Cardiac troponin T (cTnT) and troponin I (cTnI) have been suggested as new, more specific markers of myocardial cellular damage. The objective of this study was to examine how the distributions of cTnI and cTnT were affected in postinfarction left ventricular remodeled (LVR) myocardium. At 2 months postinfarct in a porcine heart failure model, both Western blot and biochemical assay analyses were performed on left ventricular myocardium from similar sized healthy (control) pigs (n = 7). Autoradiograms from Western blot analysis showed that the protein mass for cTnI and cTnT in LVR hearts decreased 80% (P < 0.001) and 40% (P < 0.02), respectively, when compared with nondiseased tissue. Similarly, the concentrations for cTnI and cTnT in LVR hearts decreased 42% (P < 0.05) and 70% (P < 0.001), respectively, compared with nondiseased normal tissue. The clinical assumption is that the appearance of cTnI and cTnT in the blood is proportional to chronic loss of cTnI and cTnT from injured myocardium associated with left ventricular remodeling.

INDEXING TERMS: heart disorders • animal models of disease • Western blot analysis • immunoassays

Troponins I and T (TnI and TnT) are two proteins of the thin filament-associated regulatory system of the contractile complex of skeletal and heart muscle [1]. TnI is encoded by three different genes that are differentially expressed by various muscle tissues, resulting in slow- and fast-twitch skeletal and cardiac TnI isoforms [2]. Cardiac troponin I (cTnI) is uniquely specific for the heart [3]. The 31 amino acids on its N terminus form a sequence not present in skeletal muscle forms. In addition, cTnI has ~40% sequence similarity with skeletal TnI [4]. The unique amino acid sequence of cTnI makes it an ideal candidate for laboratory detection of myocardial infarction (MI) and has facilitated the development of monoclonal antibodies that do not cross-react with skeletal muscle Tns [5, 6]. TnT is expressed in three different isoforms, i.e., slow- and fast-twitch muscle TnT and cardiac TnT (cTnT). Whereas several forms of TnT exist in different muscle types, the cardiac subunit is encoded by a separate gene, giving it a unique amino acid sequence and thus allowing for monoclonal antibody development and commercial production of immunoassays for use in clinical laboratories [7–9]. Published studies from various groups demonstrate the usefulness of cTnI and cTnT in diagnosing MI [5, 10–13]. Recently, reports have compared cTnT or cTnI measurements with creatine kinase MB isoenzyme (CK-MB), the most frequently used marker of acute MI [14], for the diagnosis of MI [15–21]. Both cTnI and cTnT exhibited similar detection limits but substantially better specificity than CK-MB. Recently, increased concentrations of cTnI have been shown to be more tissue-specific for myocardial damage than the other cardiac markers [11, 18–22]. Because the majority of cTns are myofibril-bound, their release in serum may correlate with the extent of cardiac necrosis following MI, providing a noninvasive method for assessing infarct size [23, 24].

The similarity of the pig heart to the human heart in size and physiology has led to the use of pigs as an experimental model of left ventricular remodeling for cardiac disease and injury [25]. It will be important to understand the distribution of cTnI and cTnT, as new markers of myocardial injury, in the healthy heart and the effects of heart failure on changes in their distribution. Alterations in the distribution of cTns could have an impact on the ability of these biochemical markers to accurately indicate, when measured in blood, the presence of injury as well as the extent of damage to myocardial cells. Therefore, we designed this study to examine
how the distribution of cTnI and cTnT was affected in postinfarction left ventricular remodeled (LVR) myocardium. We measured the changes in tissue cTnI and cTnT by quantifying the relative protein alterations by Western blot analysis and biochemical immunoassays in nondiseased and diseased cardiac tissue in a porcine model.

