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Development of Immunochemical Systems for Detection of Human Skeletal Troponin I Isoforms

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Abstract—Troponin I (TnI), together with troponin T (TnT) and troponin C (TnC), forms the troponin complex, a thin filament protein of the striated muscle that plays a key role in regulation of muscle contraction. In humans, TnI is represented by three isoforms: cardiac, which is synthesized only in myocardium, and fast and slow skeletal, which are synthesized in fast- and slow-twitch muscle fibers, respectively. Skeletal TnI isoforms could be used as biomarkers of skeletal muscle damage of various etiologies, including mechanical trauma, myopathies, muscle atrophy (sarcopenia), and rhabdomyolysis. Unlike classical markers of muscle damage, such as creatine kinase or myoglobin, which are also present in other tissues, skeletal TnIs are specific for skeletal muscle. In this study, we developed a panel of monoclonal antibodies for immunochemical detection of skeletal TnI isoforms using Western blotting (sensitivity: 0.01-1 ng per lane), immunohistochemical assays, and fluorescence immunoassays. Some of the designed fluorescence immunoassays enable quantification of fast skeletal (limit of detection [LOD] = 0.07 ng/mL) and slow skeletal (LOD = 0.1 ng/mL) TnI isoforms or both isoforms (LOD = 0.1 ng/ml). Others allow differential detection of binary (with TnC) or ternary (with TnT and TnC) complexes, revealing composition of troponin forms in the human blood.

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INTRODUCTION

Skeletal muscles accounts for up to 50% of the total body weight in adults and are crucial for movement, respiration, glucose and protein metabolism, and thermogenesis. Therefore, skeletal muscle diseases (including mechanical muscle damage, muscle atrophy, and myopathies) could significantly affect functioning of the entire body. Skeletal muscle damage could be caused by trauma, surgery, intense physical activity, or prolonged compression syndrome. Skeletal muscle diseases (myopathies) are either inherited or acquired. Inherited myopathies are mostly caused by mutations in the genes of the contractile apparatus and enzymes involved in carbohydrate and lipid metabolism. The major reasons for acquired myopathies, including inflammatory myopathies, are infections, myotoxic drugs, and various diseases. Muscle atrophy is often observed in the patients immobilized due to prolonged hospitalization and those with limb denervation, cancer, chronic heart failure, or sarcopenia [1, 2]. Specific and highly sensitive tools for detecting

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muscle fiber damage are required to successfully treat skeletal muscle diseases and/or prevent skeletal muscle degeneration [3, 4]. A common method for diagnosing skeletal muscle damage is immunochemical detection of cytoplasmic proteins released into the bloodstream during muscle fiber necrosis. Creatine kinase and myoglobin are typically used as such biomarkers [5-7]. However, apart from the skeletal muscle, these proteins are synthesized in other tissues, which reduces specificity of the diagnosis. Creatine kinase is represented by three isoenzymes – MM, MB, and BB. Their ratio is 98% MM and 2% MB in the skeletal muscles and 70-80% MM and 20-30% MB in the heart (BB is present mainly in the brain) [6]. Myoglobin is synthesized in both skeletal muscles and heart [7]. Isoforms of skeletal troponin I (TnI), which are detected only in the skeletal muscles, could serve as alternative and more specific biomarkers.

TnI is a contractile apparatus protein in skeletal and cardiac muscles. Together with troponin T (TnT) and troponin C (TnC), it forms a noncovalent complex with molar ratio 1 : 1 : 1 and participates in the Ca^{2+}-dependent regulation of muscle contraction. In humans, TnI is represented by three tissue-specific isoforms: cardiac (cTnI), which is synthesized only in myocardium; fast skeletal (fsTnI) and slow skeletal (ssTnI) isoforms, which are specific to fast-twitch and slow-twitch muscle fibers, respectively. The degree of similarity of skeletal TnI isoforms is ~60%, with the C-terminal parts being the most conserved parts of the molecules [8].

In the series of studies on drug myotoxicity conducted in rats to determine sensitivity and specificity of skeletal TnI isoforms, this marker outperformed creatine kinase not only in specificity, but also in sensitivity and diagnostic accuracy [9]. In humans, concentration of the skeletal TnI isoforms in the blood increases after intense physical exercise, rhabdomyolysis, various traumas, muscular dystrophies, and inflammatory myopathies, suggesting that these markers could be used to diagnose these conditions [9-16]. The skeletal TnI isoforms could also be used to assess severity of the course of muscular dystrophies and to monitor response to the corticosteroid supportive therapy, along with other developing treatment methods [17].

Some conditions are characterized by selective damage to certain types of muscle fibers. For instance, administration of statins predominantly induces injury to the fast-twitch fibers, whereas administration of fibrates mostly affects the slow-twitch fibers [3, 18]. Eccentric muscle contractions primarily lead to the fast-twitch fiber deterioration, which is accompanied by increase in the fsTnI concentration in blood [15, 19]. Denervation or limb immobility, spinal cord injury, prolonged bed rest, and exposure to microgravity usually induce the slow-to-fast fiber type shift, whereas sarcopenia, cachexia, starvation, and glucocorticoid administration result in the fast-to-slow fiber shift [20]. Therefore, methods that can specifically detect injury in the particular type of fibers could provide additional possibilities for differential diagnosis of some diseases. Thus, it is important to develop methods that simultaneously detect both skeletal TnI isoforms and can distinguish between fsTnI and ssTnI.

Immunochemical methods provide a convenient approach for detecting skeletal TnIs. Among these, Western blotting (WB) is suitable for qualitative and quantitative detection of skeletal TnI isoforms in cell and tissue lysates. Immunocytochemical or immunohistochemical (IHC) staining is performed to visualize proteins in the samples of fixed cells and tissues. Enzyme-linked immunosorbent assay (ELISA) and fluorescence immunoassay (FIA) are commonly used for quantitative measurement of antigens in blood to diagnose skeletal muscle injury. cTnI, which has been thoroughly examined using immunochemical methods, is released into the bloodstream not in a free form but as a complex with TnT and TnC, and is present in blood as part of the ternary or binary (with TnC) complexes [21-23]. To the best of our knowledge, there are no experimental data on the release of skeletal TnI forms. However, because of homology between the cardiac and skeletal troponins, it could be assumed that the latter are also present in the blood as complexes. Therefore, some epitopes of the skeletal TnI could be screened by TnT and TnC, which should be considered when developing diagnostic systems. Future research should focus on the assays that recognize total skeletal TnI, as well as methods that can differentiate between the various forms of fsTnI and ssTnI in blood.

In this study, we describe both approaches: assays that can detect fsTnI and ssTnI both separately and together in free form or in complex (binary or ternary), and methods that can differentially identify these complexes. Aim of this study was to obtain and characterize monoclonal antibodies (mAbs) and develop different immunochemical detection systems based on WB, IHC, and FIA for determination of the skeletal TnI isoforms.

MATERIALS AND METHODS

All reagents were purchased from Sigma-Aldrich (USA), unless stated otherwise. The following recombinant proteins (expessed in *Escherichia coli*) were used: fsTnI, fast skeletal TnT (fsTnT), fast skeletal TnC (fsTnC), ssTnI, slow skeletal TnT (ssTnT), slow skeletal/cardiac TnC (ss/cTnC), and cTnI. The following complexes were used: fast skeletal TnI-TnC complex (fsIC), fast skeletal TnI–TnT–TnC complex (fsITC),

slow skeletal TnI–TnC complex (ssIC), cardiac TnI–TnC complex (cIC), and cardiac TnI–TnT–TnC complex (cITC). Monoclonal antibodies (mAbs) specific toward cTnI (560, 4C2), TnT (TnT111), and fsTnC as part of the fsITC (TnC99A5) were from Hytest (Finland). Anti-MyHC1 mAb (antibodies specific to MHC) were purchased from Abcam (UK).

