Differential reactivity of cardiac and skeletal muscle from various species in a cardiac troponin I immunoassay

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To identify a blood test that can differentiate cardiac from skeletal muscle injury in animals, we compared tissue reactivities for various species with the use of an immunoassay for human cardiac troponin I (cTnI). Tissue reactivity varied as a function of the homology of tissue troponin with human cTnI. Cardiac reactivity in large mammals was equivalent to cTnI, 9.8 ± 0.6 mg/g, and was 2-fold, 10-fold, and 100-fold greater than in small mammals, birds, and fish, respectively. Skeletal muscle reactivity was equivalent to cTnI, 5.1 ± 0.6 µg/g, in all species except fish, in which it was 50% lower. The ratio of reactivities of cardiac and skeletal muscle was: 1800 in large mammals, 1100 in small mammals, 230 in birds, and 43 in fish. We conclude that cTnI is a powerful candidate in mammals, a possible candidate in birds, but unlikely to be of use in fish as a sensitive and tissue-selective diagnostic test for cardiac injury.

No reliable blood test for diagnosis of cardiac injury in animals is yet available [1–3]. Currently, increased serum activities of cardiac isoenzymes of creatine kinase [EC 2.7.3.2; isoenzyme MB (CK-MB)]3 and lactate dehydrogenase (EC 1.1.1.27; isoenzymes 1 and 2) are used, but the low specificity and sensitivity of these assays frequently makes them ineffective in animals if injury of skeletal muscle, such as may occur with handling, restraint, injections, or myopathies, coexists. Furthermore, the more sensitive and specific immunoassays for mass CK-MB and cardiac troponin T (cTnT) that are used to diagnose myocardial infarction in humans [4, 5] may also be unreliable with skeletal muscle injury [6–8] or chronic renal disease [6, 9–11].

Cardiac troponin I (cTnI) is a strong candidate as a cardiac biomarker in animals. cTnI is a sensitive and persistent indicator of cardiac injury in humans [8, 12, 13], with high tissue specificity in the presence of marked skeletal muscle injury [6–8] and chronic renal failure [6, 8, 9, 14]. Additionally, cTnI expression has not been detected in fetal or diseased skeletal muscle [15], although cTnT and CK-MB may be upregulated with chronic muscle injury [16, 17], and CK-MB is upregulated with endurance training [18, 19]. Increases in serum cTnI after myocardial injury are persistent; the diagnostic window after myocardial infarction in humans is at least 9 days for cTnI [20, 21], which is longer than increases of CK-MB [20, 22] and comparable with cTnT, although some evidence suggests that cTnT remains increased longer [22, 23]. Accordingly, we tested a cTnI immunoassay developed for diagnosis of cardiac injury in humans for its usefulness across a wide range of species.

Materials and Methods

ANIMALS AND TISSUES
Experiments on animals followed the guidelines established in the Guide for the Care and Use of Laboratory Animals [24] and were approved by the Institutional Animal Care and Use Committee. All tissue biopsy specimens were collected at the Ontario Veterinary College from animals without clinical signs or gross pathological indication of cardiac or skeletal muscle disease. In addition, myocardial biopsy specimens were obtained from six Doberman pinscher dogs with heart failure due to idiopathic dilated cardiomyopathy [25]. All animals used in this study were being killed for other unrelated studies conducted at the University of Guelph.

Biopsy specimens of ~1 g were collected from heart and skeletal muscle within 5 min after animals were killed by barbiturates. The left ventricle was used for cardiac studies. Biopsies were obtained from muscles of the upper
limbs for mammals, from pectoral muscles for birds, and from trunk muscles for fish for skeletal muscle studies. Heart and skeletal muscle biopsy specimens were obtained from six dogs of mixed breed, three Holstein calves, three horses (muscle biopsy obtained from two), three sheep, three Yorkshire pigs (heart biopsy obtained from two), three New Zealand rabbits (heart biopsy obtained from two), three Sprague-Dawley rats, three mice, three turkey pouls (heart biopsy obtained from two), three chickens (used for egg production), and three rainbow trout. Tissue was immediately blotted free of blood, trimmed of obvious connective tissue and fat, and ultrafrozen in liquid nitrogen. Samples were stored temporarily in polypropylene vials at $-70^\circ$C until analyzed. This temperature preserves cTnI in myocardium for at least 1 year (data not shown). Samples for analysis were shipped on solid CO$_2$ from Guelph in Ontario to the Division of Laboratory Medicine, Washington University School of Medicine, St. Louis, MO.

