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Nucleophosmin/B23 Is a Proliferate Shuttle Protein Associated With Nuclear Matrix

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Abstract It has become obvious that a better understanding and potential elucidation of the nucleolar phosphoprotein B23 involving in functional interrelationship between nuclear organization and gene expression. In present study, protein B23 expression were investigated in the regenerative hepatocytes at different periods (at days 0, 1, 2, 3, 4, 7) during liver regeneration after partial hepatectomy on the rats with immunohistochemistry and Western blot analysis. Another experiment was done with immunolabeling methods and two-dimensional (2-D) gel electrophoresis for identification of B23 in the regenerating hepatocytes and HepG2 cells (hepatoblastoma cell line) after sequential extraction with detergents, nuclease, and salt. The results showed that its expression in the hepatocytes had a locative move and quantitative change during the process of liver regeneration post-operation. Its immunochemical localization in the hepatocytes during the process showed that it moved from nucleoli of the hepatocytes in the stationary stage to nucleoplasm, cytoplasm, mitotic spindles, and mitotic chromosomes of the hepatocytes in the regenerating livers. It was quantitatively increased progressively to peak level at day 3 post-operation and declined gradually to normal level at day 7. It was detected in nuclear matrix protein (NMP) composition extracted from the regenerating hepatocytes and HepG2 cells and identified with isoelectric point (pI) value of 5.1 and molecular weight of 40 kDa. These results indicated that B23 was a proliferate shuttle protein involving in cell cycle and cell proliferation associated with nuclear matrix. J. Cell. Biochem. 90: 1140–1148, 2003.

Key words: nucleophosmin (NPM)/B23; liver regeneration; partial hepatectomy; nuclear matrix; immunohistochemistry; two-dimensional gel electrophoresis

Nucleophosmin (NPM, also called protein B23, numatrin, protein NO 38) is a major nucleolar phosphoprotein which is more abundant in cancer cells and proliferating cells than in normal resting cells [Chan et al., 1989]. It shuttles between the nucleus and cytoplasm [Borer et al., 1989], and translocates from the nucleolus to nucleoplasm during the stationary phase of cell growth [Yung et al., 1990a] or during treatment with certain anti-cancer drugs [Yung et al., 1992; Wu et al., 1995]. Its putative function is involved in the synthesis, assembly, and/or transport of ribosome [Fankhauser et al., 1991; Wang et al., 1994], in DNA synthesis [Feuerstein et al., 1990; Takemura et al., 1994], and in chromosome organization in mitosis [Hernandez-Verdun and Gautier, 1994].

The nuclear matrix is defined as the insoluble structural framework of the nucleus after sequential extraction of nuclei with non-ionic detergents, nucleases, and high-salt buffers

The study is experimental.

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[Berezney and Coffey, 1974]. It consists of protein shell, lamina, and internal meshwork of protein fibers under ultra microscopy and participates in many nuclear functions such as organization of the enormous length of chromatin [Ciejek et al., 1983], DNA replication, RNA synthesis and processing, and hormone binding in the nucleus [Barrack and Coffey, 1990]. Protein B23 was identified as a nuclear matrix protein (NMP) abundant in the malignant cells: KB (epidermoid carcinoma), HepG2 (human hepatoblastoma), and the proliferating B lymphocytes [Feuerstein and Mond, 1987; Feuerstein et al., 1988].

For increasing understanding and potential elucidation of the nucleolar phosphoprotein B23 involving in functional interrelationship between nuclear organization and gene expression, B23 expression was investigated in the regenerative hepatocytes at different periods (at days 0, 1, 2, 3, 4, and 7) after about 70% partial hepatectomy with immunohistochemistry and Western blot analysis. The experiments were done with B23 identification as NMP in the regenerating hepatocytes and HepG2 cells (hepatoblastoma cell line) after sequential extraction with immunolabeling method and two-dimensional (2-D) gel electrophoresis.

MATERIALS AND METHODS

Normal and Regenerating Rat Livers

Animals. Ten healthy Wistar male rats of 300–500 g were prepared. All the rats were kept on a controlled lighting schedule with a 12 h dark period. Food and water were available ad labium but prior to experiments the rats were fasted overnight.

