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Biomarker Discovery and Profiling

Skeletal Muscle Expression of Creatine Kinase-B in End-Stage Renal Disease

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Abstract

The tissue specificity of creatine kinase (CK-MB) has been shown not to be absolute for the myocardium. Unexplained increases of plasma creatine kinase (CK-MB) occur in patients with skeletal muscle disease, confounding the diagnosis of myocardial injury.

The purpose of this study was to examine the expression of CK-B as well as of the nuclear regulatory factor MyoD by Western blot and reverse transcriptase-polymerase chain reaction (RT-PCR) techniques in skeletal muscle biopsies obtained from end-stage renal disease (ESRD) patients.

Quantitation of CKMB mass demonstrated a 35-fold greater concentration in skeletal muscle from ESRD patients (n = 45) vs normal skeletal muscles (n = 10), ($p < 0.01$). In 22 of 45 ESRD skeletal muscles, CK-B expression was detected by Western blot analysis, molecular weight 46 kDa. In the seven CK-B Western blot positive tissues studied, one demonstrated a RT-PCR amplification product at 111 bp. In contrast, no CK-B expression was detected by either Western blot or RT-PCR in normal skeletal muscles. MyoD expression (98 bp) was found in all ESRD and normal skeletal muscles. The intensity of the MyoD expression was greatest in tissues that demonstrated a higher CKMB mass concentration.

Our findings demonstrate an increase in CK-B protein expression in human skeletal muscle obtained from patients with ESRD, that may in part be controlled by an increase in expression of mRNA for CK-B. The resulting increase in CKMB mass in skeletal muscle from ESRD patients may also be regulated by alterations in expression of the nuclear regulatory protein MyD.

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Key Words: CK-MB; end-stage renal disease; chronic hemodialysis; myogenic factor; skeletal muscle; gene expression.

Introduction

Unexplained increases of plasma creatine kinase (CK-MB) in patients with end-stage renal disease (ESRD) occur without a suspected diagnosis of myocardial injury *(1,2)*. The source of the increased CK-MB in blood is often thought to be from diseased or damaged skeletal muscle and not the myocardium *(3–5)*. Skeletal muscle is an extremely adaptable tissue. The B and M genes of CK are expressed differentially during development. The product of the B gene is expressed during early fetal development by all muscle tissues, including skeletal muscle. In fetal development, and postnatally, it is replaced by the CK-M gene product *(5,6)*. In animals and in humans, degenerative diseases of skeletal muscle, such as Duchenne's muscular dystrophy, and repetitive exercise can reactivate CK-B gene expression *(4,6–8)*. This in turn leads to an increase of CK-MB concentrations in skeletal muscle otherwise found only in adult cardiac muscle.

Two families of genes encoding transcription factors have been found to be important in regulating the expression of muscle specific genes: The myogenic regulatory factor (MyoD) family and the myocyte enhancer-binding factor 2 family *(9)*. These nuclear regulatory factor proteins activate transcription in an number of muscle specific genes *(10,11)*. Despite the extensive work on the molecular biology of these transcription factors in normal skeletal muscle development, few studies have examined for the presence of these factors in diseased human skeletal muscle tissue.

The purpose of this study was to determine the concentration of CK-MB mass and the expression of CK-B in human skeletal muscle from ESRD patients at both the protein and mRNA level. The expression of MyoD at the mRNA level in these tissues were also examined.

Methods

Subjects

Forty-five ESRD patients were recruited for participation in this study from the Regional Kidney Disease Program (RKDP) (now Davita) affiliated with Hennepin County Medical Center (HCMC). Informed consents were obtained from the patients according to the Institutional Human Subjects Research Review Board guidelines. Study samples consisted of skeletal muscle biopsies obtained prior to kidney transplantation or change of a vascular access. Skeletal muscle biopsies were obtained from all 45 ESRD patients. Histological, normal, nondiseased skeletal muscle specimens (n = 10) were obtained at autopsy within 15 h of death from subjects who expired after noncardiac-related and non myopathy related illnesses. All biopsied tissues were frozen in liquid nitrogen and stored at –70˚C until analysis.