Generation of mAbs. Female BALB/c mice ("Nursery for laboratory animals", IBCh RAS) weighing 20 g 6-8 weeks old at the start of the experiment were used to obtain mAbs. The mice were housed in open-type conventional cages with free access to food and water. Isoflurane inhalation was used to euthanize mice. All procedures were conducted in compliance with the European Community Directive 2010/63/EU. Mice were immunized with recombinant fsTnI, fsITC, or ssTnI. Spleen cells from the immunized mice were hybridized with sp 2/0 myeloma cells using a standard procedure [24]. Indirect ELISA was used to select clones that produced antibodies specific to fsTnI or ssTnI.

Indirect ELISA. For this assay, solutions of 25-100 ng of antigen in a phosphate-buffered saline (PBS; 10 mM KH_2PO_4, 150 mM NaCl, pH 7.4) were placed into 96-well polystyrene plates (Greiner, Germany) for sorption, and incubated for 40 min at 25°C with constant stirring. The wells were next washed to remove nonspecifically adsorbed components using a PBS containing 0.1% Tween 20 (PBST). Next, 0.5-1 μg/mL of antibodies in PBST were added. Serial dilutions were performed if necessary. The plates were further incubated for another 40 min at 25°C with constant stirring. Following incubation, they were washed four times and 100 μL of a solution of detection goat polyclonal antibodies targeting mouse IgG conjugated with horseradish peroxidase (Beckman Dickinson, USA) were added. After another round of incubation for 20 min at 25°C with constant stirring, the plates were washed with PBST six times. This was followed by addition of 100 μL of a substrate (3,3′,5,5′-tetramethylbenzidine in 0.1 M Na-acetic buffer, pH 4.5, containing 0.01% H_2O_2). After color developed (within 10 min or less), the reaction was stopped by adding 25 μL of 2 M orthophosphoric acid per well, and absorbance in the wells was measured at 450 nm using a Multiscan EX plate reader (Labsystems).

Epitope mapping of mAbs. Specificity of the mAbs toward epitopes was determined using an indirect ELISA with peptides conjugated with a carrier protein. A set of 20 amino acid peptides that comprised the entire fsTnI or ssTnI sequence, with an overlap of six amino acids, was synthesized by GenScript (USA). An additional cysteine residue was added to the C-terminus of each peptide (except for the last peptide, where the Cys residue was added to the N-terminus) for conjugation with a carrier protein (ovalbumin or bovine serum albumin). If the antibody recognized one peptide, the epitope of the antibody was considered to be this peptide. If the antibody recognized two neighboring peptides, the overlapping site was considered the epitope.

Sandwich FIA with europium chelate. FIA was performed according to the standard procedures [25, 26]. Solutions of mAbs in PBS were adsorbed onto 96-well polystyrene plates (Greiner, Germany) at concentration 2 μg/mL (50 μL per well) and incubated for 40 min at 25°C with constant stirring. The wells were then washed with a washing buffer (10 mM Tris-HCl, pH 7.8, 0.9% NaCl, 0.025% Tween 40, 0.05% NaN_3) on a PlateWasher (Perkin Elmer, USA) to remove non-specifically adsorbed components. After washing, 25 μL of a solution of detection mAbs conjugated with stable europium chelate in 50 mM Tris-HCl pH 7.8, 0.9% NaCl, 0.5% bovine serum albumin (BSA), 0.01% Tween 40, 0.5% NaN_3 and, optionally, 20 mM EDTA, along with 25 μL of antigen diluted in 150 mM KCl, 20 mM Tris-HCl, 7.5% BSA, and 0.15% NaN_3 were added into each well. The resulting mixture was incubated for 40 min at 25°C with stirring, and the wells were washed six times with a washing buffer. After that, 100 μL of enhancement solution (0.1 M CH_3COOH, pH 3.2, 50 μM trioctylphosphine oxide, 50 μM 4,4,4-trifluoro-1-(2-naphthyl)-1,3-butanedione, 0.1% Triton X-100) was added into each well incubated for 10 min under constant stirring. Phosphorescence intensity was next measured at an excitation wavelength of 340 nm and emission wavelength of 615 nm using a Victor 1420 Multilabel Counter (Perkin Elmer).

Sensitivity parameters of FIAs. Limit of detection (LOD) was calculated as:

$$LOD = mean_{blank} + 1.645 \times SD_{blank} + 1.645 \times SD_{low\ concentration}$$

(where $mean_{blank}$ is mean background signal, SD_{blank} is standard deviation of the background signal, and $SD_{low\ concentration}$ is standard deviation of the sample with low concentration of the analyte). Samples of fsIC and ssIC were used for calibration. To determine LOD, background signal was measured in 36 repetitions, while samples with low analyte concentrations (selected from the $[mean_{blank} + 1.645 \times SD_{blank}]$ to $4 \times [mean_{blank} + 1.645 \times SD_{blank}]$) were measured in 60 repetitions. This experiment was repeated three times and mean value was calculated. Outliers were identified using the formula: mean ± 2×SD. Data are presented as mean ± SD.

Linearity range of FIAs. To construct linear approximations, a series of dilutions of the target antigen were made in the concentration range 0.04-1280 ng/mL. Linear range was determined according to the CLSI EP6-A procedure (Evaluation of the Linearity of Quantitative Measurement Procedures:

A statistical Approach; Approved Guideline) [27] using Microsoft Office Excel 2007. Linear approximations were calculated using the "Data Analysis" add-in and the "Regression" function, which was employed to perform extrapolation using third-order, second-order, and first-order polynomials. Linear range was defined as the region where difference between the values predicted by the polynomial and those predicted by the linear model were less than 5% for all data points.

Cross reactivity of FIAs with other TnI isoforms. Cross-reactivity with other TnI isoforms was determined using sandwich FIA. Calibration curve was constructed using a series of antigen dilutions at concentrations ranging from 0.3 to 80 ng/mL. Additionally, dilutions of cross-reacting antigens were prepared at concentrations of 2000 ng/mL and 1000 ng/mL. Cross-reactivity (%) was calculated using the formula $C_{Ag}/C_{Ag\ real} \times 100$, where C_{Ag} is antigen concentration calculated from the calibration curve, and $C_{Ag\ real}$ is the real antigen concentration.

Western blotting. For immunochemical staining in WB, proteins were separated via sodium dodecyl sulfate-polyacrylamide gel electrophoresis under reducing conditions. Next, the proteins were blotted onto a 0.45 μm nitrocellulose membrane (Bio-Rad, USA) at a constant voltage of 100 V for 40 min. The membrane was blocked with PBST containing 5% skim milk and incubated overnight at 4°C in 10 mL of PBST containing 5% skim milk and biotin isothiocyanate-conjugated mAbs (1 μg/mL). After washing with PBST, the membrane was incubated with streptavidin-polyperoxidase (Thermo Fisher Scientific, USA) in PBST for 40 min at room temperature and washed again with PBST. Immune complexes were detected using a SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Fisher Scientific) with a ChemiDoc MP Imaging System (Bio-Rad). The bands were analyzed using ImageLab 6.1 software (Bio-Rad).