Partial hepatectomy. These rats were done for partial hepatectomy under ether anesthesia according to the method of Higgins and Anderson [Feuerstein et al., 1990]. The large median and the left lateral lobes were surgically excised and the right lateral and the small caudate or spigelian lobes were left. In this procedure, 65–75% of the livers were removed. These operated rats were divided into five groups with two rats in each group according to five different killing time points at days 1, 2, 3, 4, and 7 post-operation. The liver tissues of the large median and the left lateral lobes resected were used as controls as the liver samples at day 0 post-operation on the rats. The regenerating liver tissues were obtained when the rats were killed at different time points. The liver samples from each case were cut into two pieces, one piece was fixed and used for immunohistochemistry, another was first polished for hepatocyte purification by low speed centrifugation and the cell lysate was then made for SDS–PAGE gel separation and Western blot analysis.

Cell Culture

HepG2 cells was routinely cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), 100 IU/ ml penicillin, and 100 IU/ ml streptomycin. The culture was incubated at 37°C in an atmosphere of 5% carbon dioxide in air. The cells that grown on cover slip were fixed in situ with 10% neutral buffered formalin stored at 4°C for immunocytochemistry and processed with nuclear matrix extraction in situ.

Extraction Methods of Nuclear Matrix and NMP

Extraction of nuclear matrix in situ. The cells on cover slip were extracted in cytoskeleton (CSK) buffer containing 100 mM KCl, 3 mM $MgCl_2$, 1 mM EGTA, 1 mM PMSF, 10 mM PIPES pH 6.8, 300 mM sucrose, and 0.5% Triton X-100 for 15 min at 4°C. The chromatin was removed by incubation of the pellet in DNase I (100 μg/ml) and RNase A (100 μg/ml) in the buffer solution in the presence of 50 mM NaCl instead of KCl with protease inhibitor for 30 min at room temperature about 24°C and terminated by the addition of cold ammonium sulfate. The cell residues were fixed and prepared for immunofluorolabeling.

Extraction of NMPs. The NMPs were isolated by the previous method [Wang et al., 1999]. Briefly, the cultured cells and the regenerating hepatocytes were step-by-step extracted in CSK buffer and nuclease buffer solution and terminated by cold ammonium sulfate. The remaining pellet fraction was soluble in the disassembly buffer and dialyzed against the assembly buffer. The NMPs were left in the supernatant fraction after ultra centrifugation and dried by N_2 gas. The NMPs resolved in the sample buffer were prepared for 2-D electrophoresis.

Immunofluorolabeling and Immunohistochemistry

The cultured HepG2 cells or the in situ extracted cell residue were fixed and prepared for immunocytofluorescine staining. The mouse

monoclonal antibody against B23 is a gift of Prof. P.K. Chan (M.D. Anderson Cancer Center, Houston, TX). The routine method was done, same as immunocytochemistry, except the second antibody changed to fluorescine-conjugated antibody. The fluorescent signal was observed under fluorescent microscopy.

Fresh rat liver tissues were fixed and prepared for immunohistochemistry [Yun et al., 2002]. The three-step immunoperoxidase method using Strept–Avidin Biotin (Dakopatts, Glostrup, Denmark) was performed according to the routine procedure. Simply, after blocking, the sections were incubated in primary antibodies overnight and followed by incubation in secondary antibodies, and further incubated in Strept–Avidin Biotin complex (Dakopatts) and developed in chromogen DAB solution. After contrast staining, the stained sections were finally dehydrated and mounted. The brown particle in the cell was considered as positive staining. Percentages of positive stain nuclei of hepatocytes in each case were respectively counted under total ten high-power fields and the median percentages of each group were figured out.

2-D Electrophoresis and SDS–PAGE Gel Electrophoresis

High-resolution 2-D gel electrophoresis was carried out using the Mini-PROTEAN II electrophoresis system (Bio-Rad, Hercules, CA) [Jin et al., 2001]. One hundred micrograms of NMP samples were dissolved in the first dimensional gels for isoelectric focusing and then run the gels for SDS–PAGE gel electrophoresis. Two slab gels from the same samples were done, one for silver staining and another for Western blot analysis.

