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Brain tissue identification based on myosin heavy chain isoforms

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Abstract Non-muscle tissues contain 3 myosin heavy chain (MHC) isoforms; MIIA, MIIB1 and MIIB2. MIIA is a non-muscle type isoform distributed in all non-muscle tissues and smooth-muscle, while MIIB1 and MIIB2 are brain-type isoforms distributed mainly in neuronal tissues. The ratio of MIIA and MIIB (A/B ratio) differs between tissues, suggesting that this ratio may be a useful marker for tissue identification.

To apply the A/B ratio for tissue identification in forensic practice, we developed a highly sensitive ELISA for quantification of each MHC isoform. At least 100 pg of both MHC isoforms could be detected by the present method. Analysis of the A/B ratio of the cerebrum, cerebellum, liver, kidney, spleen and andrenal gland by the present method indicated that the A/B ratio of the brain tissue (< 0.5) was quite different from other tissues (>3.0). The A/B ratio could be determined from at least 8 μg of fresh tissue sample and 0.1 mg of dried tissue sample stored for 1 month at room temperature. Therefore, the A/B ratio seems to be an excellent marker for identification of the brain tissue.

Key words Brain · Tissue identification · Myosin isoform · ELISA

Zusammenfassung Nicht-Muskelgewebe enthalten drei Isoformen der schweren Kette des Myosins (MHC); MIIA und MIIB (MIIB1 und MIIB2). MIIA MHC ist eine Isoform vom Nicht-Muskel-Typ und ist in allen Nicht-Muskelgeweben und glatter Muskulatur verbreitet, während MIIB1 und MIIB2 MHC Isoformen vom Hirn-Typ sind und hauptsächlich in neuronalem Gewebe vorkommen. Die Ratio von MIIA und MIIB (A/B-Ratio) unterscheidet sich zwischen den Geweben, so daß es nahe liegt, daß diese Ratio ein nützlicher Marker zur Gewebeidentifizierung sein könnte.

Um diese A/B-Ratio zur Gewebeidentifizierung in der rechtsmedizinischen Praxis anwenden zu können, entwickelten wir einen hochsensitiven ELISA zur Quantifizierung jeder MHC Isoform. Mit dieser vorliegenden Methode konnten wenigstens 100 pg von beiden MHC Isoformen nachgewiesen werden. Die Analyse der A/B-Ratios von Großhirn, Kleinhirn, Leber, Niere, Milz und Nebenniere mit der vorliegenden Methode zeigte an, daß die A/B-Ratio von Hirn-Gewebe (< 0,5) sich gänzlich von anderem Gewebe (> 3,0) unterschied. Die A/B-Ratio konnte an frischer Gewebeprobe von mindestens 8 μg und an 0,1 mg getrockneter, 1 Monat bei Zimmertemperatur aufbewahrter Gewebeprobe bestimmt werden. Daher scheint diese A/B-Ratio ein ausgezeichneter Marker für die Identifizierung von Hirngewebe zu sein.

Schlüsselwörter Hirn · Gewebeidentifizierung · Myosin Isoform · ELISA

Introduction

It is convenient for identification of individuals by DNA polymorphisms but not for tissue identification in forensic practice that all somatic cells possess uniform genetic information within the genome. mRNAs are often transcribed in a tissue-specific manner, but are not suitable markers for tissue identification because they are extremely unstable. Therefore, tissue identification must be performed on the basis of tissue-specific proteins including tissue-specific isoforms or carbohydrate chains [1]. In murder cases or traffic accidents, tissue identification must occasionally be performed on minute tissue samples. For this purpose, histological or immunological procedures have been mainly employed. Monoclonal antibodies (mAbs) specific for marker proteins or carbohydrate chains are suitable reagents for this purpose [2, 3].

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In our previous studies concerning myosin heavy chain (MHC) isoforms in human tissues, we showed that non-muscle tissues expressed substantial amounts of non-muscle-type (MIIA) MHC and brain-type (MIIB) MHCs, and we produced mAbs specific for each MHC [4]. These mAbs seemed to be applicable for tissue identification.

In this study, we established highly sensitive enzyme-linked immunosorbent assay (ELISA) systems for quantification of each MHC and applied them for the brain tissue identification from minute and dried tissue samples.

Materials and methods

Tissue samples

Human tissue samples were obtained at autopsies. Tissues (brain, liver, kidney, spleen and adrenal gland) for fresh samples were frozen immediately and stored until use, while those for dried samples were weighed, dried and stored on glass slides at room temperature until use.