**Materials and Methods**

**Tissue Source**
Heart samples from similar sized healthy (control group) and LVR Yorkshire pigs were obtained from a previous study [25]. In that study, left circumflex coronary artery occlusion induced MI and subsequent left ventricular remodeling over a period of 2 months. Left ventricular remodeling was confirmed from Magnetic Resonance Imaging, anatomical data, and simultaneously measured left ventricular (LV) pressure. Animals were killed by a lethal injection of 10 mL of KCl introduced directly into the LV cavity with a fine-gauge needle. Hearts were rapidly excised and dissected. Selected samples were cut from zones of scar, which were always located at the postlateral area [25], and from areas remote from the scar, including the LV anterior wall from which the biopsy specimens used in this study for biochemical analysis and light-microscopic examination were taken [26–29]. These LV sections (remote from the infarct zone), used for protein extraction, were cut into 1-g cubes, placed into vials, and quickly frozen in liquid nitrogen. Samples were stored at −80°C.

**Protein Extraction**
Frozen heart samples (~50 mg) were coarsely ground in a liquid nitrogen-cooled mortar and then added to 1 mL of ice cold buffer (200 mmol/L potassium phosphate, pH 7.4, 5.0 mmol/L EGTA, 5.0 mmol/L β-mercaptoethanol, and 100 mL/L glycerol) to release both mitochondrial and cytoplasmic enzymes [30]. The samples were homogenized at 4°C for 20 s at high speed with a Polytron tissue homogenizer (Brinkmann Instruments, Westbury, NY). This was followed by a 1-h room temperature incubation with gentle agitation and subsequent centrifugation at 40,000g for 30 min at 4°C [31]. The supernatants were used immediately in biochemical analysis and Western blotting. Protein concentration was determined by a modified Lowry method [32] (Sigma Diagnostics, St. Louis, MO) standardized with bovine serum albumin.

**Antibodies**
Two different primary antibodies were selected for use in Western blotting based on preliminary tests (data not shown) that characterized antibody specificity by using purified human cTnI and cTnT proteins (gift from Spectral Diagnostics, Toronto, Canada). A mouse monoclonal antibody specific for the cTnI (11E12) [33] was obtained from Sanofi Diagnostics Pasteur-ERIA, Lyon, France, and used at 2 μg/mL. The other mouse monoclonal antibody specific to the cTnT, JS-2, was a gift from Lakeland Biomedical, Minneapolis, MN, and was also used at 2 μg/mL. This monoclonal anti-cTnT antibody was derived from hybridization of Balb/c mice splenic B cells immunized with human cTnT and myeloma line sp 2/0. It demonstrated 0% cross-reactivity with human and porcine skeletal muscle TnT (unpublished data).

**Western Blot Analysis**
Protein extracts (5 μg) were size-fractionated on 120 mL/L sodium dodecyl sulfate (SDS)–polyacrylamide gels [34] and subsequently transferred to Hybond nitrocellulose membranes (Amersham, Arlington Heights, IL) [35]. Nonspecific binding sites were blocked by incubating the membranes in a blocking buffer [50 g/L nonfat dry milk in TTBS (20 mmol/L Tris-HCl, pH 7.6, 137 mmol/L NaCl, 1 mL/L Tween 20)] for 1 h. A primary antibody was diluted in antibody buffer (10 g/L nonfat dry milk in TTBS) and incubated with the membranes for 2 h on a rotating cylinder. The membranes were washed three times in changes of TTBS buffer for 30 min. Appropriate horseradish peroxidase-labeled secondary antibodies (sheep anti-mouse) were then incubated with the membranes for 1 h. The membranes were again washed three times in TTBS buffer before a 1-min incubation with ECL™ chemiluminescent substrate (Amersham). Light emission was detected by exposure to Fuji RX autoradiography film in the presence of Cronex intensifying screens. Signal intensities within the linear range were quantitated by laser densitometry (Molecular Dynamics Inc., Sunnyvale, CA). Linearity was established by analysis of a calibrated curve generated with known amounts of total protein by Western blot (data not shown). Afterwards, membranes were stained with Ponceau S (Sigma Diagnostics) to ensure that equal amounts of total protein had been transferred in each sample lane. One control pig sample was transferred onto all the membranes, allowing for comparison between sample intensities from different membranes.