Ten microliters of the hepatocyte lysate were separated by SDS–PAGE gel electrophoresis same as above, and then the slab gels were prepared for Western blot analysis.

Western Blot Analysis and Immunolabeling

The slab gels were processed for Western blot analysis and immunolabeling as previous method [Jin et al., 2001; Yun et al., 2002]. Briefly, The gels were first equilibrated and the proteins in the gels were transferred onto blotting membranes. After blocking, the membranes were routinely incubated with mouse monoclonal antibody against B23, rabbit anti-mouse IgG (Amersham Life Science, Piscataway, NJ), and horseradish peroxidase-conjugated mouse anti-rabbit IgG. The blots were then incubated with ECL kit (Amersham) and developed for 3 min with X-ray film. The intensities of the bands in the film were measured by software "Quantity One" (Bio-Rad).

RESULTS

B23 expression were observed in the regenerative hepatocytes at different periods after partial hepatectomy. The results showed that its expression in the hepatocytes had a locative move and quantitative change during the process by immunohistochemistry (Figs. 1–3) and Western blot analysis (Fig. 4).

Using monoclonal antibody against B23, its immunochemical localization of hepatocytes showed that B23 faintly localized in nucleoli of the hepatocytes at day 0 as control. At day 1 post-operation, its nucleolar expression of the regenerative hepatocytes were strongly stained and circle-shaped, but little of its expression occurred in the nucleoplasm and cytoplasm. At day 2, its expression were increased in the nucleoplasm and cytoplasm. At day 3, B23 expression peaked, over 90% of the hepatocytes with strongly positive nucleoplasm were figured out, over 90-fold than those at day 1 and day 7. Since the mitotic hepatocytes easily found at day 3, B23 expression appeared in cytoplasms, chromosomes, spindles, and the nuclei of daughter hepatocytes (Fig. 2). At day 4, its expression were gradually declined including decreased hepatocytes with strongly positive nucleoplasm. At day 7, it was most localized in the nucleoli, scarcely in the nuclei and the cytoplasm. The results showed B23 locations were changed from nucleoli of the hepatocytes in the stationary stage to nucleoplasm, cytoplasm, and mitotic chromosomes of the regenerating hepatocytes and back to nucleoli of the regenerated hepatocytes during the process of liver regeneration. In Figure 3, statistics of the hepatocytes with positive nucleoplasm indicated that B23 semi-quantitative expression altered from at low level in the stationary stage of the hepatocytes to peak level in the regenerating hepatocytes and back to low level in the regenerated hepatocytes during the process, and at day 3 post-operation, its expression attached to peak level.

Using monoclonal antibody against B23 to detect the hepatocye lysate from the liver