Gel filtration. Proteins were separated using an AKTA Pure chromatography system (GE HealthCare, USA) equipped with a Hiload Superdex 200 16/600 pg column (Cytiva, USA). Gel filtration buffer consisted of 50 mM Tris-HCl, 150 mM NaCl, 5 mM $CaCl_2$, 0.1% BSA, and 0.1% NaN_3. The column was loaded with a 100-ng/mL recombinant antigen solution (1 mL) or 2.5 mg skeletal muscle tissue extract diluted in a gel filtration buffer (1 mL). Flow rate was set at 1 mL/min. The resulting fractions were analyzed using sandwich FIA with different mAb pairs.

Preparation of tissue sections and immunohistochemistry. To prepare tissue sections, tissue blocks from the archive of the Department of Pathological Anatomy of the I. I. Mechnikov Northwestern State Medical University of Russia were used. Heart blocks of human fetuses that died from causes unrelated to cardiac pathology at 29 and 41-42 weeks of intrauterine development, adult myocardium samples of the anterior wall of the left ventricle, and adult *m. vastus lateralis* were included. All tissue samples were collected according to the protocol approved by the local Ethical Committee of the Lopukhin Federal Research and Clinical Center of Physical-Chemical Medicine at the Federal Medical Biological Agency of Russia on 06 February 2024 (meeting protocol number, 2024/02/06).

Before archiving, the samples were subjected to a standard histological procedure: fixation in a neutral 10% formalin solution, dehydration in isopropanol, embedding in paraffin, and subsequent block preparation [28]. Histologic sections of 3-μm thickness were prepared using a Thermo Fisher HM 325 rotary microtome.

Tissue sections were de-paraffinized and rehydrated, then subjected to heat-induced antigen retrieval in a sealed container using a Trilogy® solution (Cell Marque, USA). The process was carried out in a dry heat cabinet for 12 h at 60°C. After washing with distilled water, a 3% solution of hydrogen peroxide was added to inhibit activity of endogenous peroxidases. The tissue sections were next washed with distilled water and PBS, treated with donkey serum solution (up to 5% v/v; Jackson ImmunoResearch, USA) in PBS, and incubated for 30 min. In the next step, the serum solution was removed from the tissue sections, primary mAbs were added, and the sections were placed in a humid chamber in a dry heat cabinet at 37°C for 2 h. Primary mAbs specific to troponins were diluted in an Antibody Diluent (Abcam) at a ratio of 1 : 100, anti-MyHC antibodies at 1 : 4000, and anti-MyHC1 antibodies at 1 : 500. After 2 h, the tissue slices were washed three times in PBS and treated with a N-Histofine® Simple Stain™ Max PO (Nichirei Biosciences, Japan) for 30 min at 25°C. The tissue sections were washed again in PBS and a diaminobenzidine (DAB) substrate kit (Abcam) was applied. The slices were stained with a Mayer's haematoxylin solution, dehydrated, clarified in xylene, and placed under a coverslip.

In a negative control incubation with primary mAbs specific to the target proteins was omitted from the IHC protocol to evaluate nonspecific binding. For all antibodies, two independent staining were performed for each sample.

Human skeletal muscle extract preparation. To 50-65 mg of a skeletal muscle tissue sample, 1 mL of an extraction buffer (50 mM Tris-HCl, pH 7.5, 0.4 M LiCl, 5 mM $CaCl_2$, phenylmethylsulphonyl fluoride, pepstatin A, and leupeptin) was added. The tissue was homogenized and proteins were extracted for 30 min on ice. The mixture was next centrifuged for 10 min at 4°C and 20,000*g*. The resulting supernatant, the skeletal muscle tissue extract, was used for further analysis.

Statistics. Statistical analysis and construction of graphs were carried out using Microsoft Office Excel 2007.

RESULTS

Development and characterization of mAbs. A panel of 97 mAbs that recognize fsTnI, ssTnI, or both isoforms simultaneously was obtained using the hybridoma technique. Indirect ELISA was utilized for primary antibody selection, and mAbs that interacted with only one TnI isoform (without cross-reactivity with other isoforms) or both skeletal TnI isoforms were identified. According to the epitope mapping results, mAbs recognizing regions 30-105 aa and 156-175 aa (specific to fsTnI), 2-119 aa and 142-187 aa (specific to ssTnI), and 30-49 aa and 156-182 aa (specific to both skeletal TnI isoforms) were obtained. These mAbs were tested in pairs using FIA, and combinations capable of detecting the target antigen with the highest sensitivity were selected. To select mAbs that were suitable for WB, we used the following criteria: specificity to the exact TnI isoform in WB (i.e., no cross-reactivity with other TnI isoforms), no interaction with non-target proteins in the skeletal muscle extract or serum, and sensitivity in WB. The mAb epitopes selected for further studies are shown in Fig. 1.

Specificity of mAbs in WB. The mAbs with the highest sensitivity and specificity for the target protein in WB were selected (Fig. 2). Sensitivity of the

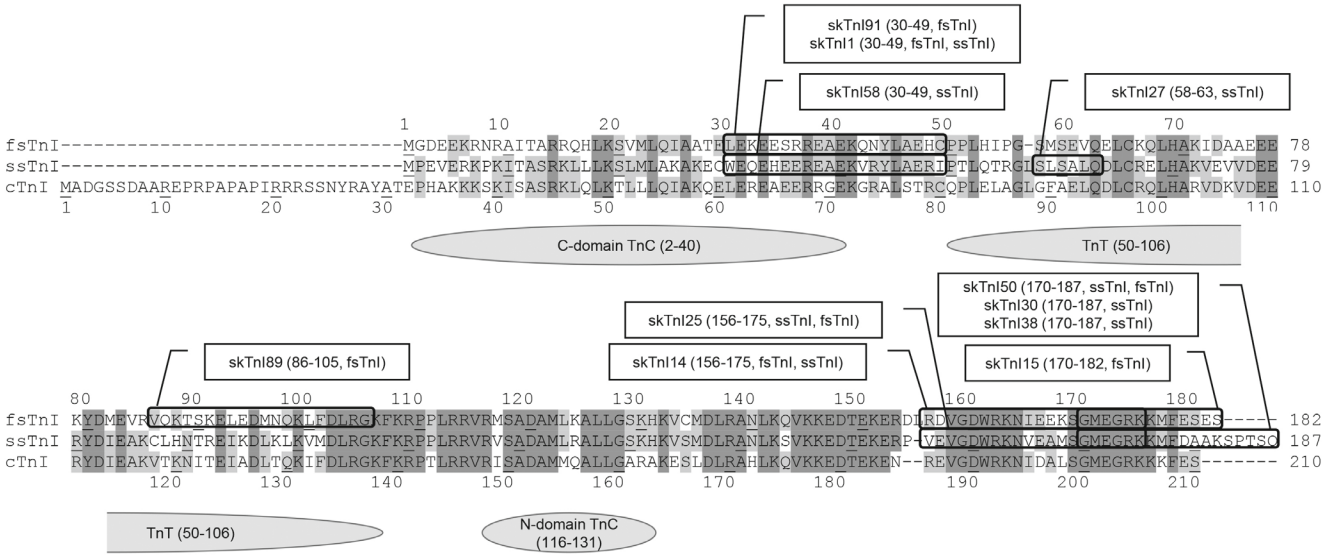

Fig. 1. TnI isoforms. Alignment of the three TnI isoforms – fsTnI (Expasy identificator P48788), ssTnI (P19237), cTnI (P19429) – was performed using Clustal Omega. Identical amino acids residues are marked in dark grey; residues identical in two isoforms are marked in light grey. Ovals indicate TnT- and TnC-binding sites (approximate numbering of residues is for the fsTnI sequence). Rectangles indicate the antibody epitopes. Footnotes indicate the name of the mAb, approximate boundaries of its epitope, and its specificity. If the obtained antibody is specific to both skeletal TnI isoforms, the epitope is indicated for the molecule that was used as an immunogen when obtaining this mAb [29-38].