Extraction of myosin from human tissues

Tissue samples were homogenized in 100 µl of 25 mM Tris-HCl buffer (pH 7.5) containing 0.3 M NaCl, 10 mM $MgCl_2$, 10 mM ATP, 1mM EGTA, 5 mM 2-mercaptoethanol and 1 mM phenylmethylsulfonyl fluoride at 4°C. The homogenates were centrifuged for 10 min at 15,000 × g and the supernatants were used for MHC analysis as crude myosin extracts. Human cerebrum and liver myosin were purified as described previously for bovine myosins [5].

Monoclonal antibodies (mAbs)

HBM1 (IgG1) and HBM3 (IgG1) which were specific for brain-type MHC isoforms and non-muscle-type MHC isoform, respectively, were produced by immunizing mice with purified human cerebrum myosin as described previously [4]. BBM4 (IgM), which reacted efficiently with the smooth muscle or non-muscle and brain-type MHC isoforms but not with the skeletal or cardiac MHC isoforms, was obtained by immunizing mice with purified bovine cerebrum myosin as described previously [5]. Biotinylation of BBM4 was performed with Sulfo-NHS-Biotin (PIERCE, IL, USA) according to the supplier's instructions.

Enzyme-linked immunosorbent assay (ELISA)

HBM1 and HBM3-coated 8-well microstrips (Labsystems, MA, USA) were prepared by incubating overnight at 4°C with 100 µl/well of HBM1 and HBM3 solution (10 µg/ml in 10 mM phosphate buffered saline, pH 7.4), respectively, and blocked with 0.3% gelatin in 10 mM Tris-HCl buffer, pH 7.4 containing 0.15 M NaCl. Myosin extracts were diluted with 10 mM Tris-HCl buffer, pH 7.4 containing 0.5 M NaCl, 0.3% gelatin and 0.05% Tween-20, and then 100 µl aliquots were incubated on the mAb-coated microstrips for 1 h at room temperature. The myosins bound to the solid-phase mAb were detected by using biotin-conjugated BBM4 as a detector antibody, and followed by β-galactosidase-conjugated streptavidin (Boehringer Mannheim, Germany) and 4-methylumbelliferyl-β-D-galactopyranoside (New Brunswick, UK) as reagents for production of fluorescent products [6]. The fluorescence intensity (EX, 360 nm; EM, 450 nm) was measured with a microplate reader (MTP-100F, CORONA, Japan).

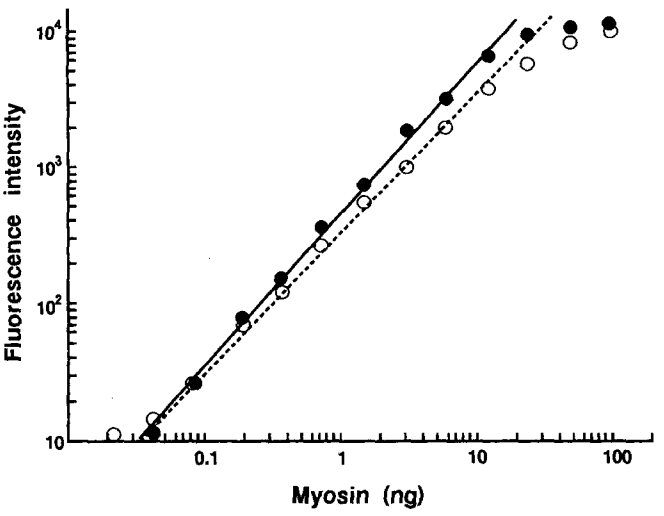

Fig. 1 Calibration curve of ELISA for non-muscle-type and brain-type MHC. Non-muscle-type (MIIA)- and brain-type (MIIB) MHCs were detected by ELISA on microstrips coated with mAbs HBM3 and HBM1, respectively. The myosin bound to microstrips was detected using biotin-conjugated BBM4, followed by β-galactosidase-conjugated streptavidin and 4-methylumbelliferyl-β-D-galactopyranoside. ●: MIIA in purified human liver myosin, ○: MIIB in purified human cerebrum myosin