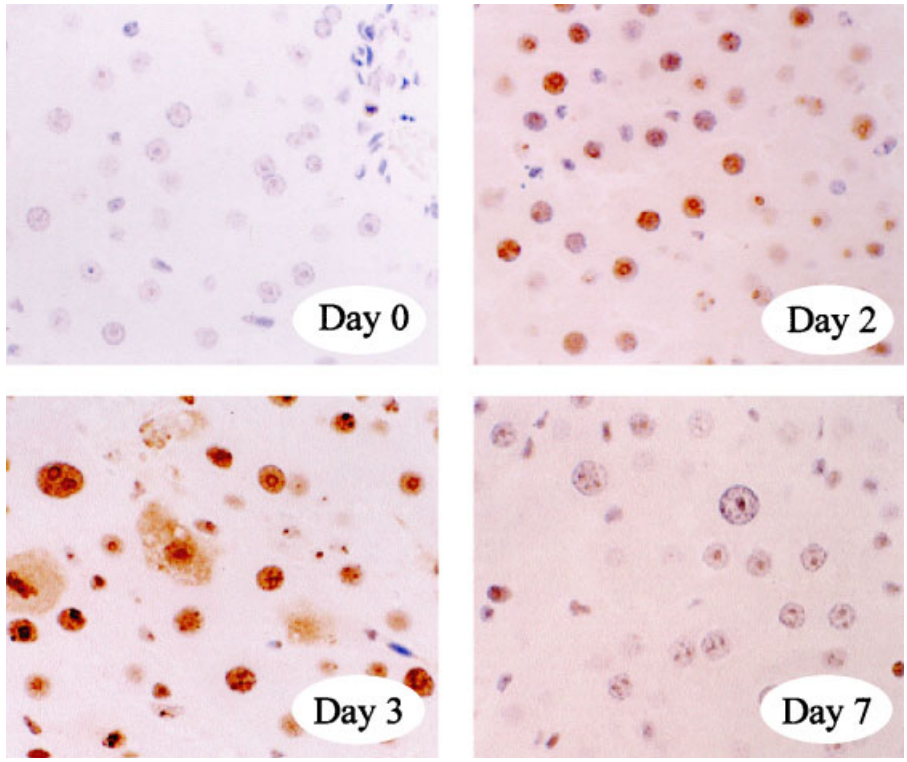

Fig. 1. The immunohistochemical pictures showed B23 positive expression in the hepatocytes from the liver samples obtained at different time points (days 0, 2, 3, 7) after partial hepatectomy. Higher nuclear expression of B23 in the liver samples at days 2 and 3 after partial hepatectomy were compared with those at days 0 and 7; 200×. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

samples obtained at different periods (at days 0, 1, 2, 3, 7) post-operation, the Western blot analysis results showed that B23 expression of the regenerative hepatocytes at day 3 was at the highest level among those five different time points, and its expression at day 7 was declined similar expressive level to that at day 1 (Fig. 4).

Another experiments were done with nuclear matrix extraction for the regenerating hepatocytes of the liver samples on day 3 post-operation and the cultured HepG2 cells (human hepatoma cell line). These results showed that B23 was detected in NMP composition extracted from the regenerating hepatocytes and the HepG2 cells, and identified with pI value of 5.1 and molecular weight of 40 kDa by 2-D gel electrophoresis and Western blot analysis. It was observed in the nuclear matrix residue of HepG2 cells after sequential extraction by immunocytofluoroscene labeling (Figs. 5–7).

DISCUSSION

The liver regeneration model after partial hepatectomy is one of the best in vivo models for cell cycle and cell proliferation. In the normal adult liver, hepatocytes are quiescent, exhibiting minimal replicate activity with rare mitosis observed in approximately 1 in every 20,000 hepatocytes. After partial hepatectomy, the hepatocytes are activated into entering cell cycle from G_0 phase (quiescent state). The maximal hepatocyte DNA synthesis occurs in 24 h with a second small peak occurring in 48 h. During liver regeneration, most of the hepatocytes are estimated to replicate once or twice. Once the original size and volume of the liver is achieved, the hepatocytes revert to their quiescent state [Michalopoulos and DeFrances, 1997].

The data from present study showed that B23 expression of hepatocytes had quantitative increase and locative alteration during the process of liver regeneration. These expressive alterations of B23 in the hepatocytes were consistent with different phases of cell cycle and hepatocyte proliferation during liver regeneration. In normal rat livers, the majority of the hepatocytes are in quiescent state or G_0 phase, B23 expression was faintly stained in the