Western Blot Analysis

As described previously *(12)*, all samples (approx 50 mg) were coarsely ground in a liquid nitrogen-cooled mortal and then added to a protein extraction 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). The samples were homogenized at 4˚C. The supernatants were used immediately for protein analysis and Western blotting. All protein extracts, 50 μg, were size-fractionated on sodium dodecyl sulfate-polyacrylamide gels using the method of Laemmli with the following modifications: 30% acrylamide and 1.1% bis-acrylamide stock solutions were used in 7.5% running gels and 3.3% stacking gels. Proteins were subsequently

transferred to nitrocellulose membrane. After the blocking step, the primary antibody was diluted in antibody buffer and incubated with the membrane. Then, the membranes were washed three times. 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 prior to a 1 min incubation with EC™ chemiluminescent substrate (Amersham). Light emission was detected by exposure to Fuji RX autoradiography film in the presence of Cronex intensifying screens (Fisher Scientific). Signal intensities within the linear range were quantitated using laser densitometry (Molecular Dynamics). Commercially prepared molecular weight standards (Bio-Rad) and purified CK-B proteins (a gift from Spectral Diagnostics, Toronto, Canada) were included in each run as controls. A primary MAb was selected for use in Western blotting, as described previously *(13)*. The mouse MAb specific for CK-B ("Mr Bill") was a gift from the laboratory of Jack Ladenson, PhD, Washington University School of Medicine, St Louis, MO, and was used at 2 mg/L.

Assays

CK-MB concentrations were assayed in muscle homogenates using a monoclonal antibody-based immunosorbant assay, that recognizes CK-MB, but neither CK-MM nor CK-BB, on the Stratus II Analyser (Dade International, Miami, FL) as previously described *(14)*. Total proteins concentrations were determined using a modified Lowry protein assay (Sigma Diagnostics, St Louis, MO). Results are reported as microgram per gram (µg/g) of total protein for CK-MB.

RNA Isolation From Patient Muscles

RNA was extracted from all skeletal muscle tissues as previously described *(15)*. Fifty to one hundred milligrams of each tissue were homogenized with a Polytron homogenizer (Brinkman Instruments, Westbury, NY, cat no. 11590), using a commercial procedure modified from the guanidine-isothiocyanate-phenol method developed by Chomczynski and Sacchi *(15)*. The cell components were disrupted in 1 mL TRIzol reagent (Gibco BRL, Grand Island, NY) and homogenates were incubated at room temperature for 30 min. Following the addition of 0.2 mL chloroform per 1 mL of homogenates, tubes were mixed by vortexing for a few seconds, and incubated at room temperature for 5 min. Samples were centrifuged at 12,000*g* for 15 min at 4°C. RNA was recovered from the upper aqueous phase (600 µL) by precipitation with 0.5 mL isopropyl alcohol. Samples were incubated at room temperature for 15 min, and centrifuged at 12,000*g* for 15 min at 4˚C. The supernatants were removed and the RNA pellets were washed twice with 1 mL 75% ethanol. The samples were mixed by vortexing, and centrifuged at 7500*g* for 5 min at 4°C. The RNA pellets were air dried 5 min at room temperature and reconstituted in 20 µL RNase free water. The purity of RNA was determined from the ratio of absorbance readings at 260 and 280 nm with a desired $A_{260/280}$ ratio between 1.8 and 2.0 indicating sufficient purity. The concentration of RNA was determined from the absorbance at 260 nm. RNA samples were kept frozen at –75°C until used.

Reverse Transcription-Polymerase Chain Reaction

As described previously *(16)*, 1 µg of total RNA was reversed transcribed using Moloney Murine Leukemia Virus (M-MLV) reverse transcriptase (SuperScript™ II, Life Technologies™) according to the supplier's protocol. Forward and reverse primers were designed using the primer design and analysis software Oligo 6.01 (Molecular Biology Insights, Inc., Plymouth, MN). The reverse-transcribed

cDNAs were amplified by polymerase chain reaction (PCR) using the following oligonucleotide primers:

Human CK-B; *(17)*

Forward:5′-CCAGCCCTGCTGCTTCCTAACTTA3′

Reverse: 5′-CCAAGGGTGACGGAAGTCTCTACA3′

Human myoD; *(18)*

Forward: 5′-CGCTCCGCGACGTAGACCTGA3′

Reverse: 5′-GAGTCGAAACACGGGTCGTCATAGA3′

The following components were combined in PCR reaction tubes: 5 µL of 10X PCR buffer solution, 200 m*M* Tris-HCL, pH 8.4; 500 m*M* KCl (Promega Corporation, Madison, WI); 3 µL of $MgCl_2$ solution, 25 m*M* (GenAmp® PCR Products, Perkin Elmer, Branchburg, NJ); 1 µL dNTP solution, 10 m*M*; 0.5 µL *Taq* Polymerase solution, 5 U/µL (Promega Corporation); 2 µL forward primer and reverse primer solutions both at 15 µ*M*; 2 µL of reverse-transcribed cDNAs; and 36.5 µL of autoclaved distilled water with a final reaction volume of 50 µL. Samples were mixed gently and two drops of silicon oil (Sigma Chemical Co., St Louis, MO) were layered over the reaction solution. The reaction tubes were heated at 94°C for 3 min for denaturation. Then, 30 or 40 cycles of PCR were performed at 94°C for 10 s and at 68°C for 45 s in a DNA thermal Cycler (GenAmp® PCR System 2400, Perkin Elmer). The amplified DNA fragments were visualized by 4% modified agarose gel (NuSieve®, FMC BioProducts, Rockland, ME) electrophoresis combined with ethidium bromide staining. Two micrograms of 100 bp DNA ladder (100–2000 bp, GibcoBRL, Life Technologies™) was used as a reference control to estimate the PCR product length. The amplification products obtained by RT-PCR from skeletal muscle mRNAs were compared using DNA sequencing (University of Maine System, Orono, ME) and a sequence comparison software program (Genetic Computer Group version 9.1, Madison, WI).

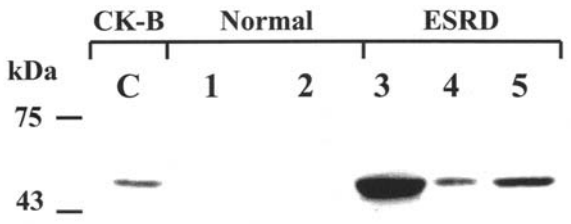

Fig. 1. Representative Western blot of CK-B protein expression of a purified CK-B protein (Control, C), nondiseased human skeletal muscles (Normal), and skeletal muscles from ESRD patients (ESRD). The protein molecular mass (kDa) markers are shown on the left.

Statistical Analysis

Values are expressed as means (SD). Data from the control nondiseased skeletal and ESRD skeletal muscles were analyzed using a student *t*-test (Statistica, StatSoft®, Tulsa, OK). For all statistical tests the 0.05 level of confidence was accepted for statistical significance.

Results

Forty five ESRD patients, on dialysis for between two and nine years, were donors of skeletal muscle biopsies in this study. Their mean age was 57 yr (range 42–63), with 60% males (n = 27) and 40% females, without significant difference between sexes. CKMB concentrations in skeletal muscles determined by mass immunoassay were 35-fold greater in skeletal muscle from ESRD patients, 491 µg/g (SD 447 µg/g), compared to skeletal muscle from normal control patients, 18 µg/g (SD 8 µg/g); $p < 0.01$. Figure 1 illustrates a representative Western blot of purified CK-B protein (control), proteins from a normal human skeletal muscle homogenate, and proteins from skeletal muscle homogenates from patients with ESRD probed with the anti-CK-B MAb. One CK-B isoform, molecular mass 46 kDa,

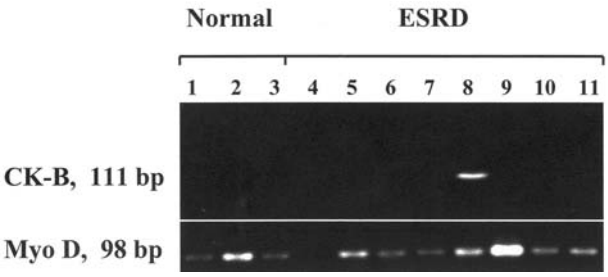

Fig. 2. PCR amplification products of CK-B (111 base pairs, bp) and MyoD (98 bp) from reverse-transcribed RNA from muscle tissues; *lanes 1–3:* nondiseased human skeletal muscle (Normal); *lanes 4–10:* skeletal muscles from patients with ESRD.