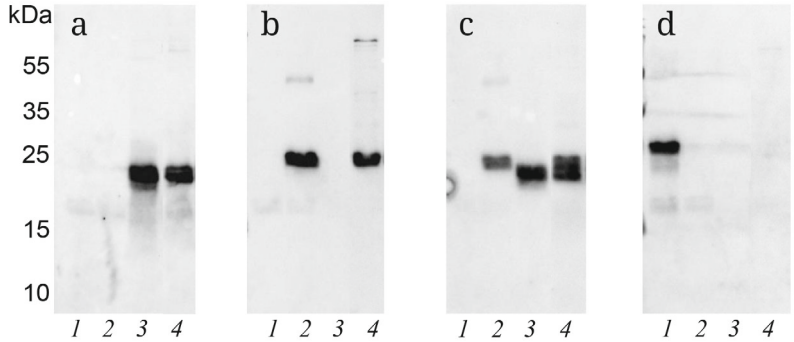

Fig. 2. Recognition of different TnI isoforms by mAbs. a) skTnI89 (fsTnI), b) skTnI38 (ssTnI), c) skTnI50 (fsTnI and ssTnI), d) 560 (cTnI). Lanes: *1*) cTnI, cTnT, ss/cTnC in 1 : 1 : 1 molar ratio, cTnI is 67 ng/lane; *2*) ssTnI, ssTnT, ss/cTnC in 1 : 1 : 1 molar ratio, ssTnI is 67 ng/lane; *3*) fsTnI, fsTnT, fsTnC in 1 : 1 : 1 molar ratio, fsTnI is 67 ng/lane; *4*) human skeletal muscle tissue extract (*m. vastus lateralis*), 42 μg of tissue/lane.

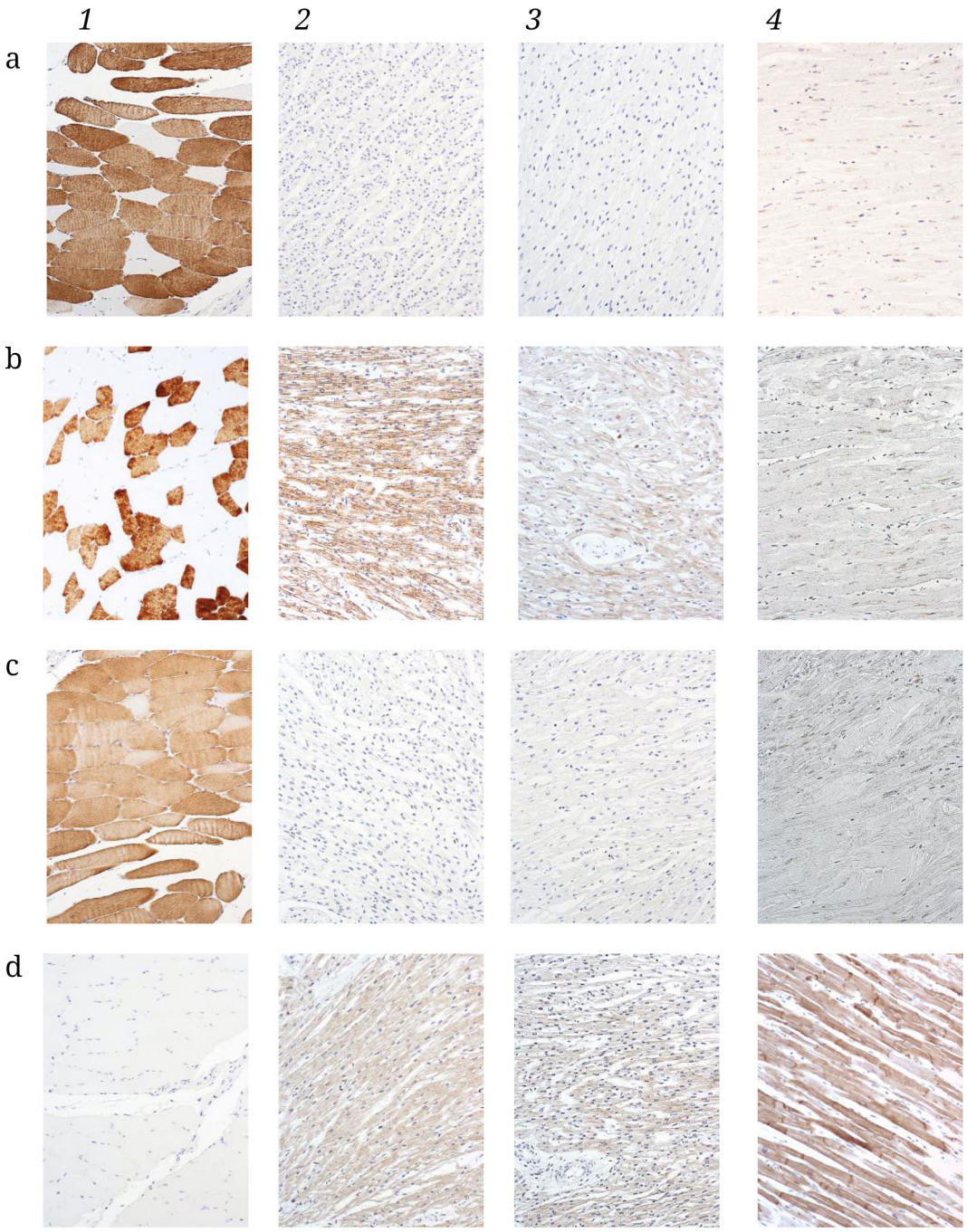

Fig. 3. Immunochemical detection of skeletal TnI isoforms in human striated muscle tissues. mAbs used for IHC staining: a) skTnI15 (fsTnI), b) skTnI30 (ssTnI), c) skTnI50 (fsTnI + ssTnI), d) 4C2 (cTnI). *1*) Adult skeletal muscle (*m. vastus lateralis*); *2*) 29-week fetal heart; *3*) 41-42-weeks fetal heat; *4*) adult heart.

skTnI89 mAb in WB was 0.01 ng fsTnI/lane and skTnI38 was 0.11 ng ssTnI/lane (Fig. S1 in the Online Resource 1). These mAbs did not interact with cardiac or other skeletal isoforms (Fig. 2, a and b). Sensitivity of the skTnI50 mAb, which is specific to fsTnI and ssTnI, was 1 ng fsTnI/ssTnI per lane (Fig. S1 in the Online Resource 1). This mAb did not interact with cTnI.