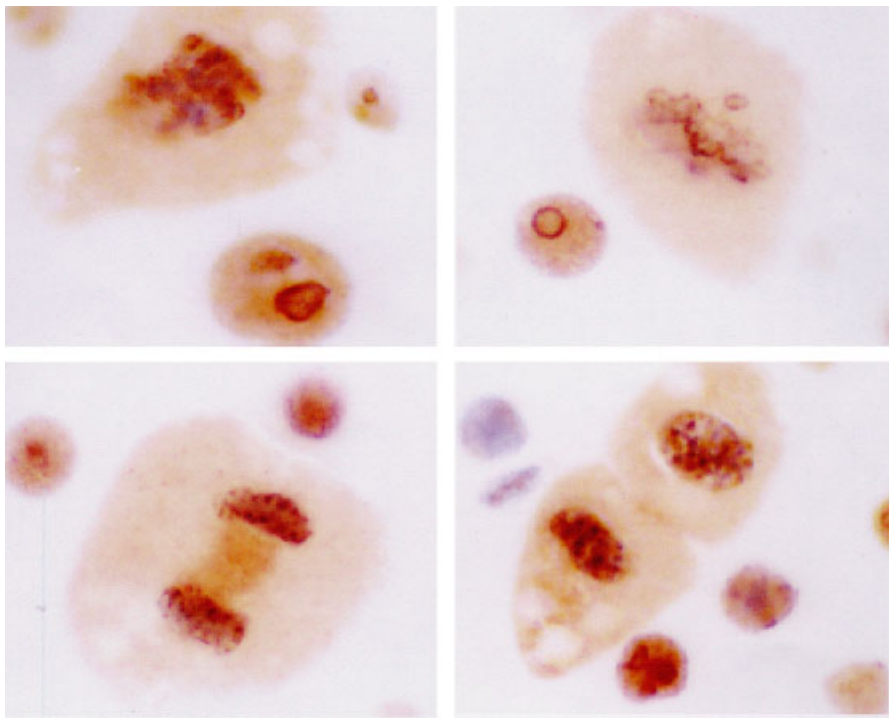

Fig. 2. B23 positive expression in the mitotic hepatocytes were labeled in brown color with hematoxylin (blue color) as counter stain by immunohistochemistry; 1,000×. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

nucleoli of hepatocytes. At day 1 post-operation, the majority of the hepatocytes entering G_1 or S phase that rRNA synthesis and its transcriptional activities increased, B23 was localized predominantly in the nucleoli of the hepatocytes. At day 3, the majority of the hepatocytes entering G_2 phase and M phase that DNA synthesis increased and cells were divided with chromosomal organization, the expression of B23 were progressively increased to the highest

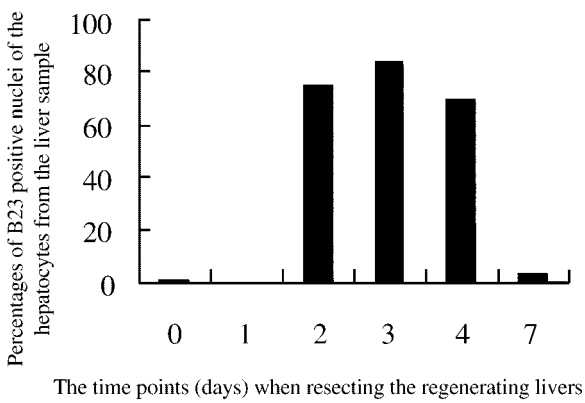

Fig. 3. The schematics showed percentages of B23 positive nuclei of the hepatocytes from the liver samples obtained at different time points (days 0, 1, 2, 3, 4, 7) after partial hepatectomy. High nuclear expression of B23 in the liver samples at days 2 to 4 post-operation were figured out, of them the highest is at day 3, over 90-fold than those at day 0, 1, or day 7.

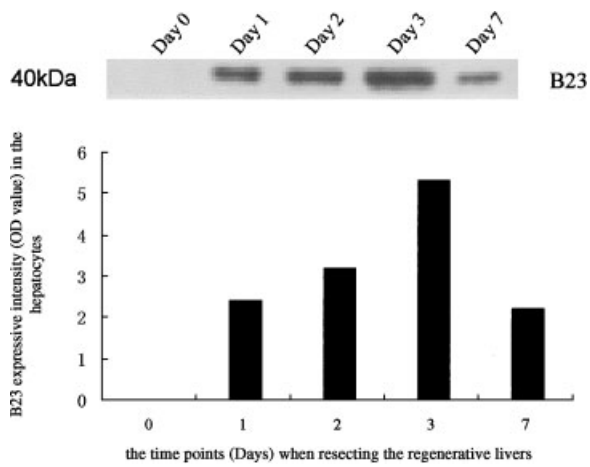

Fig. 4. The bands detected in the hepatocyte lysate by Western blot analysis (**upper**) and their OD values measured by ''Quantity One'' software (**lower**) were representative of the intensities of B23 expression in the hepatocytes from the liver samples obtained at the days 0, 1, 2, 3, and 7 after partial hepatectomy. The highest expression of B23 appeared in the liver samples at day 3 after partial hepatectomy.