Human tissue samples were also analyzed with the use of a protocol approved by the Institutional Review Board at Washington University. Informed consent was obtained from each patient before enrollment in the study. Five heart samples were obtained (three from the Washington University Autopsy Service and two from explanted failing hearts) and three skeletal muscle samples were obtained from patients undergoing back surgery. The cardiac explants and skeletal muscle biopsy specimens were obtained fresh; however, at least several hours elapsed between the time of death and attainment of the samples from the autopsy service. Accordingly, reactivity in these latter samples may underestimate cTnI content, although this underestimation is assumed to be similar for failing and nonfailing hearts because of similar delay times and processing procedures. These samples were also stored at $-70^\circ$C until analysis.

**ASSAYS**

Tissue (~100–300 mg) was weighed and minced. Ten volumes of homogenization buffer containing 75 mmol/L Tris, pH 8.0, 8 mol/L urea, and 1.0 mmol/L CaCl$_2$ were added. Homogenization was accomplished with the use of a hand-held rechargeable homogenizer with a generator bore diameter of 5 mm (Omni International, Warren ton, VA) for two 30-s bursts and an additional burst if necessary. After the homogenates were incubated for 30 min at 4 °C to extract the cTnI, they were centrifuged at 20,000g for 1 h.

To qualitatively confirm the tissue specificity of the cTnI assay in different species, Western blot analysis was performed with the use of the two cTnI-specific monoclonal antibodies of the assay (2B1.9 and 2F6.6) and a third monoclonal antibody (3C5.10) that recognizes both cTnI and skeletal muscle troponin I (sTnI) from human and dog [20]. Homogenates were prepared from tissue samples as described above and diluted 50-fold in gel buffer containing sodium dodecyl sulfate. Of this, 10 μL was applied to 165 g/L polyacrylamide gels containing sodium dodecyl sulfate [26]. After electrophoretic separation, proteins were transferred to nitrocellulose and incubated first with the anti-TnI monoclonal antibodies and then with goat anti-mouse antibodies conjugated with alkaline phosphatase. Color was developed with 5-bromo-4-chloro-3-indolylphosphate and nitroblue tetrazolium (BCIP/NBT Phosphate Substrate System, Kirkegaard and Perry) as substrates. Purified canine cTnI and sTnI was prepared from canine heart and skeletal muscle [27] and used as controls.

For measurements of tissue reactivity in the cTnI assay, supernatant of centrifuged homogenates was removed and diluted with pooled normal human sera, 100-fold for homogenates of muscle and fish hearts and 20,000-fold for homogenates of mammalian and avian hearts. The concentration of cTnI was determined with a research-application, sandwich enzyme immunoassay (Stratus I, Dade International) in which two cTnI-specific murine monoclonal antibodies [20] are used, 2F6.6 as the capture antibody and 2B1.9 as the labeling antibody. Human cTnI was used in the calibrators, its concentration determined with use of a Bradford protein assay (Bio-Rad). Because this method overestimates cTnI concentrations fivefold compared with the Lowry assay [28] (data not shown), the tissue reactivities are fivefold greater than would be obtained by the current, commercially available cTnI immunoassay (Stratus II, Dade International) [29].