Methods to detect skeletal TnI isoforms in tissues. Skeletal muscle tissue sections were subjected to IHC staining with mAbs specific toward fsTnI (skTnI15), ssTnI (skTnI30), and both skeletal TnI isoforms (skTnI50). Accumulation of DAB (intensity of staining) was observed in the skeletal muscle fibers (sarcoplasmic staining pattern) but not in the adult human cardiomyocytes (Fig. 3). Utilization of skTnI15 and skTnI30 resulted in accumulation of DAB only in some of the fibers, whereas using skTnI50 resulted in positive staining reactions in all fibers. To identify

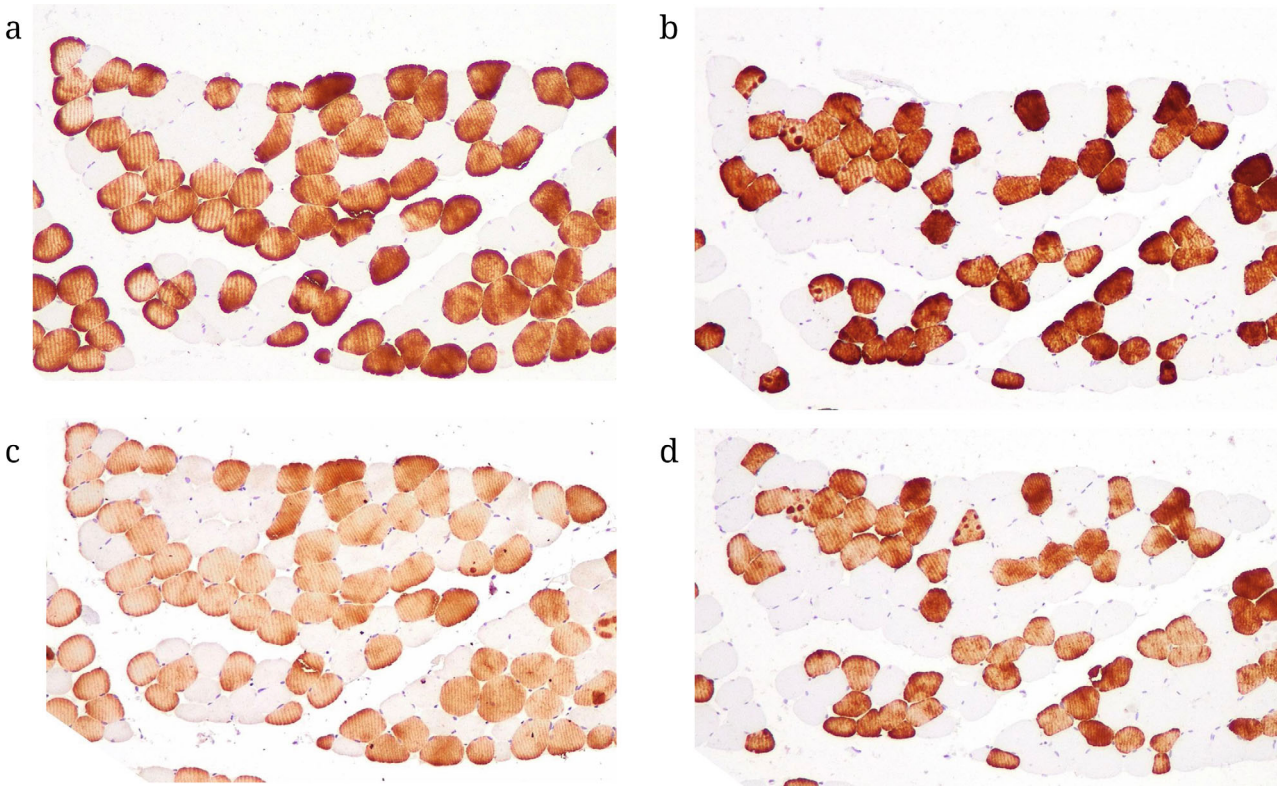

Fig. 4. Immunochemical detection of skeletal TnI and MHC isoforms in human skeletal muscle. mAbs used for IHC staining: a) skTnI15 (fsTnI), b) skTnI30 (ssTnI), c) anti-MyHC1, d) anti-MyHC.

the fast- and slow-twitch muscle fibers, we performed tissue staining with the anti-MyHC antibodies specific to the myosin heavy chain (MHC) isoform, which is present only in the slow-type muscle fibers, and with the anti-MyHC1 antibodies specific to the MHC isoform, which is synthesized in the fast-type muscle fibers. skTnI30 stained the same fibers as anti-MyHC, while skTnI15 stained the same fibers as anti-MyHC1, indicating specificity of skTnI30 and skTnI15 towards slow- and fast-twitch muscle fibers, respectively (Fig. 4).

ssTnI is expressed in heart during the prenatal period and switches to cTnI during the postnatal period [39]. IHC staining with skTnI30 (specific to ssTnI) led to DAB accumulation in the fetal hearts at 29 and 41-42 weeks of prenatal development, but not in the adult hearts (Fig. 3b). skTnI15 (fsTnI) was neither detected in cardiomyocytes of mAb fetal hearts nor in the adult myocardium (Fig. 3a). skTnI50 (specific to both fsTnI and ssTnI) did not interact with either the fetal or adult myocardium (Fig. 3c). We presume that absence of the staining of the fetal myocardium by mAb skTnI50 could be due to insufficient sensitivity of this antibody (Fig. S1 in the Online Resource 1).

Development of sandwich FIAs to detect skeletal TnI isoforms in human blood. Three immunochemical assays were developed based on the obtained mAbs for the detection of fsTnI, ssTnI, or both skeletal TnI isoforms. Table 1 lists analytical parameters of the FIAs (see also Fig. S2 in the Online Resource 1).

The developed assays showed reduced recognition of IC and ITC compared to their recognition of free TnI. Therefore, to restore intensity of the immunochemical signal, EDTA was added to the solution.

Table 1. Sandwich FIAs to detect skeletal TnI isoforms

Skeletal TnI isoforms	LOD, ng/mL (mean ± SD)	Linear range, ng/mL	Level of cross reactivity, %
skTnI89-skTnI91 (fsTnI)	0.07 ± 0.02	0.08-80	<0.004% for ssTnI and cTnI
skTnI27-skTnI58 (ssTnI)	0.10 ± 0.02	0.31-640	<0.005% for fsTnI, 0.026% for cTnI
skTnI25-skTnI50 (fsTnI and ssTnI)	0.10 ± 0.02 for fsTnI 0.11 ± 0.03 for ssTnI	0.08-320 (fsTnI) 0.31-320 (ssTnI)	<0.005% for cTnI

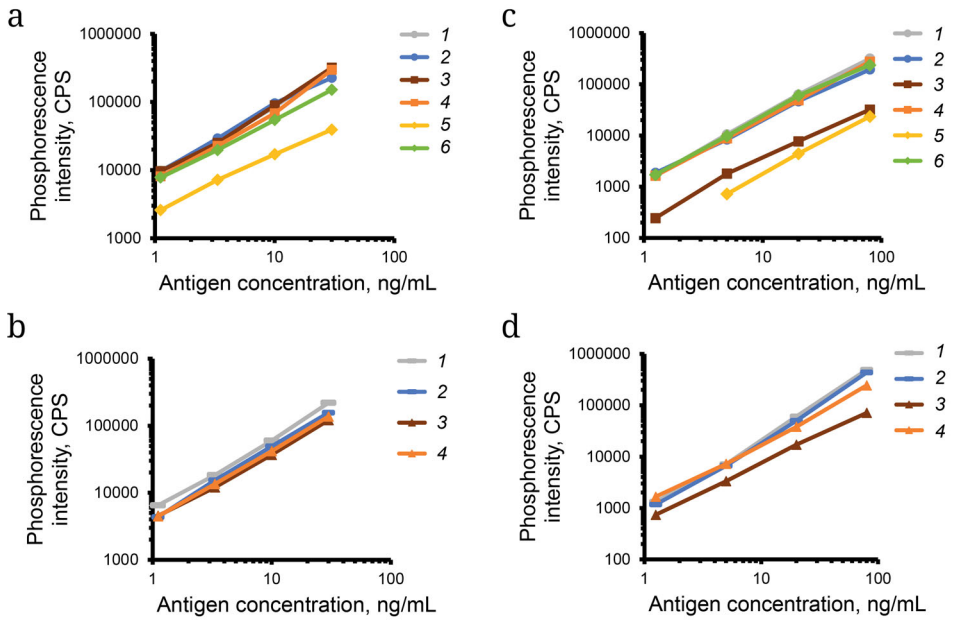

Fig. 5. Detection of free TnI, IC, and ITC with sandwich FIAs in the absence and presence of EDTA. a) skTnI89-skTnI91; b) skTnI58-skTnI27; c, d) skTnI25-skTnI50. Recombinant ssITC was not used due to its instability (dissociation into ssIC and free TnT). a, c) Curves: *1*) fsTnI in the absence of EDTA, *2*) fsTnI in the presence of EDTA, *3*) fsIC in the absence of EDTA, *4*) fsIC in the presence of EDTA, *5*) fsITC in the absence of EDTA, *6*) fsITC in the presence of EDTA. b, d) Curves: *1*) ssTnI in the absence of EDTA, *2*) ssTnI in the presence of EDTA, *3*) ssIC in the absence of EDTA, *4*) ssIC in the presence of EDTA.