**Assays**
cTnT was quantified in a second-generation immunoassay (Enzymun ES300; Boehringer Mannheim, Indianapolis, IN), which utilizes a capture antibody (M7) and a detection antibody (M11.7) that show no cross-reactivity with skeletal TnT up to 1000 ng/mL (<0.005%) [9]. Measurement of cTnI was performed in a monoclonal antibody-based immunosorbent assay (Stratus II; Baxter Dade, Miami, FL) [36]. cTnI and cTnT protein quantification assays were performed on the tissue homogenates at a total protein concentration of 1.7 μg/mL, and results for cTnT and cTnI (μg/L) were reported.

**Statistical Analysis**
Data from the control and the LVM groups of pigs were analyzed by a two-tail, unpaired t-test. Significance was set at 0.05. All results are reported as mean ± SE unless stated otherwise.
Results

Light-microscopic evaluations of myocardium of control hearts showed no evidence of fibrosis or necrosis. All specimens of the remodeled myocardium remote from the infarcted heart also showed no evidence of necrosis or fibrosis. However, the scar zone demonstrated decreased LV wall thickness with transmural scarring, extensive reorganization, and fibrosis (data not shown).

Figure 1, top and bottom, shows that the cTnI and cTnT proteins migrated to positions corresponding to ~29 and 39 kDa, respectively, on 12% SDS-polyacrylamide gels. No cross-reactivity with TnI or TnT skeletal forms or CK subunits were observed with either mouse monoclonal antibody specific for cTnI or cTnT used in the Western blot studies. Linearity was verified by laser densitometry analysis of the calibrated curves generated with known amounts of antibodies (data not shown). Fig. 2 compares the cardiac distribution of cTnI and cTnT determined by Western blot analyses for the control pigs and the left circumflex coronary artery-occluded pigs. The densitometry of autoradiograms showed that the protein mass for cTnI and cTnT in LVR hearts decreased 80% (P < 0.001) and 40% (P < 0.02), respectively, when compared with nondiseased normal tissue.

Figure 3 compares the cardiac distribution of cTnI and cTnT determined by immunoassay analyses for the control pigs and the left circumflex coronary artery-occluded pigs. The cTnI concentrations in diseased hearts were substantially decreased by 42% (P < 0.05) in the LVR myocardium [9.5 µg/L (SE 3.6)] when compared with nondiseased tissue [16.4 µg/L (SE 3.9)]. Similarly, cTnT concentrations in diseased hearts (Fig. 3) were substantially decreased by 70% in the LVR myocardium [3.5 µg/L (SE 1.8)] (P < 0.001) when compared with nondiseased tissue [11.8 µg/L (SE 3.2)].

Discussion

The main findings of our present study show that postinfarction remodeling induced a substantial decrease of the concentrations of a single isoform of cTnT and a single isoform of cTnI in the LVR pig heart when compared with nondiseased tissue (Figs. 2 and 3). The decreases in cTnI and cTnT concentrations were in remodeled myocardium remote from scar tissue that demonstrated no evidence by light microscopy of necrosis or fibrosis. Our findings as well as others [26–29] regarding lack of necrosis/fibrosis in remodeled myocardium support this as not being a
confounding factor in this study. Either a decrease in the synthesis of cTnl and cTnT through down-regulation of their respective mRNAs and (or) a chronic loss of cTnl and cTnT protein associated with LV dysfunction, decreased coronary flow reserves, and bioenergetic abnormalities [25] could be responsible for the decrease in tissue Tn. Additional studies are currently underway to examine the role of mRNA expression for cTnl and cTnT in this model.

