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Cell cycle protein profile of the hepatic stellate cells (HSCs) in dimethylnitrosamine-induced rat hepatic fibrosis

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Abbreviation: α-SMA, α-smooth muscle actin Cdk, cyclin dependent kinase DMN, dimethylnitrosamine ECM, extracellular matrix HSC, hepatic stellate cell PCNA, proliferating cell nuclear antigen PDGF, platelet-derived growth factor

Abstract

Cell cycle regulating proteins are known to have close relation with the proliferation of the mammalian cells. In injured liver, the number of HSCs is increased from proliferation. However, the expression of cell cycle proteins of HSCs during proliferation remains unevaluated. Therefore, cell cycle protein profiles of HSCs were studied in dimethyl-nitrosamine (DMN)-induced rat liver fibrosis model. Sprague-Dawley rats were intraperitoneally injected of DMN and the animals were sacrificed every week up to 4 weeks. HSCs were separated and the number of the cells in S phase was counted to evaluate the cell proliferation by flow cytometry. The expression of cyclin A, cyclin B, cyclin D1, cdk2, cdk4, cdc2, proliferating cell nuclear antigen (PCNA), $p21^{Cip/WAF1}$, and p27 was examined with immunoblotting analysis. Portion of S-phase cells peaked 7days after DMN injection. At that time, cyclin A, and PCNA showed significant increase in HSCs compared to untreated HSCs (114% and 116%, respectively, $P < 0.001$). $p21^{Cip/WAF1}$ was decreased significantly in DMN-treated HSCs compared to control cells (88%, $P < 0.001$). The increase of cyclin A, and PCNA and the decrease of $p21^{Cip/WAF1}$ seem to play important roles in the proliferation of HSCs during the early period of DMN treatment.

Keywords: cell cycle; cell cycle proteins; dimethylnitrosamine; hepatic fibrosis; liver regeneration; rat

Introduction

Fibrosis induced by low doses of dimethylnitrosamine (DMN) in the rat has been shown to be a good reproducible model, inducing an initial central hemorrhagic necrosis of the liver with rapid formation of septa and fully established micronodular cirrhosis after 3 weeks of treatment. The model appears appropriate for the study of the early events associated with the development of fibrosis (Jenkins *et al.*, 1985; Jezequel *et al.*, 1987; Jezequel *et al.*, 1989; George *et al.*, 1996; George *et al.*, 2001).

Although the pathogenic mechanism of liver cirrhosis is not fully understood, this seems to involve a marked accumulation of extracellular matrix (ECM) components, activation of cells capable of producing matrix materials, cytokine release, and tissue remodeling. Hepatic stellate cells (HSCs) are known as the primary cellular source of the matrix components in liver fibrosis (Marc *et al.*, 1997; Gressner, 1998). HSCs in the space of Disse have two known functions: primary storage of the body's retinoids in intact liver lobules and the production of ECM components in the injured liver. Following liver injury of any etiology, hepatic stellate cells undergo a response known as “activation,” which is the transition of quiescent cells into proliferative, fibrogenic, and contractile myofibroblasts (Shiba *et al.*, 1998; Shimizu *et al.*, 1999).

Stellate cell activation is a remarkably pleiotropic yet tightly programmed response occurring in a reproducible sequence (Friedman, 2000). This sequence consists of ‘initiation’ and ‘perpetuation’. Initiation encompasses rapid changes in gene expression and phenotypes that render the cells responsive to cytokines and other local stimuli. Perpetuation involves key phenotypic responses mediated by increased

cytokine effects and remodeling of ECM (Friedman, 2000). Discrete phenotype responses of stellate cell perpetuation include: 1) proliferation; 2) contractility; 3) fibrogenesis; 4) matrix degradation; 5) chemotaxis; 6) retinoid loss; and 7) cytokine release and white blood cell chemoattraction.