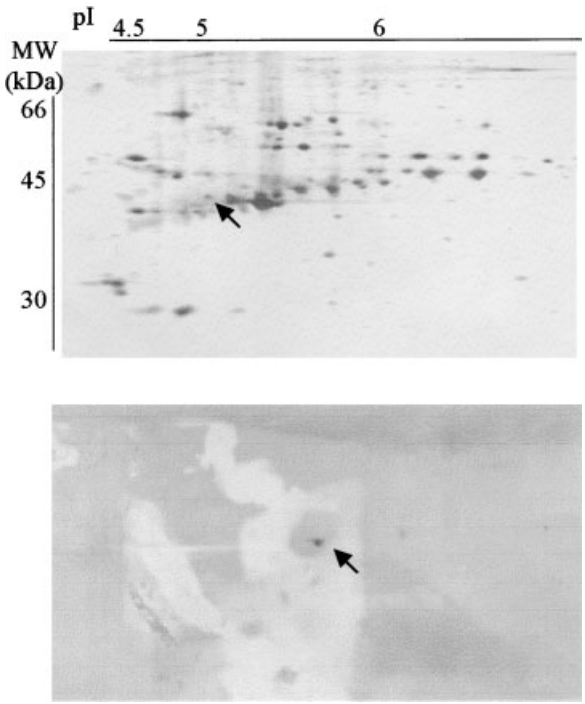

Fig. 5. Using two-dimensional (2-D) gel electrophoresis (**upper**) and Western blot analysis (**lower**), B23 signal was detected in the nuclear matrix protein (NMP) composition extracted from the regenerating hepatocytes obtained from the liver samples at day 3 after partial hepatectomy (indicated by arrow).

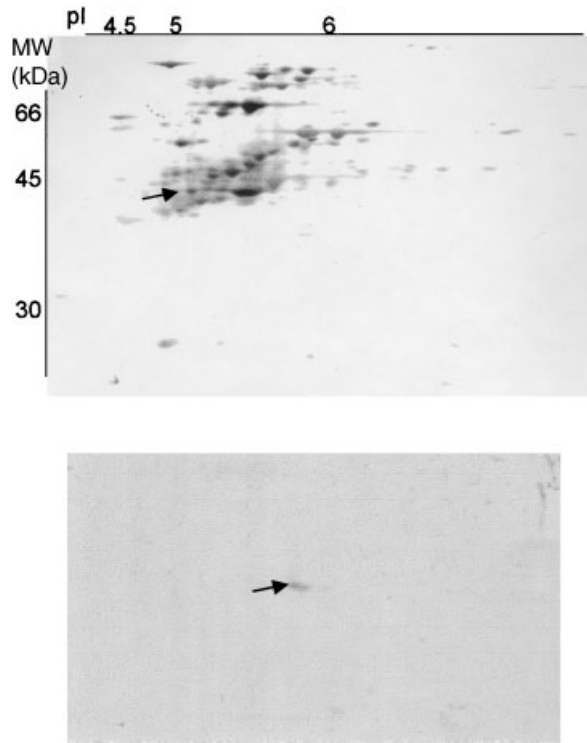

Fig. 6. Using 2-D gel electrophoresis (**upper**) and Western blot analysis (**lower**), B23 signal was detected in the NMP composition extracted from the HepG2 cells (indicated by arrow).

levels and localized either in nuclei of the hepatocytes or in the mitotic hepatocytes. At day 7, majorities of the hepatocytes reverted to enter the G_0 phase or quiescent state, B23 expression decreased back to the normal liver level and localized in the nucleoli of hepatocytes. The results showed that protein B23 shuttled from the nucleoli of the hepatocytes in the stationary stage to nuclei, cytoplasm, and mitotic chromosomes of the regenerating hepatocytes and back to the nucleoli of the regenerated hepatocytes during the process of liver regeneration after partial hepatectomy. It implied that B23 played different roles in different phases of cell cycle, including involvement with rRNA and DNA synthesis and chromosomal organization.

