Transient Expression of a Brain/Embryonic-Type Myosin Heavy Chain Isoform (MIIB2) in Regenerating Rat Liver

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The expressions of non-muscle-type (MIIA) and brain/embryonic-type (MIIB2) myosin heavy-chain isoforms in regenerating rat liver were examined. In regenerating liver after partial hepatectomy, the level of MIIA was nearly constant, while that of MIIB2 increased transiently. The level of MIIB2 was very low in normal livers, and increased gradually and then declined with a peak at the 4th day, when the regeneration was almost completed. The level of proliferating cell nuclear antigen was highest at the 2nd day. Serine dehydratase activity in the liver decreased on partial hepatectomy, began to increase at the 5th day, and reached 71% of the control at the 7th day. These results suggest that MIIB2 plays a role in reconstruction or differentiation of the regenerating tissues rather than in proliferation of hepatocytes.

Key words: brain-type myosin heavy chain, cell growth, liver regeneration, non-muscle-type myosin heavy chain, PCNA.

Vertebrate tissues express at least three non-muscle-type myosin heavy chain (MHC) isoforms, a non-muscle-type MHC of 196 kDa (MIIA or NM3) and two brain-type MHCs of 200- and 198 kDa (MIIB1 or NM1 and MIIB2 or NM2, respectively) (1-3). The MIIB1 MHC (brain-type) is supposed to be an alternatively spliced product of the MIIB2 MHC (brain/embryonic-type) gene with an inserted sequence of about 2 kDa in the head region (4). MIIA isoform is widely expressed in various non-muscle tissues (1-3). Whereas the MIIB1 isoform is expressed almost exclusively in the brain, the MIIB2 isoform is also expressed in small amounts in other non-brain tissues such as kidney and adrenal gland (1-3). The restricted expression of MIIB1 in the brain suggests its contribution to specialized neuronal functions (2). Several lines of evidence suggest that the MIIA is involved in cellular proliferation, especially in cytokinesis (5-7). Recent studies indicate that many fetal and neonatal tissues (8, 9) as well as the neointima of atherosclerotic arteries (9, 10) express considerable levels of the MIIB2 isoform, suggesting that MIIB2 is also involved in cellular proliferation, although its role is not known.

It is well known that the liver regenerates very rapidly after partial hepatectomy; the remnant liver recovers its original tissue weight within 10 days after 70% hepatectomy (11). Therefore, the regenerating liver seems to be a very suitable experimental model for analysis of biochemical events that occur during cell growth in vivo. In this study, we have analyzed the expression of MIIB2 in the liver following partial hepatectomy to elucidate the function of MIIB2 in cellular proliferation.

MATERIALS AND METHODS

Animals and Partial Hepatectomy—Female 7-week-old Wistar rats weighing 170 to 190 g were employed for partial hepatectomy and sham operation. These rats were fed ad libitum on a nutritionally balanced rodent diet and water. For 70% hepatectomy, the medial and left hepatic lobes were excised under ether anesthesia as described by Higgins and Anderson (11). Histological examination indicated that tissue specimens from the livers of both control and partially hepatectomized rats did not contain necrotic lesions to any appreciable extent (data not shown).

Monoclonal Anti-Myosin Antibodies—BBM4 (IgM), which reacted efficiently with the smooth muscle, non-muscle and brain-type MHC isoforms but not with the skeletal or cardiac MHC isoform, was obtained by immunizing mice with purified bovine cerebrum myosin as described previously (1). HBMI (IgGl) which was specific for brain-type MHC isoforms was produced by immunizing mice with purified human cerebrum myosin as described previously (2). SMemb, which was specific to embryonic and brain-type MHCs (10), was a generous gift from Yamasa Shoyu (Chiba).

Immunohistochemical Detection of Proliferating Cell Nuclear Antigen—The remnant livers were dissected out from the rats, weighed, fixed in formalin, and then embedded in paraaffin. After deparaffinization of the liver sections, endogenous peroxidase was inactivated with 3% H2O2. Proliferating cell nuclear antigen (PCNA) (12, 13) in liver sections was stained with a peroxidase-conjugated monoclonal antibody (PC10) to PCNA (Daco) and 3-aminol-9-ethylcarbazole (Vector) according to the manufacturer's

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Abbreviations: ELISA, enzyme-linked immunosorbent assay; MHC, myosin heavy chain; PCNA, proliferating cell nuclear antigen; SDH, serine dehydratase.