cTnl and cTnT have attracted increasing interest in recent years as markers for MI and damage, because of their cardiac specificity [3, 8]. The clinical assumption is that the amount of these macromolecules appearing in the bloodstream is proportional to the extent of the myocardial injury. This study is the first to report myocardial distribution of cTnT and cTnl in LVR pig myocardium. The distribution of cTnT in nondiseased and diseased human and canine myocardium has been described previously in our laboratory and others [23, 24]. Release of cTnT and CK-MB into serum occurs following canine coronary artery occlusion similar to that in humans; however, the myocardial distribution of the cytosolic cTnT pool in dogs was less than that found in humans (2% vs 8%, respectively). Parallel to the serum increases of cTnT, both cytosolic and myofibril cTnT concentrations decreased in heart tissue after coronary artery occlusion in dogs and after acute MI in humans. Infarct sizing in dog hearts initially did not correlate well with serum cTnT or CK-MB concentrations. Our results based on direct quantification of cTnT mass in the pig model of LV remodeling are thus consistent with this previous reported change in cTnT (Figs. 2 and 3). Recently, a study attempted to compare cTnl release with the results of other independent methods for quantifying infarct size in living patients [37]. The authors demonstrated cTnl release in patients with first-time MI who was substantially correlated with scintigraphic estimates of myocardial scarring. cTns are clearly known to increase in blood postinfarction; release and loss of Tns in chronic diseased hearts support serum evidence of increased cTnl and cTnT in acute myocardial ischemia, unstable angina, or non-Q-wave MI patients pointing toward poor prognosis [38, 39].

Certain genes that are expressed during normal fetal cardiac growth are reexpressed during pathological cardiac hypertrophy and in end-stage heart failure [40]. Given both the developmental changes in isoform composition during development and functional importance in regulating muscle contraction, the Tn complex forms a potential site for alterations associated with adult heart failure. In a study by Sasse et al. [41], skeletal Tnl mRNA was not detectable in any of the 17 explanted hearts in end-stage heart failure resulting from dilated cardiomyopathy, ischemic heart disease, or primary pulmonary hypertension. The authors concluded that there was no qualitative change in Tnl isoform expression associated with end-stage heart failure. Therefore, alterations in Tnl isoform content cannot be invoked as an underlying mechanism for the altered characteristics of contractility associated with the failing ventricle. In contrast, alterations in TnT expression may be associated with adult heart failure in humans because the presence of a TnT isoform that is usually present during fetal cardiac development has been reported in the adult pathological heart [42, 43]. Recently, Cummins et al. [44] examined four models of experimental cardiac hypertrophy and heart failure for alterations in Tn isoform expression, particularly in the reexpression of the fetal isoforms. Cardiac protein extracts from experimental and sham-operated control rats were analyzed by one-dimensional gel electrophoresis, followed by Western blotting and detection with antibodies specific to Tnl and TnT. No alteration in protein profile was observed for these proteins between control, hypertrophied, and heart failure samples. The authors concluded that the reversion to the fetal pattern of Tn expression is not a feature of experimental cardiac hypertrophy and heart failure in the rat.

Our findings obtained with specific monoclonal antibodies showed a decrease of cTnl and cTnT in remodeled myocardium associated with acute MI, when compared with nondiseased tissue in the remodeled pig heart (Figs. 2 and 3). In agreement with others [41] using monoclonal antibodies specific to skeletal isoforms of Tnl and TnT, we were unable to detect skeletal isoforms of Tns in damaged heart or nondiseased tissues (data not shown). Whereas extensive experimental and clinical literature has demonstrated that ventricular remodeling occurs after myocardial damage [45–47], the biochemical and molecular consequences of LV remodeling, which may partially explain the transition to failure, are not completely understood at this point.

Certain limitations of the present study must also be addressed. First, measurements were not performed to quantitate cTnT and cTnl in blood from these experimental pigs to correlate with loss of proteins observed in the diseased myocardial tissue. Second, no direct measurements of infarct size were gathered to allow us to correlate loss of cardiac markers with infarct size. Third, no Northern blotting was performed to quantitate mRNA of TnT and Tnl to correlate with the Western blotting protein profile.

The clinical implications of our findings imply a chronic loss of cTn protein in remodeled myocardium, potentially allowing for increased serum cTnl and cTnT concentrations. Whereas these findings might be misinterpreted as acute MI, they would imply chronic release from injured myocardium. Blood studies involving heart failure patients without acute MI are necessary to assist the clinician in interpretation of serum cTn concentrations and patient management decisions.

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