Among these, 'proliferation' is increased numbers of stellate cells in injured liver, which arise in part from local proliferation in response to polypeptide growth factors. Although several peptides and signal transduction molecules including platelet derived growth factor, nuclear factor-κB (NFκB), transforming growth factor β (TGF-β), tumor necrosis factor α (TNF-α) and phosphoinositol 3-kinase have been suggested to contribute to the regulation of HSC proliferation (Marra *et al.*, 1997; Pinzani *et al.*, 1998; Elsharkawy *et al.*,1999; Saile *et al.*, 1999; Lang *et al.*,2000), the precise mechanisms related to the proliferation of HSC remain inconclusive.

Several immunohistochemical studies concerning HSC proliferation has been reported in the DMN induced cirrhosis (Paolucci *et al.*, 1990; Mancini *et al.*, 1992). Those data indicate that HSC proliferate mainly in the 1st week of DMN treatment and consecutively its proliferative activity decreases with progressive fibrogenesis in later period. These findings suggest that the proteins expressed in the first week of DMN treatment may play an important role in the HSC proliferation. Cell cycle regulating proteins seem to be a key event for the proliferation of the mammalian cells. However, the expression of cell cycle proteins related to the proliferation of HSC remains unevaluated.

Therefore, we separately isolated HSCs from rat liver in DMN-induced fibrosis and examined the expression of various cell cycle regulatory proteins to elucidate the cell cycle protein profile, which is related to HSCs proliferation.

Materials and Methods

Chemicals

DMN, α-SMA and DNase were purchased from Sigma (Louis, MO). Pronase, collagenase A and protease inhibitor were obtained Boehringer Mannheim (Mannheim, Germany). Antibodies against Cdk2, cdk4, cdc2, cyclinA, cyclinB, cyclinD1 and PCNA were purchased from Santa Cruz Biotechnology Inc. (California); anti-mouse Ig horseradish peroxidase link whole antibody (NA931), anti-rabbit Ig horseradish peroxidase link whole antibody (NA934) and ECL kit were from Amersham Life Science (Buckinghamshire, UK). Bicinchonic acid (BCA) protein assay kit was purchased from Pierce Chemical Co. (Rockford, IL), nitrocellurose membrane was from Bio-RAD (California), Histostain sp bulk kit and liquid DAB substrate kit from Zymed (San Francisco, California), HBSS (Hanks' balanced salt solution) from GIBCO BRL (Grand Island, NY), and DMEM (Dulbecco's modified Eagle medium) from Seoul National University Hospital Clinical Research Institute.

Animals and induction of hepatic fibrosis

Six-week-old male Sprague-Dawley (SD) rats were obtained from Korea Laboratory Animals. All animals received humane care and the Committee of Laboratory Animals of Seoul National University College of Medicine approved the study protocol according to the institution's guideline. Hepatic fibrosis was induced by intraperitoneal injections of DMN in doses of 10 μl/kg body weight in normal saline. The injections were given on the first three consecutive days of each week over a period of 4 weeks. Treated animals were perfused on days 7, 14, 21, and 28 of the experiment and sacrificed on 7 and 28 days to obtain liver tissue. Livers were removed and immediately weighed. Liver section from the left and right median, and right lobes were taken and fixed in 10% neutral buffered formalin. The remaining liver was freshly frozen in liquid nitrogen and stored at -80°C.

