT- or ST-fiber domi-
nant, but this issue is unresolved. Eccentric or concentric
contraction-injured muscle may be preferential for FT
(8–10, 14) or ST (10, 12–14) fibers. Vijayan et al. (11)
proposed that selective recruitment of fiber types ex-
plains, in part, fiber-specific injury. In addition, the biopsy
procedure itself can produce damage, mimicking exercise-
induced injury (24). Moreover, because ultrastruc-
tural injury is generally patchy, involving a small per-
centage of cells, the limited amount of tissue sampled
(compared with that of the whole muscle) may lead to
over- or underestimation of the extent of injury. In addi-
tion, histologic techniques are believed to be too insensi-
tive to detect low-level cellular injury (25).

Unlike exercise, ischemia–reperfusion causes similar
degrees of injury in FT and ST fibers (26). Targeting of
isform-specific proteins may overcome the problems
associated with classic methods of detecting fiber-type-
specific damage. Our finding that only fsTnI was detected
in the blood of patients presenting with activity-induced
injury is identical to that reported recently in rats with
diaphragmatic fatigue and respiratory failure caused by
breathing against an inspiratory resistive load (27). These
results suggest that activity injures only FT fibers.

In our patients, we observed no relationships between
CK concentrations, either mean or peak, and the extent or
nature of proteolysis (Fig. 1B). If CK concentrations in-
deed reflect the amount of injured muscle (28), then
proteolysis may be related not just to the amount of tissue
damage, but also (or rather) to the nature of the stress to
which the muscle cells are exposed.

Another issue related to reliance on CK is that its tissue
of origin may not always be readily apparent. Skeletal
muscle injury may not be diagnosed when traditional
serum markers such as CK are used because of “contam-
ination” of the skeletal signal by cardiac injury (e.g., see
Fig. 3A). Thus, one could wrongly infer that the increase
in CK was a result of the myocardial infarction (increased
cTnT) rather than skeletal muscle injury.

To our knowledge, only 2 companies have tried to
develop a skeletal muscle assay using sTnI as a biomar-
ker, but neither assay could differentiate between skeletal
and cardiac muscle injury because of extensive cross-
reactivity of the mAbs with the cardiac isoform. Fur-
thermore, these assays could not differentiate between the 2
skeletal isoforms. Takahashi et al. (5) and Sorichter et al.
(2) used these assays to investigate skeletal muscle inju-
y/recovery. In both studies, the focus was on acute
injuries, but Takahashi et al. (5) also investigated serum
sTnI concentrations in patients with Duchenne muscular
dystrophy and polymyositis, both examples of progres-
se degenerative muscle diseases. However, both studies
were limited because fiber-specific injury cannot be tracked. In addition, although both of the aforementioned assays possess analytical sensitivity, their diagnostic sensitivity could have been compromised by PTMs affecting the antibody affinity of sTnI, as shown in this study (Fig. 2A).

In conclusion, skeletal muscle injury led to the release of one or both isoforms of sTnI and their modified products into serum; activity-induced injury preferentially injured FT fibers. Because sTnI, unlike cTnI, can be released from any skeletal muscle, its source cannot be determined, but a careful history should reveal the likely source(s). Detection of different forms and isoforms of sTnI raises the possibility that we may be able to determine not just the origin but also the nature of the injury and its severity.

Our results emphasize the need to develop an assay that can exploit the potential of sTnI as a diagnostic marker.

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