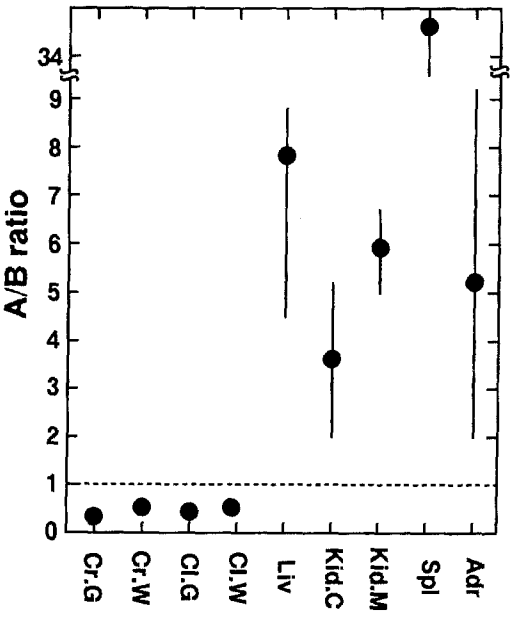

Fig. 2 A/B ratios of human tissues. A/B ratios in the crude myosin extracts from human tissues were determined by ELISA. Each point and bar depict mean and SE (standard error) in triplicate experiments. Cr.G: gray matter of the cerebrum, Cr.W: white matter of the cerebrum, Cl.G: gray matter of the cerebellum, Cl.W: white matter of the cerebellum, Liv: liver, Kid.C: cortex of the kidney, Kid.M: medulla of the kidney, Spl: spleen, Adr: adrenal gland

Results

ELISAs for quantitative analysis of non-muscle-type (MIIA) MHC and brain-type (MIIB) MHCs were established. The mAbs HBM3 and HBM1 which were specific

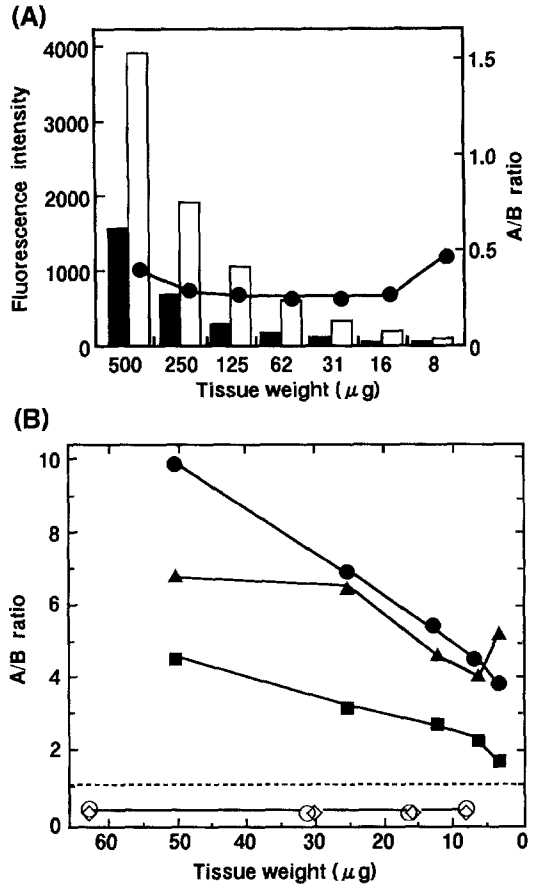

Fig. 3 The effects of quantity on detection of MHCs and A/B ratios of human tissues. (A) MHC levels and A/B ratios were determined by ELISA on serial dilutions of crude myosin extracts from minute human cerebral tissue samples (1 mg). Tissue weight was calculated from dilutions. The solid and open bars express fluorescence intensities of MIIA and MIIB, respectively. The closed circles express A/B ratios. (B) A/B ratios were determined by ELISA on serial dilutions of minute human tissue samples (1 mg). □: cerebrum, ◇: cerebellum, ●: liver, ■: kidney, ▲: adrenal gland