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instructions. Counter-staining of the specimens was performed with hematoxylin solution. The index of PCNA expression was determined by counting more than 2,000 nuclei in photomicrographs of three randomly selected fields taken under the light microscope.

**Assay of Serine Dehydratase (SDH) Activity**—Livers were excised from the rats at the indicated days after partial hepatectomy or sham operation, and 300 mg of each liver was homogenized with 1 ml of 0.05 M potassium phosphate buffer, pH 7.2, containing 0.15 M KCl and 1 mM EDTA. The homogenates were centrifuged for 20 min at 10,000 × g, and SDH activity of the supernatants was measured by the method of Suda and Nakagawa (14). One unit of the activity is defined as the activity forming 1 μmol of pyruvate per min at 37°C. Protein was measured by the method of Lowry et al. (15).

**Extraction of Myosins from Rat Tissues**—Livers were excised from the rats at the indicated days after partial hepatectomy or sham operation, and 500 mg of each liver was homogenized for 30 s in 4 ml of a low-salt solution containing 10 mM imidazole-HCl (pH 7.0), 5 mM EGTA, 5 mM EDTA, 5 mM 2-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride, 1 mM benzamidine-HCl, and 0.1 mg/ml each of pepstatin A, leupeptin, aprotinin, and soybean trypsin inhibitor. The homogenates were centrifuged for 10 min at 15,000 × g and the supernatants were discarded. The pellets were extracted as above with 1 ml of 25 mM Tris-HCl buffer (pH 7.5) containing 0.3 M NaCl, 10 mM MgCl₂, 10 mM ATP, 1 mM EGTA, 5 mM 2-mercaptoethanol, and the protease inhibitors, and the extracts (crude myosin extracts) were subjected to MHC analysis. The above extraction procedures were carried out at 4°C. Where indicated, crude myosin extracts were prepared from the cerebellum and neonatal livers by the same procedures as described above.

**Enzyme-Linked Immunosorbent Assay (ELISA)**—HBM1-coated 96-well plates were prepared by incubation overnight at 4°C with 100 μl/well of HBM1 solution (10 μg/ml in 10 mM phosphate-buffered saline, pH 7.4) and blocked with 0.3% gelatin in 10 mM Tris-HCl, pH 7.4, and 0.15 M NaCl. The crude myosin extracts (10 μg protein in 100 μl of 10 mM Tris-HCl, pH 7.4, containing 0.5 M NaCl, 0.3% gelatin, and 0.05% Tween-20) were incubated on the HMB1-coated plates for 1 h at room temperature. The myosins bound to HBM1 were detected by using biotin-conjugated BBM4 as a detector antibody followed by avidin-horseradish peroxidase and o-phenylenediamine as reagents for color development. The bound myosins were calibrated with purified human brain myosin as a standard.

**Immunoblotting**—SDS-PAGE (7.5% acrylamide) was carried out as described by Laemmli (16). The polypeptides separated on the gels were transferred electrophoretically (0.8 mA/cm² for 1 h) onto polyvinylidene difluoride (PVDF) membrane sheets (Millipore) with a semidy-type blotter and then blotted with monoclonal anti-myosin antibodies. The primary antibodies bound to the membrane sheets were detected with biotin-conjugated anti-mouse IgG or IgM (CALTAG) followed by streptavidin-horseradish peroxidase conjugate (Amersham) and 4-chloro-1-naphthol. The blot with BBM4 was analyzed densitometrically by using an image scanner, JX-320M (Sharp) with NIH Image 1.54.