Ochs et al. [1983] found that protein B23 was localized in the nucleolus of rat–Kangaroo Ptk2 cell. As the nucleolus disappeared in prophase, the distribution of protein B23 became nucleoplasmic. Throughout the metaphase and anaphase, protein B23 was found to be associated with chromosome. When the nucleolus reformed during telophase, it did not appear in the nucleolus until late telophase or early G_1 phase. Yung et al. [1990b] discovered that B23 shifts from nucleolus to nucleus of HeLa cells with increasing doses of actinomycin D and longer incubation periods. The degree of translocation of protein B23 from nucleoli to nucleoplasm was dependent on the amount of the drug used and the duration of incubation. Their data showed that B23 translocation was closely associated with the states of cell growth and inhibition of RNA synthesis. Zatsepina et al. [1997] used a new monoclonal antibody against B23 (20B2) to observe B23 expression in the cell cycle of cultured cell lines. B23 was mainly located within the nucleolus at interphase and associated with a few cellular domains during mitosis. Its expression presented within the nucleoplasm at prophase before the nuclear envelop breakdown and within the cytoplasm from prometaphase. Neverova et al. [1999] studied post-mitotic reconstruction of nucleoli in culture cells with UV-microbeam photoinactivated centrosome. Protein B23 was present in the mitotic poles and at the chromosome surface. In the anaphase, it dispersed in the cytoplasm of cells in early G_1 period. The typical nucleolar structures were formed. It appeared

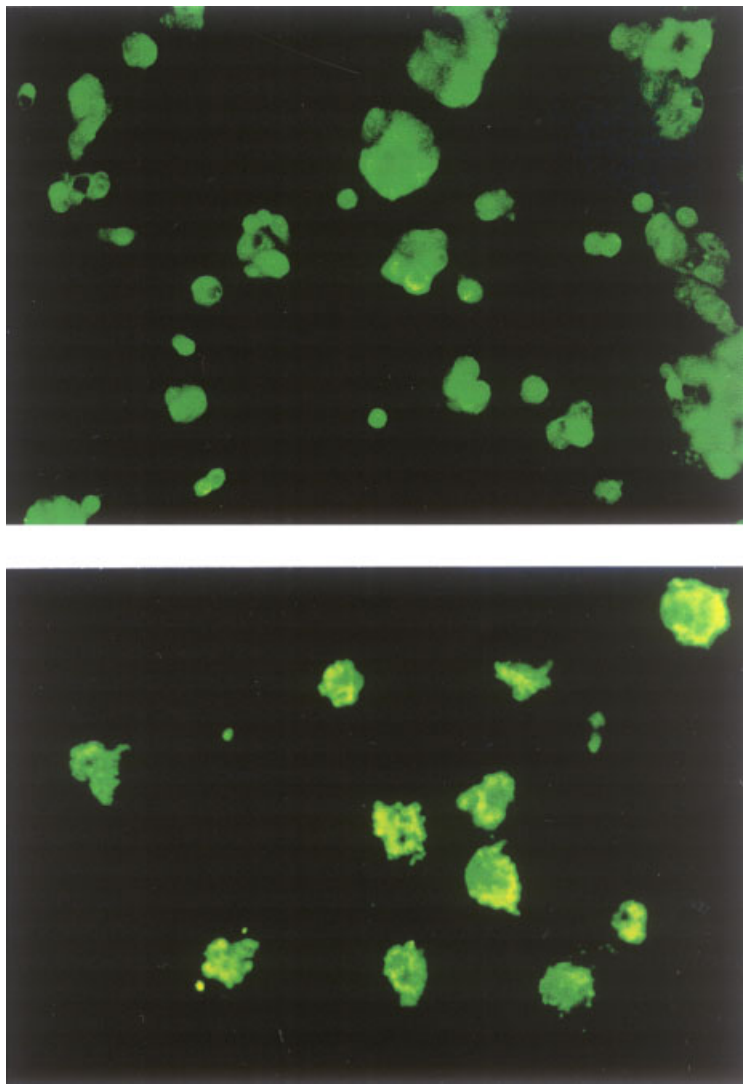

Fig. 7. Using immunocytofluoroscene labeling, B23 signals in green color were detected in nucleoplasm of the HepG2 cells before nuclear matrix extraction (**upper**) and in nuclear matrix residue of the cells after nuclear matrix extraction (**lower**); 200×. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

