

## Up-regulation of Human nm23-H2/NDP Kinase B in Early Phase of Regenerating Transgenic Mouse Hepatocyte

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**ABSTRACT** : The kinetics and subcellular localization of nm23-M1 and -M2 was reported in *in vivo* regenerating mouse liver cells after partial hepatectomy (Lee *et al.* 1997a). On the human nm23-H2 transgenic mice, the kinetics of murine types of nm23 and mitotic index were very similar to results from ordinary mice B6, whereas the kinetics of human type of nm23 was responded strongly and shortly in early phase of regeneration. In subcellular study, all fractions of nm23-H2 protein at 6hr after partial hepatectomy were increased to very high level and decreased soon day 1. These studies suggest that exogenous DNA sequence nm23-H2 expressed immediately in early phase of hepatocyte regeneration, and in short term duration comparing with native murine type of nm23. This analysis might include that another cellular cofactor(s) is(are) necessary for human type nm23 expression in regenerating mouse hepatocyte in playing a role as a transcription factor, or that is not associated with any cellular cofactors in organ crisis.

**Key words** : nm23/NDP kinase, Transgenic mice, Regeneration, Mouse, Hepatocyte, Transcription effect

### INTRODUCTION

In mammals such as mice, rats, and humans, the presence of two isotypes of nm23/NDP kinase has been identified (Urano *et al.* 1992, Okada *et al.* 1996, Ishikawa *et al.* 1992). These two isotypes encode 17 kDa proteins that are high homology of 82~89% in amino acid bases and nucleotide bases (Okada *et al.* 1996, Gilles *et al.* 1991) and display quite similar NDP kinase activity *in vitro* (Stahl *et al.* 1991) and form hexamers with a random association of two isotypes in the cells (Postel *et al.* 1996).

The nm23/NDP kinase expression correlates inversely with the metastatic potential of several rodent

tumors (Steeg *et al.* 1988) and human breast carcinoma and human hepatocellular carcinoma (Bevilacqua *et al.* 1989). Transfection of nm23 cDNA into highly metastatic murine K-1735-TK melanoma cells results in significant reduction of metastasis. However in colorectal carcinoma, no significant association was found between nm23 expression and metastatic potential, whereas in childhood neuroblastoma, overexpression and mutation of nm23 were associated with advanced tumors.

Increased expression of nm23-H2/NDP kinase associated with cellular proliferation has been observed in activated lymphocytes (Gilles *et al.* 1991, Caligo *et al.* 1995) and in a variety of cell lines (Igawa *et al.* 1995, Keim *et al.* 1992). The facts that the differentiation of murine myeloid leukemia was inhibited by nm23/NDP kinase (Okabe-Kado *et al.* 1995), and differentiation of human hematopoietic cell lines was

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associated with decreased expression of the gene (Yamashiro *et al.* 1994) suggest that nm23/NDP kinase participates in cellular proliferation/differentiation.

The nm23-H2/NDP kinase B encodes PuF, which transactivates c-myc oncogene, catalyzes the shuttling of  $\gamma$ -phosphates (Postel *et al.* 1996). In this study, the activation of exogenous DNA sequence nm23-H2/NDP kinase B in transgenic mouse was analyzed. To induce proliferation of hepatocyte, partial hepatectomy ( $50 \pm 2\%$ ) was done. In early phase of regeneration at 6hr after partial hepatectomy, increased nm23-H2/NDP kinase B molecule was found, especially distinguished level in the all fractions including nuclear fraction.

## MATERIALS AND METHODS

### Liver tissue

8-week-old B6 male mice and nm23-H2 transgenic mice were partially hepatectomized ( $50 \pm 2\%$ ) under anesthesia (Higgins and Anderson 1931). The transgenic mice were kindly provided from Department of Oncology, Nagasaki Medical School. Six hours, 12 hr, 1d, 3d, 5d, and 15d after partial hepatectomy, liver tissues were taken out. Sham operation was done for control in which just the abdominal wall was opened and the liver was exposed to air.

### Cell fractionation and protein extraction

A detailed fractionation procedure has been described elsewhere (Dignam *et al.* 1983). Briefly, for the nuclear fractionation, washed cells or tissues were minced in the lysis buffer I [0.5% NP-40, 0.01 M Tris-HCl (pH 7.5), 0.15 M NaCl, 0.01 M  $MgCl_2$ , 1.5 mM phenylmethyl-sulfonylfluoride (PMSF) (Sigma, St. Louise, MO)]. The lysate was centrifuged for 10 min at 3,500 rpm in a microcentrifuge at 4°C. The pellet was resuspended in the lysis buffer I and sonicated at 50% of duty cycle for 15 sec twice. After cen-

trifugation for 15 min at 12,000 rpm, the supernatant was used as a nuclear fraction. For cytoplasmic and membrane fractions, washed cells or tissue were minced in the lysis buffer II (same as the lysis buffer I except NP-40) and sonicated, then centrifuged for 10 min. The supernatant was centrifuged for 40 min at 15,000 rpm. The supernatant was used as a cytoplasmic fraction. The pellet was resuspended in the lysis buffer I and centrifuged again for 5 min at 12,000 rpm. The supernatant was used as a membrane fraction.