Cell isolation

Hepatic stellate cells were isolated according to the original method of Alpini (Alpini *et al.*, 1994). All procedures were performed with the animals under ether anesthesia. Initially livers were perfused in site via portal veins at 37°C for 10 min of Ca^{2+}-free Hank's Balanced Salt Solution (HBSS, GibcoBRL) and with 0.04% collagenase dissolved in Ca^{2+}contained HBSS for 15 min. The liver was then gently homogenized and was incubated in Dulbecco's modified Eagle medium (DMEM), containing 0.02% collagenase, 0.05% pronase, 0.001% DNase for 20 min at 37°C under constant shaking. This suspension was filtered through nylon gauze (mesh size 106 μm) and centrifuged for 2 min at 600 *g* to remove remaining hepatocytes. Sinosoidal cells in the supernatant were recovered by centrifugation for 10 min at 2,000 *g*. The cells were resuspended in DMEM, centrifuged once for 10 min at 2,000 *g* and resuspended in the presence of 28% Nycodenz stock solution. The final concentration of Nycodenz at this stage was 11.5%. This was loaded onto a 17.2% Nycodenz solution and covered the layer with 1 ml of HBSS. Following centrifugation for 15 min at 3,000 *g*, the cells at the top of the Nycodenz were collected, and washed by centrifugation (Hendriks *et al.*, 1985; Vyas *et al.*, 1995; Geerts *et al.*, 1998).

Isolated cells from control animal showed eccentric nuclei, large fat vacuoles and a few cell organells in

cytoplasm, which were characteristics of Ito cell. After 7 days of incubation, these cells revealed stellate appearance and strong positivity for desmin, vimentin, and α-SMA immunohistochemistry (data not shown).

Collagen content evaluation

Two sections of liver were cut at a approximately 15 μm thick, resulting in a tissue area of 50-100 mm^2. The sections were deparaffinized with successive incubation with xylene and dehydrated with ethanol (99.9%, 90%, 80%, 70% and water). The samples were then kept in distilled water at 4°C and stained as follows. Individual slices were incubated in aluminum foil-covered test tubes in the presence of a 0.04% solution of Fast green FCF in saturated picric acid for 15 min. The sections were then washed thoroughly with distilled water until the supernatant was clear; they were then incubated in the presence of Fast green FCF 0.1% and Sirus red F3B 0.04% for 30 min and washed as described above. One ml of 0.05% NaOH in 50% aqueous methanol was the added and each tube was gently mixed. The eluted color was read in a spectrophotometer at 530 and 605 nm (corresponding to the maximal absorbance of Sirius red and Fast green, respectively). To quantitate the absorbance found for each dye, the color equivalences determined from the relationship obtained between the absorbance using the colorimetric and the chemical methods of collagen and total protein determination of the same liver section were used. These color equivalences were 2.08 for Fast green and 38.4 for Sirius red. Non-collagenous protein (mg) was counted as absorbance at 605 nm per 2.08. Collagen (μg) was calculated with difference between absorbance at 540 nm and 0.26 × absorbance at 605 nm per 38.4. Finally, collagen content was estamated with collagen (μg) per the sum of collagen (μg) and non-collagenous protein (mg).

Immunoblotting

Isolated HSCs were lysated in 50 μl of lysis buffer (20 mM Tris, 137 mM NaCl, 10% glycerol, protease inhibitor). The lysate was then centrifuged at 9,000 *g* for 20 min and the supernatant fraction was collected. The protein concentration was determined by BCA protein assay. Aliquots of 30 μg were diluted into SDS sample buffer, boiled and run immediately on 12% or 15% acrylamide SDS-PAGE gels. Proteins were transferred electrophoretically to nitrocellulose, the membrane was blocked in 5% skim milk/Tris-buffered saline. This was followed by incubation with specific primary antibodies (2 mg/ml in each case) for 2 h in Tris buffered saline plus 1 mg/ml of BSA. Proteins were detected using the ECL system (Amersham, Arlington Heights, IL) with horseradish peroxidase conjugated secondary antibody (Bio-Rad, Hercules, SA) at a dilution of 1:5,000. Immunoblots were quantitated using a MCID software program (Imaging Research Inc., Ontario, Canada).

Flow cytometry

Isolated HSCs were fixed with 100% methanol for 10 min and washed two times with PBS. Subsequently cell membranes were lysated in 0.1% Triton X-100 for 5 min on the ice. Nuclear DNA content was measured on a FACScan (Becton Dickinson, New Jersey, USA).