in the karyoplasm and accumulated in the nucleoli and disappeared from the cytoplasm and karyoplasm. However, several studies reported the presence of two forms of the protein (B23.1 and B23.2), which arised from a single gene via alterative splicing. They had different location, B23.1 predominantly found in nucleoli, whereas B23.2 in cytoplasmic fractions, and the two isoforms may be engaged in very different functions [Wang et al., 1993].

Recent studies accumulated to prove that the phosphorylation of B23 is correlated with cellular proliferative activities. It is highly phosphorylated by cdc kinase during mitosis, and this phosphorylation most probably has a role in initiating and controlling the entry of cells into mitosis [Peter et al., 1990]. The serine phosphorylation site of B23 is phosphorylated by cdc2 kinase during the entry of mitosis [Jiang et al., 2000]. In addition, B23 is considered as a target of CDK2/cyclin E in the initiation of centrosome duplication. It is associated specifically with unduplicated centrosomes, and dissociated from centrosomes by CDK2/cyclin E-mediated phosphorylation. The anti-B23 antibody can block this phosphorylation and suppress the initiation of centrosome duplication in vivo [Okuda et al., 2000]. Centrosome-bound B23 dissociates from centrosome upon phosphorylation by CDK2/cyclin E, which in

turn triggers initiation of centriole duplication. The duplicated centrosomes remain free of B23 till mitosis. During mitosis, it re-localizes to centrosomes. Upon cytokinesis, each daughter cell receives one centrosome bound by B23, which again dissociates from the centrosome upon exposure to CDK2/cyclin E at mid-late G_1 phase of the next cell cycle. Thus, it would constitute one of the licensing systems for centrosome duplication, ensuring the coordination of centrosome and DNA duplication, which limiting duplication once per cell cyclin [Okuda, 2002]. These previous studies partly elucidated the phenomenon in the study about B23 expression in the regenerating hepatocytes such as in the mitotic hepatocytes and its quantitative and locative alteration in the hepatocytes during the process of liver regeneration.

Nuclear matrix is defined as a non-chromatic protein complex resistant to extraction with detergent, nuclease, and high-salt, which are involved in many cellular activities. B23 is a very abundant protein in the internal nuclear matrix of HeLa S3 cells [Mattern et al., 1996]. Mattern et al. [1997] furthered to study that B23 was one major internal NMPs common to different human cell types such as HeLa S3 (human cervix carcinoma) cells, T24 (human bladder carcinoma) cells, NT2/D1 (human embro carcinoma) cells, and K562 (human myelogenous leukemia) cells. Nakayasu and Berezney [1991] identified that B23, also called numatrin, is a NMP. Protein B23 and protein kinase CK2 are associated with the nuclear matrix, and phosphorylation of nuclear matrix-associated B23 is directly affected by changes in nuclear matrix-associated CK2 activities [Tawfic et al., 1993]. In present study, B23 was biochemically characterized as a NMP extracted from the regenerating hepatocytes of the liver samples on day 3 post-operation. It implied that protein B23 associated with nuclear matrix played a role in the cycling cells, including rRNA and DNA synthesis and chromosomal organization. Also, B23 was biochemically characterized as a NMP extracted from the cultured HepG2 cells and morphologically localized in the nuclear matrix residue of the cells. It indicated that the protein B23 associated with nuclear matrix played a role in the cancer cells.

Protein B23 quantitative and locative alteration during liver regeneration, along with its characteristics of nuclear matrix, inferred that it played important roles in cell cycle and cell proliferation either in the normal cells or in the cancer cells. Although the biological functions of B23 require further study, it would be worthwhile to identify functional elements and dissect their functions especially in cancer cells.

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