### Antibodies and Western blot analysis

Expressions of nm23-M1, -M2, and -H2 were fractionated by SDS-PAGE and analyzed by immunoblotting with monoclonal antibodies (Sambrook *et al.* 1987). Antibodies specific for each isotype of nm23/NDP kinase were prepared as described (Baba *et al.* 1995) and kindly provided from Dr. Shiku, Department of 2nd Internal Medicine, Mie Medical School; anti nm23-M1, M1-45; anti nm23-M2, M2-435; anti nm23-H2, H2-439.

### Mitotic index in regenerating hepatocytes

The mitotic index was checked after partial hepatectomy. Four hours before the animal was sacrificed, 1  $\mu$ g of colchicin/g of body weight was injected intraperitoneally. Mitotic metaphase cells were counted from the portal to central hepatic area in liver acinus.

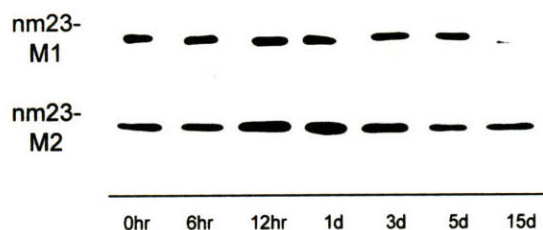
## RESULTS

### Changes of nm23-M1 and -M2 levels during the hepatic regeneration

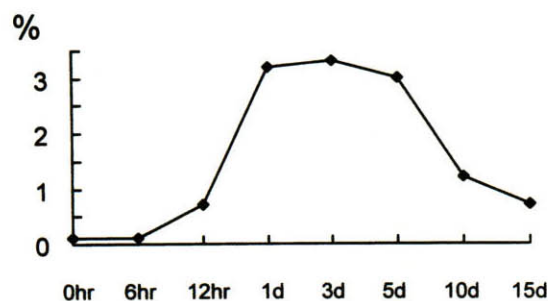
Experimental animals, B6 mice and exogenous human gene nm23-H2 in germ line were transferred. The native murine nm23-M1 and M2 genes were well expressed in B6 mice. After partial hepatectomy, the expressions of nm23-M1 and M2 in regenerating liver tissues were analyzed by Western blotting (Fig. 1). In the early phase of the regeneration (before day

3), the expressions of two isotypes rapidly increased, then slowly decreased to the original level. Expression of nm23-M1 reached the peak on 12 hr, while that of nm23-M2 reached a peak on day 1. Especially, the kinetics of nm23-M2 was very similar to that of following mitotic index.

The number of metaphases in the regenerating mouse liver tissue was also counted (Fig. 2). The mitotic response to the partial hepatectomy was in general compatible with the changes of expression of both isotypes of nm23. Mitotic index (MI) at sham operation and at 6 hr were 0.06~0.1%. A highly increased level of MI, 0.7% was found at 12hr and then reached maximum levels, 3.2% at day 1, and was maintained until day 5, then declined by day 15.



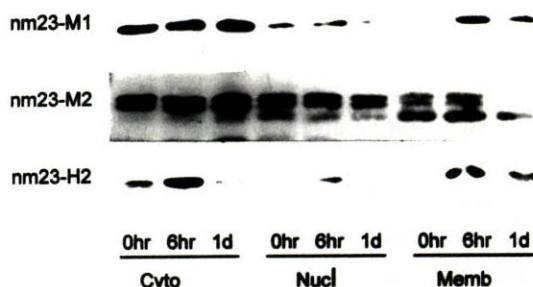
**Fig. 1.** Expressions of nm23-M1 and -M2 during liver regeneration after partial hepatectomy with time course. Hepatocyte lysate were prepared at indicated points after partial hepatectomy. Ten  $\mu$ g of protein extracts were separated on a 12% gel and immunoblotted as described in Materials and Methods.



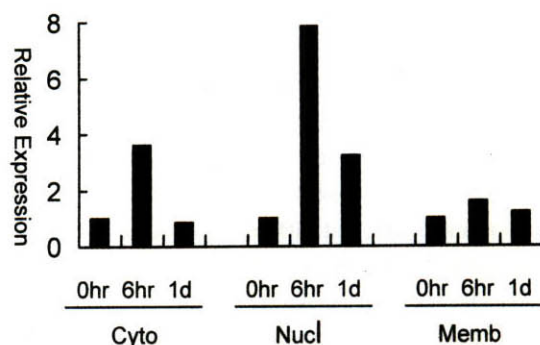
**Fig. 2.** Mitotic index with time course after partial hepatectomy. Percent of cells at metaphase were counted at the time indicated as described in Materials and Methods.