Binding of the proteins within the troponin complex is Ca^{2+}-dependent, and previous studies on cardiac isoforms have shown that addition of EDTA leads to dissociation of the ternary cITC and binary cIC; this results in restoration of immunochemical signal in the FIAs based on antibodies specific to free TnI [14, 21, 40]. Additionally, according to our results, incubation of fsITC and fsIC with EDTA results in the complete loss of immunochemical activity in the skTnI14-TnC99A5 assay (see next paragraph), indicating dissociation of fsITC and fsIC (Fig. S3 in the Online Resource 1). Moreover, when fsITC was incubated in the buffer solution containing different concentrations of Ca^{2+} and EDTA, and samples were analyzed at different time intervals in the skTnI89-skTnI91 assay (Fig. S3 in the Online Resource 1), we observed minimal increase in the signal in the presence of Ca^{2+}, and maximal increase in the presence of EDTA. This could indicate both conformational changes and dissociative processes that lead to epitope unveiling. Therefore, we hypothesized that addition of EDTA to the developed FIAs led to a conformational change or dissociation of the complex and, consequently, to recovery of immunochemical activity in detection of the skeletal TnI isoforms (Figs. 5 and 6). After addition of EDTA, immunochemical signals of the skTnI89-skTnI91 (Fig. 5a, curves *5* and *6*) and skTnI25-skTnI50 (Fig. 5c, curves *3*-*6*; Fig. 5d, curves *3* and *4*) assays were recovered in the samples containing binary or ternary complexes. The recombinant ssITC (slow skeletal ITC) obtained *in vitro* was

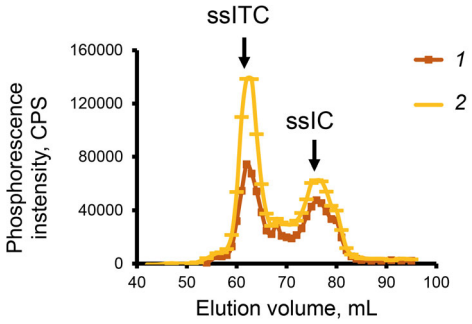

Fig. 6. Detection of troponins in human skeletal muscle extract via sandwich FIA in the absence and presence of EDTA. Fractions obtained from skeletal muscle extract after gel filtration on the Superdex 200 16/600 column were analyzed using the skTnI58-skTnI27 assay via sandwich FIA in the absence (*1*) and presence (*2*) of EDTA. Elution volumes of ssIC and ssITC standards are indicated by arrows.

unstable and dissociated into ssIC and free TnT; hence, the recombinant protein cannot be analyzed with the aforementioned method. Therefore, we studied activity of the skTnI58-skTnI27 assay using human skeletal muscle extracts after separation by gel filtration. When analyzing chromatography profile using this assay, we observed increase in the signal of fractions containing endogenous ssITC after EDTA addition (Fig. 6).

Sandwich FIAs to detect different forms of TnI. An assay capable of recognition of only fsTnI and fsIC, skTnI89-skTnI1, was selected to study the fsTnI forms.

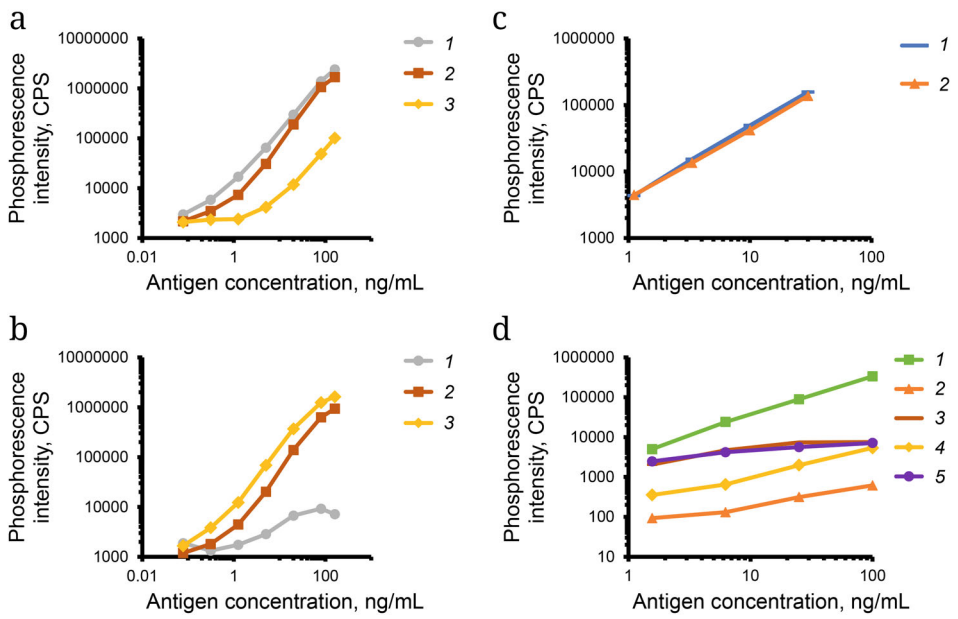

Fig. 7. Specificity of sandwich FIAs for fsTnI (a, b) and ssTnI (c, d) form analysis. a) skTnI89-skTnI1; b) skTnI14-TnC99A5; c) skTnI58-skTnI27; d) TnT111-skTnI38. a, b) Curves: *1*) fsTnI, *2*) fsIC, *3*) fsITC; c) curves: *1*) ssTnI, *2*) ssIC; d) curves: *1*) skeletal muscle extract (the source of ssITC), *2*) ssIC, *3*) ssTnT, *4*) fsITC, *5*) fsTnT.

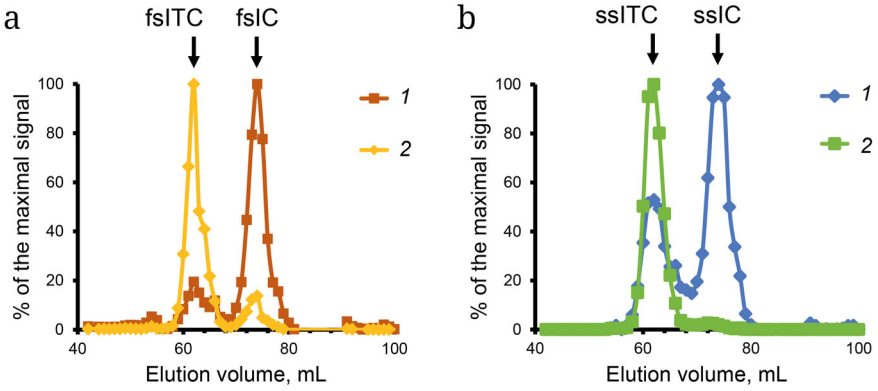

Fig. 8. Forms of skeletal TnI isoforms in human skeletal muscle extract. Immunoreactivity profile of the fractions obtained with gel filtration (using Superdex 200 16/600 column), analyzed via sandwich FIAs using different antibody pairs. a) Sandwich FIAs for detecting fsTnI forms; curves: *1*) skTnI89-skTnI1, *2*) skTnI14-TnC99A5; b) Sandwich FIAs for detecting ssTnI forms; curves: *1*) skTnI58-skTnI27, *2*) TnT111-skTnI38.