Statistical method

All results were expressed as mean value ± standard

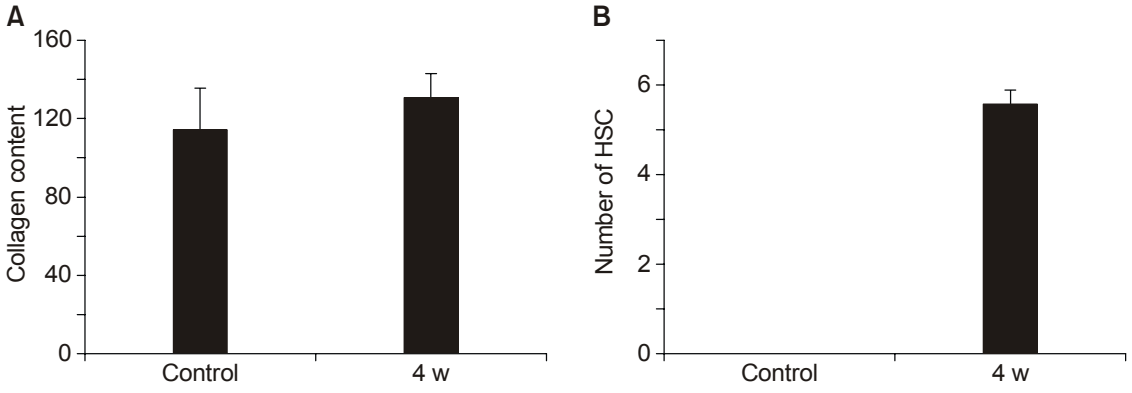

Figure 1. (A) Collagen content in control and 4th week. Hepatic collagen content was significantly increased in the DMN-treated group (4th week) compared to untreated control group. (B) α-smooth muscle positive cells in control and DMN-treated group (4th week). After DMN treatment, number of α-smooth muscle positive cells are found about 5.61 for each 1.81 mm^2 area. In contrast, no α-smooth muscle positive cells are noted in control group (Figure 1B).

deviation. The differences between controls and DMN-treated animals were assessed by the non-parametric Man Whitney test: $P < 0.05$ was considered significant.

Results

Liver fibrosis

In light microscopic examination, there was lobular architecture with thin bands of reticulin joining central areas and with some nodule formation. To evaluate the fibrosis, collagen content was determined by colorimetric evaluation in control and sample at 4th week. A highly significant increase in hepatic collagen content was observed among the DMN-treated groups compared to untreated control tissue (Figure 1A). Increased collagen content indicated that DMN-treated animal had fibrosis in hepatic parenchyma. Also α-smooth muscle positive cells at 4th week after DMN treatment are found 5.61 for each 1.81 mm^2 area in fibrotic septa and hepatic parenchyma, but no α-smooth muscle positive cells are seen in control group (Figure 1B). This result showed that DMN-treated liver tissue had significant increased number of hepatic stellate cells.

Proliferation of HSCs

To evaluate the time-dependent proportion of proliferating stellate cells, the number of S-phase HSCs was counted by flowcytometry (Figure 2A). Portion of S-phase cells were peak on the 7th day (26.32%) (Figure 2B). These indicate that HSCs remained relatively quiescent during first 7 days after DMN treatment and then their cell cycle started to progress and entered the peak of the S phase on 7th day. After that, portion of S-phase cells remained at its nadir