**CALCULATIONS AND STATISTICAL ANALYSIS**

Tissue reactivity in the cTnI assay was defined as the concentration of human cTnI that gave the equivalent reactivity. Data were analyzed with the use of Instat 2.0 software and graphed with Prism (GraphPad, San Diego, CA) and are reported as mean ± SE. For comparisons of 3 or more mean values, one-way ANOVA was used. Data were log$_10$ transformed before statistical analysis when standard deviations were >10-fold different according to Bartlett’s test for homogeneity of variances. When the F statistic indicated between-group differences in a comparison, the Student–Newman–Keuls multiple comparisons test was performed. To test for differences when there were only two groups, the two-sided, unpaired Student’s t-test was used, or if Bartlett’s test indicated different standard deviations, Welch’s alternative t-test was used. A decrease in tissue reactivity with heart failure was tested with the use of a one-sided, unpaired Student’s t-test.

For comparative purposes, data were grouped into large mammals (dogs, calves, horses, sheep, and pigs), small mammals (rabbits, rats, and mice), birds, and fish. Differences between groups were considered significant for $P < 0.05$. For the species in which the TnI sequence was known, tissue reactivity in the cTnI immunoassay was compared with the degree of homology of that TnI with human cTnI [30].
Results

All heart homogenates, except those for fish, showed dark staining in Western blot analysis with the cardiac-specific antibodies (2B1.9 and 2F6.6) used in the immunoassay (Fig. 1, panels A and B). Additionally, the antibody specific to both cTnI and sTnI (3C5.10) stained all heart homogenates (Fig. 1, panel C). All skeletal muscle homogenates also showed dark staining with the latter antibody, although the intensity of this staining was lighter for fish, but no staining with the cTnI-specific antibodies. The apparent molecular masses for cTnI and sTnI were similar for all species, except for fish, which had lower molecular masses.

The human cTnI concentrations equivalent to the reactivities of heart and skeletal muscle tissue from each species studied are listed in Table 1. They are ordered according to decreasing reactivities in hearts. Two outlying values, one from a rat heart (10.9 mg/g) and the other from a dog with heart failure (3.3 mg/g), were excluded. These values were >3 SD from the mean of the remainder of their group. The identity of these samples was thought to have been interchanged because of a transcription error. In hearts, the mean reactivity varied across species by >100-fold; skeletal muscle showed at most a fourfold difference between species.

The reactivities of hearts in the cTnI assay were ~10-fold greater for each species of mammals than for each species of birds and 6- to 10-fold greater for each species of birds than for fish. Mammals could be divided into two subgroups that differed widely in body size and had similar cTnI reactivities. One subgroup comprised dogs, calves, horses, and sheep; the other subgroup comprised laboratory animals (rabbits, rats, and mice). Values for pigs were intermediate to those for the two other groups of mammals but, for statistical analyses and figures, were grouped with values for other large mammals because of their size.

Figure 2 indicates the reactivities of cardiac (top panel) and skeletal muscle (middle panel) in the cTnI immunoassay for the four groups of animals, large and small mammals, birds, and fish. Cardiac reactivity was significantly different between groups (P <0.001). Large mammals had reactivity 1.7-fold greater than small mammals, 10-fold greater than birds, and 100-fold greater than fish. In contrast, there was no significant difference between mammals and birds in skeletal muscle reactivity (Fig. 2, middle panel), although in fish it was 50% lower than in other species (P <0.0004). No other difference between groups was detected for muscle cTnI.

The reactivity of hearts relative to muscle varied substantially across groups (Fig. 2, lower panel). In large mammals, the reactivity was 2-fold greater than in small mammals, 8-fold greater than in birds, and 42-fold greater than in fish.

In Fig. 3 the relationship between homology of the TnI gene sequence with human cTnI is indicated for those species for which this information was available [30]; log reactivity is plotted against the percentage homology for a large overlapping segment of the protein sequence comprised of ~200 amino acids (of a possible 209). Sequence homologies with human cTnI are 96.4% for bovine heart, 92.8% for mouse and rat heart, 91.4% for rabbit heart, 71.5% for chicken heart, and 57.1% for human skeletal muscle [30]. This sequence homology and log tissue reactivity were sigmoidally related (r² = 1, P <0.05).

Compared with healthy control dogs, cardiac reactivity was 32% decreased in the dogs with heart failure (P <0.002; Fig. 4). Cardiac reactivity of dogs with heart

Table 1. Human cTnI concentrations equivalent to the reactivities of heart and skeletal muscle tissue from different species.