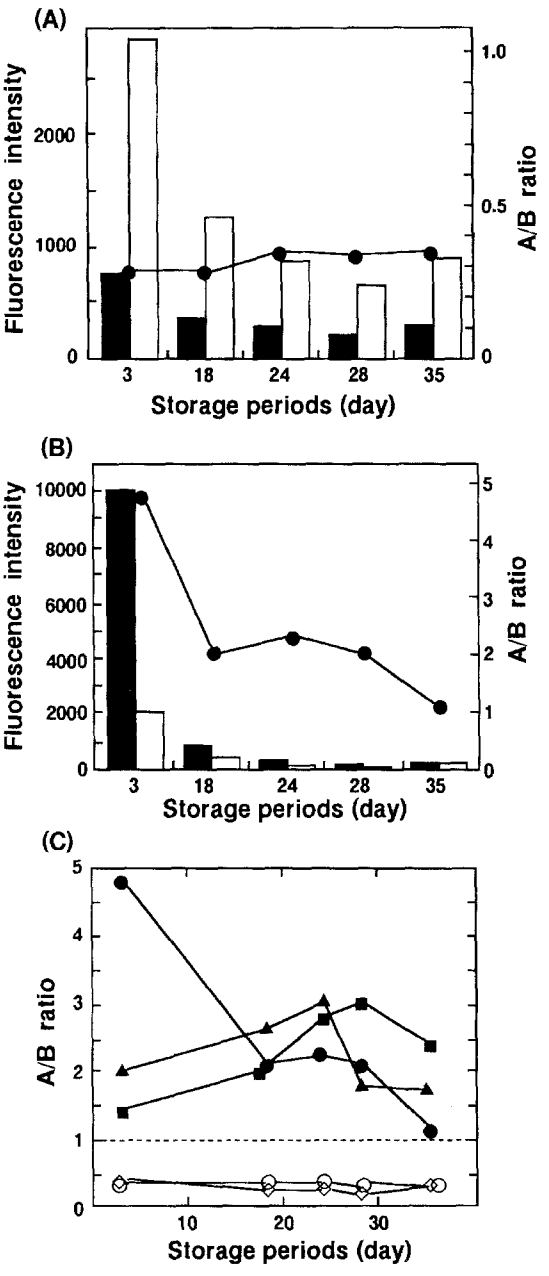

Fig. 4 The changes of the detection levels of MHCs and the A/B ratios of the human tissues during storage at room temperature. MHC levels and A/B ratios of the human cerebrum (A) and liver (B) were determined by ELISA at the indicated days after storage. The solid and open bars express fluorescence intensities of MIIA and MIIB, respectively. The closed circles express A/B ratios. (C) Changes in A/B ratios in the human tissues during storage at room temperature. ○: cerebrum, ◇: cerebellum, ●: liver, ■: kidney, ▲: adrenal gland

for MIIA and MIIB, respectively [4], were used as solid-phase antibodies for specific capture of each MHC isoform. MHCs captured by solid-phase antibodies were detected by the biotin-conjugated mAb BBM4 reactive to both MHCs. Figure 1 shows detection of MIIA and MIIB in purified human liver and cerebrum myosin, respectively, by this ELISA. MIIA and MIIB were detected in at least 100 pg of purified myosin from each tissue. The calibration curve of each isoform showed linearity between 100 pg and 10 ng.

Quantities of MIIA and MIIB in crude extracts from various human tissues were measured by the present method for determination of the ratio of MIIA and MIIB (A/B ratio) in each tissue. The A/B ratio was expressed as the ratio of fluorescence intensity of MIIA to MIIB in ELISA found in this study. As shown in Fig. 2, A/B ratios of the cerebrum and the cerebellum (< 0.5) were quite different from those of other tissues (liver, kidney, spleen and adrenal gland; >3.0), indicating that brain tissue could be discriminated from other tissues on the basis of the A/B ratio.

To assess the effects of quantity of specimens, A/B ratios of serial dilutions of extracts from minute tissue samples were determined by the present method. Fluorescence intensity declined gradually with dilution of extracts. However, no significant change of A/B ratio was observed at least to a dilution corresponding to 8 μg of cerebral tissue (Fig. 3A). The A/B ratio of the brain tissue never exceeded 0.5. However, in other tissues the A/B ra-

tio declined gradually by dilution of extracts but never went below 1.7 at least to a dilution corresponding to 3μg of tissues (Fig. 3B).

A/B ratios from dried tissue specimens (about 0.1 mg) stored at room temperature were determined periodically for 40 days. The detection level of MHCs in the dried cerebral tissue gradually declined during storage. However, the A/B ratio did not change significantly for 35 days (Fig. 4A). In the dried liver tissue, the detection level of MHCs declined faster than in other tissues, and the A/B ratio changed significantly, but was never below 1.0 (Fig. 4B). In other dried tissues, the A/B ratio did not change significantly (Fig. 4C). On day 40 the MIIA isoform could not be detected in the brain tissue, and neither MIIA nor MIIB were detected in the liver tissue or adrenal gland tissues (data not shown). These results indicated that the brain tissue could be identified from the A/B ratio from at least 0.1 mg of dried tissue specimen after storage for 1 month.

