Blood Cells, Molecules, and Diseases 48 (2012) 62–67

Contents lists available at SciVerse ScienceDirect

Blood Cells, Molecules, and Diseases

journal homepage: www.elsevier.com/locate/ybcmd

High levels of brain-type creatine kinase activity in human platelets and leukocytes: A genetic anomaly with autosomal dominant inheritance

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ARTICLE INFO

Article history:
Submitted 13 October 2011
Revised 18 October 2011
Available online 16 November 2011

(Communicated by M. Lichtman, M.D., 18 October 2011)

Keywords:
Creatine kinase
Platelets
Ectopic expression
CKB gene expression

ABSTRACT

The ectopic expression in peripheral blood cells of the brain-type creatine kinase (CKB) is an autosomal dominant inherited anomaly named CKBE (MIM ID 123270). Here, we characterized the CK activity in serum, platelets (PLT) and leukocytes (WBC) of 22 probands (from 8 unrelated families) and 10 controls. CK activity was measured by standard UV-photometry. Expression of the *CKB* gene was analyzed by real-time PCR and Western blotting. DNA sequencing including bisulfite treatment was used for molecular analysis of the *CKB* gene. Serum CK levels were comparable between probands and controls. CKBE probands revealed significantly higher CK activity in PLT (3.7 ± 2.7 *versus* 179.2 ± 83.0 U/10^{12} PLT; $p<0.001$) and WBC (0.4 ± 0.3 *versus* 2.6 ± 2.1 U/10^9 WBC; $p=0.004$). Inhibitory anti-CKM antibodies did not affect CK activity indicating that the CK activity is generated exclusively by the CK-BB isoenzyme. CKB mRNA and protein levels were significantly higher in PLT and WBC from probands compared to controls. Re-sequencing of the entire *CKB* gene and methylation analysis of a CpG island revealed no alteration in CKBE probands. The genetic basis of CKBE remains unclear, however, we propose that a de-methylated *CKB* gene is inherited that leads to high CKB expression levels in myeloic precursor cells in the bone marrow.

Introduction

Creatine kinase (CK, ATP: creatine phosphotransferase, EC 2.7.3.2) is present in tissues with high energy turnover such as muscle and nervous tissues. Because of the dimeric structure of human CK, three different cytosolic isoenzymes CK-MM, CK-MB and CK-BB can be distinguished. In skeletal muscle, nearly 100% of the activity results from the MM isoenzyme, in heart muscle, 5–25% from the MB hybrid form and in brain and nerve tissue nearly 100% are of the BB isoenzyme. For diagnostic Monitoring of CK activity in serum or plasma is widely used in the diagnosis of myopathies and encephalopathies [1–3].

During a newborn screening program for Duchenne muscular dystrophy in the late 1970s [4] using a dry spot test of whole blood [5] some newborns with a positive test with respect to this screening program were found. However, the serum CK was in the normal range and no muscle disease present, that is, the tests were "false positive". To our surprise, we found in one family unusually high levels of CK-BB within the erythrocyte fraction and this anomaly was autosomal dominant transmitted [6]. In some families unusually high levels of this enzyme within platelets were identified. Meanwhile, in this screening program 501,000 newborns were tested and 132 were found "false positive" with respect to the screening program (unpublished data). This ectopic expression of CKB (CKBE, MIM ID 123270) in blood cells is an autosomal dominant inherited anomaly with an estimated frequency of about 1 in 5000 persons. The underlying CKBE mutation has been mapped to chromosome 14q32 where the *CKB* gene is located [7]. However, the molecular basis of this phenomenon remains unknown.

In this study, we re-investigated CK activity in serum, platelets and leukocytes from CKBE probands of eight unrelated families and in normal controls. We further characterized the isoenzyme using inhibitory antibodies against CKM. The CKB expression in platelets and leukocytes was determined at the mRNA and the protein level in probands and normal controls. The promoter, silencer and the coding region of the *CKB* gene were analyzed by re-sequencing. In addition, DNA methylation was determined by bisulfite sequencing.

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doi:10.1016/j.bcmd.2011.10.005

Subjects and methods

Subjects

Because of the need for rapid separation of the blood cells, only families living in Southern Germany were included. Therefore, it was achievable to start cell separation within 3 h of blood withdrawal. From eight unrelated families 22 probands with previously diagnosed high levels of CK activity were included. In addition, we analyzed a control group with three normal individuals from the families and seven unrelated controls (healthy volunteers). For blood collection standard systems (Sarstedt, Nümbrecht, Germany) were used to obtain serum, EDTA blood for automated cell counting (Cell-Dyn 3700; Abbott), and citrated whole blood for isolation of platelets and leukocytes. The baseline characteristics of all study individuals are summarized in Table 1.

Isolation of blood cells

Platelets were isolated and leukocyte-depleted according to a protocol published earlier [8]. Briefly, platelet-rich plasma (PRP) was obtained from citrated blood after centrifugation for 20 min at 150 *g*. PRP was leukocyte-depleted with the use of Purecell PL leukocyte removal filters (Pall Medical GmbH; Bad Kreuznach, Germany). Leukocytes (WBC) were obtained from the residual citrated blood after lysis of the red blood cells. Cell numbers of the platelet (PLT) and the WBC fraction were determined using automated cell counting.

Creatine kinase assays

CK activity was measured in an optical test using a standard test system (CK; Roche Diagnostics, Mannheim, Germany). A similar test for determination of the CK-MB isoenzyme included a mixture of four monoclonal antibodies (mouse) against human CKM with an inhibiting capacity of more than 99% (CK-MB; Roche). In both tests the reduction of NADP was followed at 340 nm in a spectral photometer (Hitachi Life Science; Mannheim, Germany).