<table>
<thead>
<tr>
<th>Species</th>
<th>Cardiac reactivity, mg/g tissue</th>
<th>Skeletal muscle reactivity, µg/g tissue</th>
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</thead>
<tbody>
<tr>
<td>Dog</td>
<td>11.6 ± 0.6 (6)</td>
<td>7.3 ± 0.4 (3)</td>
</tr>
<tr>
<td>Calf</td>
<td>10.5 ± 1.9 (3)</td>
<td>8.7 ± 1.4 (3)</td>
</tr>
<tr>
<td>Horse</td>
<td>9.1 ± 0.1 (3)</td>
<td>6.0 ± 3.0 (2)</td>
</tr>
<tr>
<td>Sheep</td>
<td>8.8 ± 1.2 (3)</td>
<td>4.3 ± 2.0 (3)</td>
</tr>
<tr>
<td>Pig</td>
<td>7.2 ± 0.9 (2)</td>
<td>5.0 ± 1.2 (3)</td>
</tr>
<tr>
<td>Rabbit</td>
<td>6.1 ± 0.9 (2)</td>
<td>6.0 ± 2.3 (3)</td>
</tr>
<tr>
<td>Rat</td>
<td>5.7 ± 1.0 (2)</td>
<td>6.7 ± 3.3 (3)</td>
</tr>
<tr>
<td>Mouse</td>
<td>5.7 ± 0.2 (3)</td>
<td>3.3 ± 1.5 (3)</td>
</tr>
<tr>
<td>Turkey</td>
<td>1.1 ± 0.01 (2)</td>
<td>4.3 ± 0.7 (3)</td>
</tr>
<tr>
<td>Chicken</td>
<td>0.92 ± 0.09 (3)</td>
<td>4.3 ± 1.8 (3)</td>
</tr>
<tr>
<td>Trout</td>
<td>0.10 ± 0.03 (3)</td>
<td>2.3 ± 0.3 (3)</td>
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Fig. 1. Western blots of cardiac (C) and skeletal muscle (S) homogenates from various species.

Proteins of diluted tissue homogenates were separated electrophoretically, transferred to nitrocellulose, and reacted with monoclonal antibodies to cTnI (2B1.9 and 2F6.6, panels A and B) and sTnI (3C5.10, panel C). Purified dog cTnI and sTnI (50 ng) were applied as controls, and low-range prestained molecular mass calibrators (MW; Bio-Rad) were used as markers.
failure was not significantly different from that of small mammals.

Mean reactivity of the three nonfailing human hearts was 5.7 ± 0.6 mg/g, similar to that observed for small mammals. Reactivity in the two failing human hearts was 4.02 ± 1.0 mg/g, a decrease of 30% compared with the nonfailing hearts (P = 0.09). Reactivity of skeletal muscle was 8.4 ± 2.7 µg/g, not significantly different from that of other mammals. Heart/skeletal muscle ratio of reactivities was estimated as 680 ± 75 and was similar to that for small mammals.

Discussion

Our data indicate that cTnI is an excellent candidate biomarker of cardiac injury in mammals of all species. Mammalian myocardium has high reactivity in the cTnI immunoassay, and this reactivity is highly selective for myocardium, being >1000-fold higher than in skeletal muscle. Myocardial reactivity (and hence anticipated release of cTnI into blood with acute or ongoing injury) was shown to vary within mammals according to body size, cTnI gene sequence homology to the human gene, and the presence of cardiac disease. Myocardial cTnI is ~60% lower in small mammals compared with large mammals and 30% lower in dogs with heart failure because of idiopathic dilated cardiomyopathy compared with those with nonfailing hearts. Although these instances are unlikely to restrict the applicability of the cTnI assay in mammals, its use is restricted in nonmammalian species. Compared with its utility in mammals, the cTnI immunoassay would be substantially less effective as a cardiac biomarker in birds and probably ineffective in fish, because of much lower cardiac reactivity and heart to skeletal muscle ratio of reactivities. Reactivity of skeletal muscle was relatively constant across species, although it was 50% lower in fish.