**RESULTS**

Figure 1 shows the time courses of the changes in the liver weights and the expression of PCNA, a molecular marker for the cells in S-phase (12, 13), after 70% hepatectomy. Concomitantly with the increase in the expression of PCNA, the livers increased in weight rapidly, and gained more than 80% of the original weight within 4 days after partial hepatectomy. The liver weight continued to increase thereafter. The PCNA was highest at the 2nd day and then declined. The specific activity of serine dehydratase [EC 4.2.1.13] (SDH) in crude extracts from the remnant livers decreased to less than 15% of the original level at the 2nd day, began to increase again at the 5th day, and reached 71% of the original level at day 7 after partial hepatectomy (Fig. 2). Crude myosin extracts were prepared from the livers of control and partially hepatectomized rats and from the cerebellum and neonatal livers, and these extracts were subjected to SDS-PAGE and immunoblotting (Fig. 3). Immunoblotting with BBM4, which reacts with both non-muscle and brain-type MHC isoforms, indicated that
non-muscle MHC (MIIA) is the most abundant isoform in the livers of both control and partially hepatectomized rats and that the relative levels of the isoforms, mostly MIIA isoform, in a fixed quantity of the tissue samples did not change to an appreciable extent following partial hepatectomy (Fig. 3A). On the other hand, immunoblotting with SMemb, which recognizes both embryonic and brain-type MHC isoforms, revealed that, whereas the levels of embryonic or brain-type MHC were very low in the extracts from control rats and the rats at 1 day after partial hepatectomy, the level of MHC increased at 2 to 4 days after partial hepatectomy and then declined after 5 days (Fig. 3B). Crude extracts from neonatal rat livers contained a considerable level of SMemb-reactive MHC. Comparison of the electrophoretic mobility of the SMemb-reactive MHC in the extracts from regenerating livers with those from the cerebellum and neonatal livers indicated that the MHC was identical or very similar to the MIIB2 isoform. Immunoblotting with another brain-type isoform-specific anti-myosin antibody HBM1 gave a similar result (data not shown). These results indicate that MIIB2 is expressed transiently in regenerating livers. The changes in the level of MIIB2 during liver regeneration were analyzed quantitatively by ELISA. As shown in Fig. 4, the level of MIIB2 was highest at 4 days after hepatectomy, in accordance with the results obtained by immunoblotting. The level of total MHC (MIIA plus MIIB2) did not appear to change greatly.

**DISCUSSION**

Kawamoto and Adelstein (17) observed a transient increase and decrease of the expressions of mRNAs for MIIA and MIIB2, respectively, in cultured fetal serum-stimulated chicken embryo fibroblasts which had been starved in a serum-free medium. These changes in the expressions of the mRNAs occurred within a few hours (G1-phase) after addition of fetal bovine serum, and the levels of the mRNAs returned to the respective initial levels within approximately 20 h. These changes were followed by an increase in DNA synthesis (S-phase), suggesting that the changes in the mRNAs are related to the proliferation of the cells, although the changes of the mRNAs were not affected by pretreatment of the cells with mitomycin C, which blocked the synthesis of DNA. The relative concentration, but not the total content, of MIIA did not increase or increased only slightly in regenerating livers (Figs. 3 and 4). The relative concentration as well as the total content of MIIB2 began to increase one day after partial hepatectomy and reached the highest level at the 4th day. The level of PCNA was highest at the 2nd day (Fig. 1). The level of PCNA determined in this report was consistent with the previous observation that the synthetic rate of DNA as determined by the incorporation of 5-bromo-2-deoxyuridine into the tissue samples was maximal at 36 h after 70% hepatectomy (18). The peak of the expression of MIIB2 thus appeared to be delayed by approximately 2 days from the peak of DNA synthesis (Fig. 3). Since the synthesis of DNA is usually followed by G2- and M phases within 6 h, the synthesis of MIIB2 appears to increase after completion of the cell division of the majority of hepatocytes. In fact, the increase in the tissue weight was almost completed within 4 days after partial hepatectomy. However, regenerated hepatocytes seemed to be still immature at the 4th day. The specific activity of SDH, a key enzyme of gluconeogenesis (19) in the regenerating livers was still very low at the 4th...
day and began to increase at the 5th day (Fig. 2), suggesting that differentiation of hepatocytes in the regenerating livers begins at the 5th day after partial hepatectomy. The transient increase of MIIB2 in the regenerating livers seems to occur after cell division and during or at the beginning of differentiation of the hepatocytes. Thus, MIIB2 does not appear to be prerequisite for the growth of the hepatocytes, unlike MIA, which appears to be indispensable for cell growth (5-7). Although the meaning of the transient increase of MIIB2 isoform in regenerating liver is not known, the isoform is likely to play a role in reconstruction or differentiation of the hepatic tissues. Brain myosin has been shown to possess different physical and enzymatic properties from those of smooth muscle and other nonmuscle myosins (20). Furthermore, brain myosin was shown to exhibit a considerable magnitude of contractile activity in vitro in response to phosphorylation by Ca\(^{2+}/\)calmodulin-dependent myosin light chain kinase (21). Whereas these results obtained previously with purified bovine cerebrum myosin now appear to reflect the properties of a mixture of the MIIB1 and MIIB2 isoforms and of MIA isoform as well, some of these properties presumably reflect those of the MIIB2 isoform itself. The MIIB2 isoform thus may be involved in some special motility processes which occur not only in brain tissues but also in other non-brain tissues under certain developmental conditions, such as in regenerating liver and fetal and neonatal tissues.

REFERENCES