Relative CKB mRNA quantification by real-time PCR

Total platelet RNA was extracted as described before [9]. From leukocytes total RNA was extracted using a commercial kit (RNeasy Mini Kit; Qiagen, Hilden, Germany). First strand cDNA synthesis was performed using Transcriptor First Strand cDNA Synthesis Kit (Roche). Real-time PCR for detection of the house keeping genes *GAPDH* and *YWHAZ* as well as the *CKB* gene was performed on a LightCycler 480 system (Roche) using universal probes according to standard protocols. C_p values were used for normalization and relative quantification of mRNA levels according to geNorm algorithms [10].

Relative CKB protein quantification by Western blotting

Total protein extracts from platelets and WBCs of probands and controls were processed for Western blotting using standard procedures. The CKB protein was detected by a goat polyclonal antibody (clone N-20; Santa Cruz Biotechnology, Inc., Heidelberg, Germany). For relative quantification the same blots were stripped and re-hybridized using a monoclonal anti-actin antibody (clone C-11; Santa Cruz Biotechnology). Images from ECL detection of protein bands were taken by a 12-bit CCD camera and further processed using image analysis software (LabWorks 4.5; UVP Ltd., Upland CA, USA).

DNA sequencing of the CKB gene

For promoter and exon re-sequencing genomic DNA was prepared from EDTA-anticoagulated blood samples using a common salting-out method. PCR primers were developed with the help of the primer3 software [11]. Due to high GC content PCR amplification of the promoter region and exon 1 was achieved using specialized kits (GC-rich PCR system, Roche). PCR fragments were sequenced directly after purification in both directions by applying the same amplifying primers or additional primers (Table 2) and the dideoxy chain termination method (DYEnamic ET Kit, GE Healthcare, Munich, Germany) on a MegaBACE500 sequencer. For bisulfite sequencing genomic DNA was isolated from EDTA-anticoagulated blood samples using a commercial kit (QIAamp DNA Blood Mini Kit; Qiagen). Bisulfite conversion was done using EpiTect Bisulfite Kit (Qiagen). The CpG island was amplified and sequenced using primers given in Table 2.

Statistical evaluation

The statistical comparison of blood and CK activity values as well as *CKB* mRNA and protein expression levels was performed using the SPSS statistical software (SPSS v 12.0; SPSS, Munich, Germany). P-values <0.05 represented significant differences.

Results

Inheritance of the CKBE phenotype in eight unrelated families

The CKBE phenotype could be confirmed by a positive test CK activity in blood cells and normal CK activity in the serum in 22 probands from eight unrelated families. All pedigrees indicate an autosomal dominant mode of inheritance (Fig. 1). Three of the probands

Table 1
Baseline characteristics and CK activity of study individuals.

Parameter	Probands (n = 22)	Controls (n = 10)	p-value*
Age, mean ± SD (range)	35.8 ± 20.3	45.7 ± 15.5	0.183
Male, % (n)	68% (15)	30% (3)	0.062
Blood values			
RBC, mean ± SD [M/µl]	5.4 ± 0.7**	4.7 ± 0.2	0.005
HGB, mean ± SD [g/dl]	14.7 ± 2.0	14.2 ± 0.6	0.468
PLT, mean ± SD [K/µl]	282.5 ± 71.3	279.4 ± 34.9	0.896
WBC, mean ± SD [K/µl]	6.9 ± 1.3	6.1 ± 1.1	0.117
Creatine kinase activity			
Serum CK, mean ± SD [U/L]	55.3 ± 33.6	36.2 ± 28.5	0.130
PLT CK, mean ± SD [$U/10^{12}$ PLT]	179.2 ± 83.0	3.7 ± 2.7	<0.001
WBC CK, mean ± SD [$U/10^{9}$ WBC]	2.56 ± 2.09	0.35 ± 0.27	0.004

* Comparison of the two groups by *t*-test or Fisher's exact test (for gender); significant differences showed p-values<0.05.
** Significantly higher RBC counts in probands were due to one Turkish family (four individuals) with thalassemia minor.

Table 2
Primers for re-sequencing and bisulfite sequencing of the *CKB* gene.

Region	Forward (5′–3′)	Reverse (5′–3′)
Silencer	ACAGATGAGGGTCCCTGATG	ACTCGAGGACGCTTGGGTTC
Promoter	TGCGGGCACCTTGAGGAAG	CTTTGCACGCAGCGCCCCTAGCC
Exon 1	CCAATGGAATGAATGGGCTA	CAGCTTCAGTGCGTTGTG
Exon 2	CTGAGTGGTACGCGGGAG	TGCAGGTTGTCGGGGTTG
Exon 3–4	CTTCACGCTGGACGACG	GGGCGACGTAAACAAAAGC
Exon 5	ACTGGACGCCCGCAGAT	GAGAGGGAAAGCGGAGGTAG
Exon 6–8	TGGGAAGTTCCTTTGGTCTG	GGCACCACTCAGACGCA
Additional sequencing primers		
Silencer	CCAGCTCAAAGCCAGCAATG	
Exon 2		GGGACGGGGACAGTGAC
Exon 3	GCCACCCGTACATCATG	TGCAGGTTGTCGGGGTTG
Exon 7	CCTGTAGGGGTTTCAGGCA	CTGGGGAGACAGCAAGTCA
Bisulfite sequencing		
CpG	ATCCAAGGACCTGAGGTTTCGG	GGGACGCGGCCAAGGTCAGCG