The differences across phyla for cardiac reactivity in
the cTnI immunoassay can be attributed primarily to species differences in the primary structure of the protein [30] (Fig. 3). In addition, the differences among mammals likely also result, in part, from differences in myofibril content. Myocardial cTnI content of large mammal species (9.43 ± 1.71, n = 5) was 1.6-fold greater (P < 0.01) than that of small mammal species (5.83 ± 0.22, n = 3). Compared with large mammals, the volume fraction of cardiac myocytes composed of myofibrils is less for small mammals, although the volume fraction of mitochondria is greater, apparently because of a greater metabolic rate [31].

In ventricular and myocyte remodeling during the development of heart failure, a moderate reduction in the content of myofibrils, of which cTnI is a component, occurs [32]. In Doberman pinscher dogs with heart failure, the 32% lower (than healthy controls) cTnI concentration can be attributed to lower myofibril content of myocardium [25] and matches the 30% lower concentration of cTnT found in myocardium of the same breed of dogs with the same cardiomyopathy [25]. Myocardial content of cTnI was also decreased by 30% in failing human hearts, compared with nonfailing hearts.

Estimation of the effectiveness of potential cardiac biomarkers in animals requires consideration of their assay reactivity and tissue selectivity, especially since the primary limitation of the use of existing cardiac biomarkers in animals is their relatively low activity and lack of selectivity for cardiac vs skeletal muscle [1–3]. Release of skeletal muscle proteins in association with animal handling and stress can severely restrict the value of biomarkers with low cardiac selectivity [1–3].

Although high cardiac reactivity and specificity reveal cTnI to be an excellent candidate biomarker of cardiac injury in mammals, they indicate that cTnI would be ~100-fold and ~5000-fold less effective in birds and fish, respectively. In fish, as a consequence of this low cardiac reactivity and specificity and also because of small heart to muscle mass ratio, skeletal muscle injury should release more apparent cTnI into blood than should cardiac injury. However, in birds, the cardiac reactivity and specificity of cTnI may be sufficient for its effective use as a biomarker of cardiac injury if skeletal injury is only mild.

Comparison of the results of this study with a similar one conducted by one of us (P.J.O’B.) with the use of cTnT (second-generation assay, Boehringer Mannheim) [33] suggests that the cTnI immunoassay has ~10-fold greater heart to skeletal muscle ratio of reactivities than the cTnT immunoassay and 10-fold greater heart activity for mammals relative to other phyla than the cTnT immunoassay. Skeletal muscle had ~1% of the reactivity of cardiac muscle in the cTnT immunoassay [33], but in this study we find only 0.05–0.1% cardiac muscle reactivity in the cTnI immunoassay. Also, cardiac concentration of cTnT varied by <10-fold across phyla [33], whereas cTnI concentration varied by up to 100-fold. Although not specifically tested in this study, these differences in reactivity of the different species in the cTnI compared with the cTnT immunoassays support the proposal that the immunoassays recognize different proteins and that cross-reactivity of cTnT in the cTnI immunoassay is minimal and would not restrict its use.

LIMITATIONS

Several limitations should be considered when the results of this study are interpreted. Although high cardiac reactivity and heart to skeletal muscle ratio of reactivities identify cTnI as a candidate biomarker of cardiac injury, other factors may affect its effectiveness. For example, species variations may exist in the kinetics of cTnI release into the blood with various forms of cardiac injury and in the kinetics of clearance from the blood that could restrict its use [3]. However, on the basis of the wide diagnostic window of cTnI in humans, it seems likely that the duration of increased cTnI following cardiac injury in other mammals, and in birds, will be sufficient to provide valuable diagnostic and prognostic information.

In conclusion, our data identify cTnI as the most specific cardiac biomarker described to date for mammals and birds and as a candidate for the specific and sensitive diagnosis and prognosis of cardiac injury in these phyla.

Biopsy specimens used in this study were obtained at the Ontario Veterinary College while P.J.O’B. was on staff there. We thank Hua Shen for her assistance in obtaining the biopsy specimens. Supported in part by a grant from Dade International.

References