was recognized in 22 of 45 skeletal muscle biopsies obtained from patients with ESRD. No CK-B was found in any of the normal skeletal muscles. Figure 2 shows the RT-PCR amplification products found using specific primers for CK-B and MyoD. No RT-PCR amplification products were found for CK-B in normal skeletal muscle samples. Only one of the seven skeletal muscle specimens tested from patients with ESRD showed an amplification product at the expected length (111 bp) for CK-B. RT-PCR amplification products for MyoD (98 bp) were found in all normal skeletal muscle samples and in all skeletal muscle specimens from patients with ESRD. Stronger MyoD amplification intensities were found in skeletal muscle samples from ESRD patients that demonstrated higher CKMB concentrations by immunoassay. Using CK-M protein and skeletal troponin T amplification as invariant controls to assess possible degradation of protein and mRNA that could occur during sample preparation, both levels were consistent within skeletal muscles from both ESRD patients and controls; demonstrating no substantial degradation (data not shown).

Discussion

Our results showed that in skeletal muscle from ESRD patients CK-MB concentrations were 35-fold greater than in normal skeletal muscle from control subjects. Increased CKMB concentrations are supported by demonstrating CK-B protein expression using Western blot analysis, as well as mRNA amplification using RT-PCR.

We demonstrated that one CK-B isoform, molecular mass 46 kDa, was expressed in 22 on 45 skeletal muscle biopsies obtained from ESRD patients and a CK-B mRNA RT-PCR amplification product in one of seven skeletal muscle samples tested. Unfortunately, our study was limited to RT-PCR analysis in only 7 of 45, because of lack of specimen availability. No RT-PCR amplification products were found in normal skeletal muscle samples. Our current findings are in agreement with the increased expression of CK-B and or CK-MB in skeletal muscles obtained from patients with known underlying muscle diseases, such as muscular dystrophy, polymyositis, and dermatomyositis *(5,6,8)*. Further the expression of both CK-MB and CK-B mRNA in skeletal muscle has also been described in gastrocnemius muscle from treadmill trained rats *(7)*, with muscle CK-MB content increasing 220% in exercised-trained skeletal muscle compared to controls; coinciding with an increase of 40% in CK-B subunit mRNA in exercised-trained skeletal muscle. Renal disease is known to be associated with peripheral myopathy *(19)*. Studies have indicated the impaired function of skeletal muscle, exemplified by lower muscle oxidative capacity, correlates with the degree of renal impairment *(20–22)*. The increased presence of CK-MB (CK-B) may indicate specific expression in newly formed (embryonic) muscle fibers as occurs in regeneration, and may serve as a useful marker of regenerating muscle fibers in pathological conditions. However, a limitation of the current study was that no histological analysis was performed on the skeletal muscles from the ESRD patients. Expression of CK-B appears to be partially controlled at the level of tran-

scription, and thus responsible for the increase content of CK-MB in skeletal muscle.

MyoD expression, which is limited to skeletal muscle, coincides with the initiation of myogenic differentiation *(9,23)*. Activation of the myogenic regulatory factors has been found to activate downstream transcription of muscle specific genes such as CK-M, actin, TnT, and myosin light and heavy chains. Despite the extensive work on the molecular biology of these transcription factors in normal skeletal muscle development, few studies have examined the role of these factors on muscle regulation in diseased human skeletal muscle. Our findings show that a MyoD amplicon was detected in all skeletal muscle specimens from patients with ESRD. A more intense signal was found in samples that contained the highest concentrations of CK-MB mass. While we recognize that the molecular evidence presented is preliminary, the clinical implications regarding the nonspecificity of CK-MB for the heart are demonstrated by our findings of increased CK-MB and CK-B content in the skeletal muscle of ESRD patients; therefore weakening the clinical utility of CK-MB as a specific marker for myocardial injury.

References

1 Medeiros LJ, Schotte D, Gerson B. Reliability and significance of increased creatine kinase MB isoenzyme in the serum of uremic patients. Am J Clin Pathol 1987;87:103–108.

2 Jaffe AS, Ritter C, Meltzer V, Harter H, Roberts R. Unmasking artifactual increases in creatine kinase isoenzymes in patients with renal failure. J Lab Clin Med 1984;104:193–202.