Cross-reactivity with fsITC was ~4% (Fig. 7a). When analyzing the fractions obtained from gel filtration of the skeletal muscle extract, only fsIC was detected, as there was no free fsTnI in the extract (Fig. 8a). The second antibody pair, skTnI14-TnC99A5, predominantly interacted with fsITC (Fig. 7b). Cross-reactivity of this assay with free fsTnI was ~4%, and that with fsIC was ~37%. When analyzing fractions obtained after gel filtration of the skeletal muscle extract, the skTnI14-TnC99A5 assay mediated detection of fsITC (Fig. 8a, curve *2*) with an elution volume of ~62 mL. In the process, cross-reactivity with fsIC did not interfere with identification of fsITC because these complexes had different elution volumes (Fig. 8a).

For the ssTnI forms, two assays were selected: the TnT111-skTnI38 assay, which recognizes only ssITC and the skTnI58-skTnI27 assay, which recognizes all forms of the slow skeletal isoform – ssTnI, ssIC, and ssITC (Fig. 7, c, d). Upon analysis of the fractions of skeletal muscle extract produced with gel filtration, these pairs enabled differential detection of ssIC and ssITC (Fig. 8b).

DISCUSSION

Immunochemical detection of skeletal TnI isoforms is of great importance as they are skeletal

muscle-specific proteins and are biomarkers of skeletal muscle pathologies. In the present study, we obtained a panel of mAbs that specifically recognized the skeletal TnI isoforms, fsTnI, ssTnI, or both. We selected the mAbs with the highest sensitivity and specificity that were suitable for detection of the isoforms across various applications (see Table S1 in the Online Resource 1).

Troponins are known to undergo post-translational modifications such as phosphorylation. In particular, the fsTnI isolated from rabbit skeletal muscle is in a partially phosphorylated form [41-43]. However, based on the analysis of the existing data, we could speculate that the proportion of phosphorylated forms of skeletal TnI in the blood is negligible [44-46], and, therefore, would not significantly affect skeletal troponins detection by antibodies [8]. Thus, recombinant fsTnI and ssTnI expressed in *E. coli* without additional modifications were selected as suitable immunogens for mAb production.

The mAbs that specifically bind to skeletal TnI isoforms in WB were selected. Sensitivity of the developed methods allows reliable detection of the target proteins in tissue samples. Specifically, sensitivity of detection with skTnI89 (fsTnI) is 0.01 ng/lane, skTnI38 (ssTnI) is 0.11 ng/lane, and skTnI50 (fsTnI and ssTnI) is 1 ng/lane, while the TnI content in tissue was estimated to be 20 mg per 100 g of tissue (data were obtained for cardiac TnI isoform) [47]. Thus, sensitivity of these mAbs in WB is sufficient to detect skeletal TnI isoforms in approximately 0.05-5 μg of muscle tissue. The mAb specific to fsTnI (skTnI89) recognizes central region of the molecule, which allows detection of not only the full-length protein but also its proteolytic fragments. The mAbs skTnI58 (specific to ssTnI) and skTnI50 (recognizes both skeletal TnI isoforms), which are specific to the C-terminal regions of the molecules, allow detection of the full-length proteins and skeletal TnI isoforms that are proteolytically cleaved at their N-terminal regions.

The developed mAbs could be used for IHC detection of the skeletal troponins; they effectively recognize skeletal muscle fibers but do not stain adult cardiomyocytes. Specificity of the skTnI15 and skTnI30 toward fast- and slow-twitch fibers, respectively, was confirmed by typing skeletal muscle sections with mAbs that interact with different isoforms of MHC. This is a common method to distinguish muscle fiber types [48]. Thus, our mAbs could be used as an alternative method for muscle-fiber typing. During prenatal development, ssTnI is predominantly expressed in the heart and is completely replaced by cTnI during the postnatal period [39]. Our data are consistent with this finding: the mAb skTnI30 recognized ssTnI in the tissue sections of human fetal hearts (29-week- and 41-42-week-old) but did not stain adult cardiomyocytes. Thus, it could also be possible to use this mAb for specific detection of ssTnI in the non-terminally differentiated human cardiomyocytes to determine the extent of cardiac tissue maturation. To date, various approaches for heart transplantation have been developed, including generation of cardiac tissue from the donor cells using induced pluripotent stem cell (iPSC) technology. However, reaching maturity is a challenge for these cardiomyocytes. Many researchers have recognized the need for a differential marker and considered the ratio of cTnI and ssTnI as a potential candidate [49-51]. Currently, WB is used to assess the degree of maturity, but only cTnI has been detected in the cells and tissues. However, the ssTnI-specific mAbs could be combined with the cTnI-specific mAbs to determine the cTnI : ssTnI ratio in non-terminally differentiated human cardiac muscle tissue and the degree of maturity.

One of the most common diagnostic methods is measurement of biomarkers in biological fluids, including blood. Basal concentration of the skeletal TnI isoforms can reach the values of the tenth of nanograms to nanograms per mL [11, 13, 14], and their increased levels in blood indicate skeletal muscle fiber damage. cTnI is released into the bloodstream and is present in the blood not in its free form but as part of various complexes with cTnT and ss/cTnC: ternary complexes with cTnT and ss/cTnC and binary complexes with ss/cTnC. The ratio of these forms changes over time [21, 52-54]. Therefore, detection of cTnI in all its possible forms (complexes) is considered to be the most reliable approach. To the best of our knowledge, there are no experimental data describing the forms in which the skeletal TnIs are released into the human bloodstream or how their composition and ratio change over time. In the present study, we developed immunoassays that differentially interact with the free skeletal troponins and troponins within complexes. For fsTnI, we designed an assay that could detect free fsTnI and fsIC but not fsITC (skTnI89-skTnI1), along with the antibody pair that interacted predominantly with fsITC (skTnI14-TnC99A5) but not with the binary complex or free fsTnI. For ssTnI, the assay that interacts with all forms of the protein was developed (skTnI58-skTnI27), along with the antibody pair that only detects ssITC (TnT111-skTnI38). Combination of these FIAs with the preliminary separation by gel filtration allows differential detection of various forms of skeletal TnIs and could be further used to analyze troponin composition in the blood samples. It would be interesting to compare the data on the content of skeletal troponin complexes in blood with the extensively researched dynamics of cTnI. Moreover, the ratio of skeletal TnI isoforms could vary in different skeletal muscle pathologies and could depend on whether the skeletal muscle damage is a single event, as in trauma,

or a prolonged process characteristic, for example, to dystrophies or muscle atrophy. Different compositions of troponin forms in blood could be present in different diseases, and differential recognition of these forms could serve as a diagnostic marker for the detection of some pathological conditions in the skeletal muscle.

In addition to differential detection of various troponin forms, we developed FIAs that could detect fsTnI and ssTnI individually and simultaneously. The epitopes of mAbs that detect fsTnI (skTnI89-skTnI91) and ssTnI (skTnI58-skTnI27) are specific to the central regions of the proteins. These are the most variable parts of the molecules, and utilization of the mAbs specific to these regions facilitates development of the methods that do not cross-react with other TnI isoforms. In contrast, the mAbs for detection of both skeletal TnI isoforms (skTnI25-skTnI50) are specific to the C-terminal region of the proteins. Although this is the most conserved part of the TnI isoform, it does not cross-react with cTnI. FIAs that specifically detect fsTnI or ssTnI could be used to diagnose diseases in which muscle fibers of a particular type are selectively damaged. This may be important for differential diagnosis of certain pathologies because fast and slow muscle fibers have different regeneration patterns and require different therapeutic approaches [55]. An assay that detects both skeletal TnI isoforms could be used to detect general muscle damage.