Discussion

The contractile protein myosin is well known as a force-generating protein in muscle tissues. Many isoforms of muscle-type MHC have been identified and well characterized [7, 8]. Recent progress in studies of myosin in non-muscle tissues have shown that almost all non-muscle tissues show considerable amounts of myosin [4, 5, 9, 10]. There are at least 3 MHC isoforms; non-muscle-type MHC of 196 kDa (MIIA or NM3) and 2 brain-type MHCs of 200 and 198 kDa (MIIB1 or NM1 and MIIB2 or NM2, respectively) in non-muscle tissues [4, 9]. The MIIA isoform is widely expressed in various non-muscle tissues. Whereas the MIIB1 isoform is expressed almost exclusively in the brain, the MIIB2 isoform is also expressed in the brain and in small amounts in other non-muscle tissues, especially in the kidney and adrenal gland [4, 9, 10]. MIIB1 is assumed to be an alternatively spliced product of the MIIB2 MHC gene with an inserted sequence of about 2kDa [10]. The MIIB1 isoform seems to be the most suitable marker protein of the brain tissue, however, no mAb specific for this unique sequence in MIIB1 has yet been obtained. It is likely that the unique sequence in MIIB1 is hidden in the global head region of the myosin molecule [10]. Although MIIB2 is expressed in most tissues, the level of the expression in the brain is much higher than in other tissues. Therefore, the ratio of MIIA and MIIB (MIIB1 and MIIB2) in brain tissue differs from those in other tissues. Indeed, it was evident in the newly developed ELISA for quantification of MHCs that A/B ratios of brain tissues (< 0.5) differed from those in other tissues (> 3.0) (Fig. 2). These results are consistent with those obtained by northern blot analysis of chicken tissues [10]. Furthermore, the quantity of specimens did not significantly affect the A/B ratios in these tissues (Fig. 3B). Therefore, it can be said that tissue showing an A/B ratio below 0.5 is brain tissue. Since both MHCs are not rare constituents in the brain tissue (~ 0.5% of total protein) [11], brain tissue can be discriminated from other tissues by the present method from as little as 8 μg of specimen. It seems to be very difficult or impossible to perform tissue identification on such minute specimens by histological procedures. Detection levels of MHCs from dried tissue samples declined gradually during storage at room temperature (Fig. 4A, B). However, the A/B ratio of the brain never exceeded 0.5, and that of other tissues never went below 1.0 after storage for 35 days (Fig. 4C), suggesting that there was no significant difference in stability of both MHCs. Brain tissue could be identified from its A/B ratio for at least 1 month from 0.1 mg tissue samples. However, it must be noted that specimen storage conditions would affect the detection limits of MHCs by the present method.

Conclusion

A highly sensitive ELISA for quantification of MHCs was developed. This method enabled us to determine ratios of MIIA and MIIB (A/B ratio) from several μg of tissue samples. The A/B ratio of the brain (<0.5) differed markedly from those of other tissues (>3.0). Therefore, brain tissue can be identified from its A/B ratio in minute tissue samples.

References

1. Seo Y, Takahama K (1992) A sandwich enzyme immunoassay for liver-specific antigen and its forensic evaluation. Jpn J Leg Med 46:169–176
2. Kimura A, Matsumura F, Sodesaki K, Tsuji T (1991) Production and characterization of monoclonal antibodies against tissue specific epitopes on ABO blood group substances in saliva. Int J Leg Med 104:193–196
3. Kimura A, Matsumura F, Sodesaki K, Osawa M, Ikeda H, Tsuji T (1991) ABO blood grouping of semen from mixed body fluids with monoclonal antibody to tissue-specific epitopes on seminal ABO blood group substance. Int J Leg Med 104:255–258
4. Kimura A, Nakashima S, Uda T, Ikeda H, Yasuda S, Tsuji T, Matsumura S (1993) Heavey-chain isoforms of non-muscle myosin in human tissues. Eur J Biochem 213:5–10
5. Kimura A, Tsuji T, Matoba R, Fujitani N, Ohmori K, Matsumura S (1991) Tissue-specific and non-specific heavy-chain isoforms of myosin in the brain as revealed by monoclonal antibodies. Biochim Biophys Acta 1118:59–69
6. Mukaida N, Kasahara T, Ko Y, Kawai T (1988) Establishment of highly sensitive enzyme-linked immunosorbent assay for interleukin-1α employing a fluorogenic substrate. J Immunol Methods 107:41–46
7. Emerson CP Jr, Bernstein SI (1987) Molecular genetics of myosin. Annu Rev Biochem 56:695–726
8. Warrick HM, Spudich JA (1987) Myosin structure and function in cell motility. Annu Rev Cell Biol 3:379–421
9. Murakami N, Mehta P, Elzinga M (1991) Studies on the distribution of cellular myosin with antibodies to isoform-specific synthetic peptides. FEBS Lett 278:23–25
10. Kawamoto S, Adelstein RS (1991) Chicken nonmuscle myosin heavy chains; Differential expression of two mRNAs and evidence for two different polypeptides. J Cell Biol 112:915–924
11. Burridge K, Bray D (1975) Purification and structural analysis of myosins from brain and other non-muscle tissues. J Mol Biol 99:1–14