3 Kloosterboer HJ, Stoker-de Vries SA, Hommes FA. The development of creatine kinase in rat skeletal muscle. Changes in isoenzyme ratio, protein, RNA and DNA during development. Enzyme 1976; 21:448–458.

4 Lyons GE, Mühlebach S, Moser A, Masood R, Paterson BM, Buckingham ME, et al. Developmental regulation of creatine kinase gene expression by myogenic factors in embryonic mouse and chick skeletal muscle. Development 1991; 113:1017–1029.

5 Goto I. Creatine phosphokinase isozymes in neuromuscular disorders. Arch Neurol 1974; 31:116–119.

6 Apple FS, Rogers MA, Casal DC, Sherman WM, Ivy JL. Creatine kinase-MB isoenzyme adaptations in stressed human skeletal muscle of marathon runners. J Appl Physiol 1985;59: 149–153.

7 Apple FS, Billadello JJ. Expression of creatine kinase M and B mRNAs in treadmill trained rat skeletal muscle. Life Sciences 1994;55:585–592.

8. Yasmineh WG, Ibrahim GA, Abbasnezhad M, Awad EA. Isoenzyme distribution of creatine kinase and lactate dehydrogenase in serum and skeletal muscle in Duchenne muscular dystrophy, collagen disease, and other muscular disorders. Clin Chem 1978;24:1985–1989.

9 Cox DM, Quinn ZA, McDermott JC. Cell signaling and the regulation of muscle specific gene expression by myocyte enhancer-binding factor 2. Exerc Sport Sci Rev 2000;28:33–38.

10 Ordahl CP. Developmental regulation of sarcomeric gene expression. Cur Top Dev Biol 1992; 26:145–168.

11 Lassar AB, Buskin JN, Lockshon D, Davis RL, Apone S, Hauschka SD, et al. MyoD is a sequence-specific DNA binding protein requiring a region of myc homology to bind to the muscle creatine kinase enhancer. Cell 1989; 58:823–831.

12 Ricchiuti V, Voss EM, Ney A, Odland M, Anderson PA, Apple FS. Cardiac troponin T isoforms expressed in renal diseased skeletal muscle will not cause false-positive results by the second generation cardiac troponin T assay by Boehringer Mannheim. Clin Chem 1998;44:1919–1924.

13 Hoang CD, Zhang J, Payne RM, Apple FS. Post-infarction left ventricular remodeling induces changes in creatine kinase mRNA and protein subunit levels in porcine myocardium. Am J Pathol 1997;151:257–264.

14 Voss EM, Sharkey SW, Gernert AE, Murakami MM, Johnston RB, Hsieh CC, Apple FS. Human and canine cardiac troponin T and creatine kinase-MB distribution in normal and diseased myocardium. Infarct sizing using serum profiles. Arch Pathol Lab Med 1995; 119:799–806.

15 Chomczynski P, Sacchi N. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal Biochem 1987;1:156–159.
16 Ricchiuti V, Apple FS. RNA expression of cardiac troponin T isoforms in diseased human skeletal muscle. Clin Chem 1999;45:2129–2135.
17 Mariman EC, Broers CA, Claesen CA, Tesser GI, Wieringa B. Structure and expression of the human creatine kinase B gene. Genomics 1987;1:126–137.
18. Pearson-White SH. Human MyoD: cDNA and deduced amino acid sequence. Nuc Ac Res 1991;19:1148.
19 Floyd M, Ayyar DR, Barwick DD, Hudgson P, Weightman D. Myopathy in chronic renal failure. Quart J Med 1974;43:509–524.
20 Diesel W, Noakes TD, Swanepoel C, Lambert M. Isokinetic muscle strength predicts maximum exercise tolerance in renal patients on chronic hemodialysis. Am J Kid Dis 1990; 16:109–114.
21 Diesel W, Emms M, Knight BK, Noakes TD, Swanepoel CR, van Zyl Smit R, et al. Morphologic features of the myopathy associated with chronic renal failure. Am J Kid Dis 1993; 22:677–684.
22 Tarasuik A, Heimer D, Bark H. Effect of chronic renal failure on skeletal and diaphragmatic muscle contraction. Am Rev Resp Dis 1992;146:1383–1388
23. Wright WE, Sassoon DA, Lin VK. Myogenin, a factor regulating myogenesis, has a domain homologous to MyoD. Cell 1989;56:607–617.