Based on the similarity of skeletal TnI isoforms with cTnI, it could be assumed that the skeletal TnI is also present in the blood as part of ternary or binary complexes. Therefore, one of the goals of this study was to develop immunochemical systems that would effectively recognize all possible forms of TnI. However, during the development of sandwich FIAs, recognition of ITC and IC complexes in some assays was significantly lower than that of the free TnI. To achieve comparable recognition of free TnI and its complexes, we adopted a previously established approach: addition of EDTA to the antibody dilution buffer, which presumably leads to dissociation of the troponin complex or changes in its conformation so that the TnI epitopes are exposed for interaction with antibodies [14, 21, 56]. In the presence of EDTA, the sandwich FIAs that detect fsTnI and ssTnI recognize not only free proteins, but also TnI as part of the IC and ITC complexes. Necessity for EDTA addition leading to dissociation/conformational changes is consistent with the epitope specificity of the mAbs we obtained. In particular, according to the data on the structure of troponins within the troponin complex (Fig. 1), mAb $skTnI89_{86\text{-}105}$ (fsTnI) and mAb $skTn27_{58\text{-}63}$ (ssTnI) recognize the site of interaction with TnT, which was confirmed by the recovery of immunochemical signals when EDTA was added to the samples containing fsITC (Fig. 5a, curves *5* and *6*) and ssITC (Fig. 6). Accuracy of the epitope mapping of $skTnI91_{30\text{-}49}$ (fsTnI) and $skTnI58_{30\text{-}49}$ (ssTnI) did not allow us to determine whether these mAbs bind to TnI at the interaction site with TnC. However, according to the results obtained in this study, addition of EDTA had no effect on immunochemical signal in the samples containing fsIC (Fig. 5a, curves *3* and *4*) and ssIC (Fig. 5b, curves *3* and *4*). Hence, we could assume that the epitopes of these mAbs are not shielded by TnC.

The results obtained for the skTnI25-skTnI50 assay could not be clearly interpreted. Immunochemical activities of the binary (fsIC and ssIC) and ternary (fsITC) complexes changed after EDTA addition (Fig. 5). This is despite the fact that both epitopes of the mAbs are specific to the C-terminal parts of the molecules, which are not shielded by other troponins (Fig. 1). We hypothesized that this could be due to conformational changes that affect C-terminal region of TnI when it interacts with TnC and TnT. However, this requires further investigation.

Data on the basal concentrations of skeletal TnI isoforms are controversial. Some studies did not detect skeletal TnI in the healthy individuals [10, 14, 19, 56, 57], which could be associated with insufficient sensitivity of the assays used. In other studies, concentrations of the skeletal TnI in the healthy volunteers measured with the assay recognizing both isoforms were: 1.74 ± 0.27 ng/mL, 0.5 ng/mL (interquartile range, 0.3-0.9 ng/mL), and 2.5 ± 0.9 ng/mL [11, 13, 15]. The reported data regarding troponin levels under various conditions accompanied by the skeletal muscle damage, provide the following mean concentrations of the skeletal TnI: after high intensity exercise – 62.2 ± 139 ng/mL [11], 6 h after running downhill – 27.3 ng/mL (interquartile range, 8.5-43 ng/mL), 6 h after level running – 6.6 ng/mL (3.7-9 ng/mL), and 24 h after eccentric contractions of the *quadriceps femoris* muscle – 6.8 ng/mL (3.1-14.9 ng/mL) [12]. The median concentrations of skeletal TnI isoforms obtained within 24 h after injury were reported as 15.3 ± 2.4 ng/mL after orthopedic injury and 10.4 ± 1.8 ng/mL after soft tissue injury [13]. Median concentration of the skeletal TnI isoforms in the patients with inflammatory myopathies was 8.6 ng/mL (interquartile range, 3.2-33.5 ng/mL) [14]. These data are summarized in Table S2 in the Online Resource 1. LODs of the developed test systems were in the range 0.07-0.11 ng/mL, and their linear ranges were between 0.08-0.31 and 80-640 ng/mL (Table 1). Considering the aforementioned data, sensitivity and linear range of the sandwich FIAs designed in this study are sufficient for reliable quantitative detection of the skeletal TnI isoforms in blood samples of the patients with skeletal muscle injuries.

CONCLUSION

The present study aimed to develop different methods for specific immunochemical detection of the human skeletal TnI isoforms for various applications, including WB, IHC staining, and FIA.

We developed specific and sensitive methods for detection of fast and slow skeletal TnI isoforms (together and separately) by WB. Our mAbs also recognize the skeletal TnI isoforms in human tissues by IHC, differentiated slow and fast fibers, and detected slow skeletal isoforms in the nondifferentiated cardiomyocytes.

Considering that the skeletal TnI isoforms are potential markers of the skeletal muscle damage of various etiologies, we developed methods based on FIA to determine concentrations of the fast and slow isoforms separately and together. Sensitivity of these assays is sufficient to differentiate healthy individuals from those with skeletal muscle injuries. To date, it is still unclear whether the skeletal TnI isoforms are released into the human bloodstream as free proteins or in complexes with TnC and TnT. Several assays have been designed to detect skeletal TnI in human blood, both in the free form and as part of binary and ternary troponin complexes.

In addition, we designed immunoassays that could differentially detect various forms of skeletal TnI isoforms, including binary IC complexes with TnC or ternary ITC complexes with TnT and TnC. These methods could facilitate investigation of the presence of different forms of skeletal TnI isoforms in the human bloodstream.

Abbreviations. Anti-MyHC, antibodies specific to MHC; cIC, cardiac IC; DAB, diaminobenzidine; ELISA, enzyme-linked immunosorbent assay; FIA, fluorescence immunoanalysis; fsTnI, fast skeletal troponin I; fsIC, fast skeletal IC; fsITC, fast skeletal ITC; IC, binary TnI–TnC complex; ITC, ternary TnI–TnT–TnC complex; LOD, limit of detection; mAb, monoclonal antibody; PBS, phosphate buffered saline; PBST, PBS containing 0.1% Tween-20; SD, standard deviation; ss/cTnC, slow skeletal/cardiac troponin C; ssIC, slow skeletal IC; ssITC, slow skeletal ITC; ssTnI, slow skeletal troponin I; TnC, troponin C; TnI, troponin I; TnT, troponin T; WB, western blotting.

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Contributions. I. A. Katrukha and A. G. Katrukha conceived and supervised the study; A. P. Bogomolova, A. V. Bereznikova, A. M. Emelin, R. V. Deev, and A. I. Zabolotsky carried out experiments; A. P. Bogomolova, I. A. Katrukha, and O. S. Lebedeva discussed the results of experiments with input from all authors; A. P. Bogomolova, I. A. Katrukha, A. M. Emelin, and R. V. Deev wrote the manuscript; I. A. Katrukha, A. P. Bogomolova, and O. S. Lebedeva edited the manuscript.

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Ethics approval and consent to participate. This study was conducted in accordance with the current version of the Declaration of Helsinki. All samples and tissues were collected according to a protocol approved by the local Ethical Committee at the Lopukhin Federal Research and Clinical Center of Physical-Chemical Medicine at the Federal Medical Biological Agency of Russia on February 06, 2024 (meeting number, 2024/02/06).

Conflict of interest. The authors declare no conflicts of interest.

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