### Subcellular distribution of nm23 in the regenerating hepatocytes

As shown in Fig. 3, the subcellular distribution of nm23 protein was determined. The relative expressions of nm23-M1, -M2, and -H2 increased in all proliferative fractions when compared 0 hr samples. All nuclear fractions of three genes showed a peak increased value at 6 hr and returned at day 1, whereas cytoplasmic and membrane fractions were sustained. Exogenous human nm23-H2 expression showed a peak value at 6 hr in all fractions and returned to sham operation level on day 1. In densitometry, 3.62 fold in



**Fig. 3.** Subcellular distribution of nm23-M1, -M2, and -H2 proteins during mouse liver regeneration. Western blotting of fractionated proteins; Fractionation and immunoblotting were performed as described in Materials and methods. Cyto, cytoplasmic fraction; Nucl, nuclear fraction; Memb, membrane fraction.



**Fig. 4.** Relative subcellular distribution levels of human nm23-H2 protein based on the bands in Fig. 3.

cytoplasmic fraction, 7.84 fold in nuclear fraction, and 1.65 fold in membranous fraction were shown (Fig. 4).

## DISCUSSION

Increased expression of nm23/NDP kinase molecules in association with cellular proliferation in various cells and cancer progress has been reported (Igawa *et al.* 1995, Keim *et al.* 1992). In *in vitro* transcription effect of nm23/NDP kinase on c-myc oncogene for proliferation, murine nm23 worked in murine cell lines, but human nm23 (Lee *et al.* 1997b). In *in vivo* model, human nm23 gene transferred mice, the association of exogenous human nm23 expression with murine hepatocyte proliferation and intracellular localization of two isotypes of nm23/NDP kinase was studied by utilizing isotype specific monoclonal antibodies. Murine nm23/NDP kinase isotypes increased in regenerating tissue in close association with cellular proliferation including mitotic index. However, the transferred human gene nm23-H2 expressed with different kinetics from murine nm23, in this study, all fractions of human nm23-H2 molecule responded shortly and strongly. These data suggest that depending on murine or human nm23, different cellular factors are necessary (Lee *et al.* 1997b). In transfectant experiments, murine nm23 transfectant into murine cells has a transcription effect on c-myc and prolongs S phase of cell cycle, whereas those effects in human nm23 transfectant were not shown. That is postulated that cellular cofactor is necessary for nm23/NDP kinase to transactivate the c-myc gene and control the cell cycle. In this study, transcription effect of nm23 was postulated from increased nuclear fraction molecule, and different kinetics of cytoplasmic and membrane protein of transferred human gene nm23-H2 in early phase of hepatic regeneration might show additional functions with cellular cofactor. The difference of their regulatory elements has been reported in both human and rat genomes (Postel

*et al.* 1996). And the two isotypes showed distinct patterns of kinetics indicating independent regulation of their gene expression.

Other observations, excluding PuF and awd gene homology, described previously stated that nm23 genes linked to cell growth as follows: 1) anti-nm23 antibodies recognize mitotic spindle microtubules (Lakshmi *et al.* 1993); 2) nm23 is highly homologous to the p19 protein, a neuroblastoma cell proliferation-related protein (Hailat *et al.* 1991); 3) microinjection of an nm23-specific antibody inhibits cell division in rat embryo fibroblasts (Sorscher *et al.* 1993). However, a direct proof of nm23 involvement in the cell cycle is still missing.

The transgenic approach involves the permanent introduction of an exogenous DNA sequence into the genome of a host animal. Induction of organ crisis (proliferation) was done by partial hepatectomy, it took around two weeks for recover depending molecular kinetics of nm23 and mitotic index. In terms of regeneration of liver, two isotypes of murine and human nm23 have regulatory cellular elements respectively. However, organ crisis might bring mobilization and share of different species regulatory elements of nm23/NDP kinase due to close homology between species.

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## nm23-H2/NDP kinase B 유전자이입된 쥐에서 간세포 재생 초기의 조절

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**간추림** : 쥐의 간 부분절제후 간세포의 재생과정에 따른 nm23-M1과 -M2 표현의 동역학과 세포내 분포 변화 등은 분열지수와 함께 Lee 등(1997a)에 의해 보고되어있다. 사람형의 nm23-H2가 쥐의 배자에 도입된 생쥐 (transgenic mice)에서 간 재생 과정에 따른 쥐형의 동역학은 위 보고와 아주 유사하였으나 도입된 유전자인 nm23-H2의 발현은 재생 초기에 대단히 강하고 짧은 기간이었다. 세포분획에서는, 세포질에서도 간부분 절제후 6시간에 발현의 강한 증가를 보였으나 핵에서 그 변화가 두드러져 간 재생 초기에 외부에서 도입된 사람형 nm23-H2의 전사인자로서의 역할이 추정되었다. 이런 변화는 실험실에서 이루어진 결과(Lee 등 1997b)와는 달리 중식이 강하게 시작되는 시기 혹은 위험기(crisis)에는 이 유전자가 전사인자로서 역할을 함에 있어서 다른 유사 세포성 인자와 결합하거나 단독으로 이루어지는 것으로 사료된다.

**찾아보기 낱말** : nm23/NDP kinase, Transgenic mice, 생쥐, 간세포, 재생, 전사효과