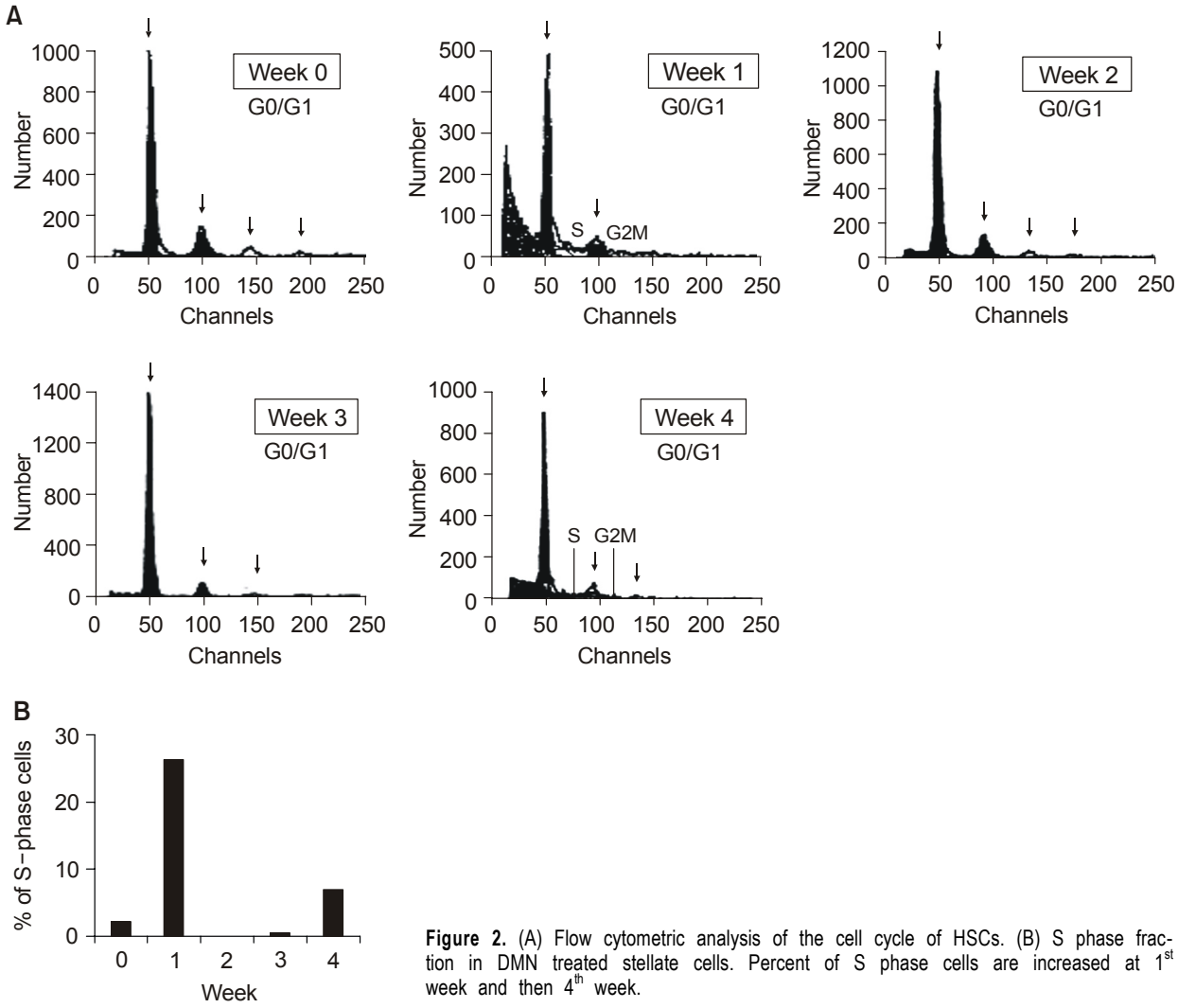

Figure 2. (A) Flow cytometric analysis of the cell cycle of HSCs. (B) S phase fraction in DMN treated stellate cells. Percent of S phase cells are increased at 1st week and then 4th week.

until 2nd peak of 4th week. These designate that proliferation of HSCs was repressed during 2th and 3th weeks of DMN treatment.

Expression of cell cycle relating proteins

Data concerning Western blots and relative band densities are shown in Figure 3 and Table 1. The percent of cell cycle related protein expression compared to untreated control (mean of relative band density of treated each samples / that of untreated control) is shown as bar chart in Figure 4.

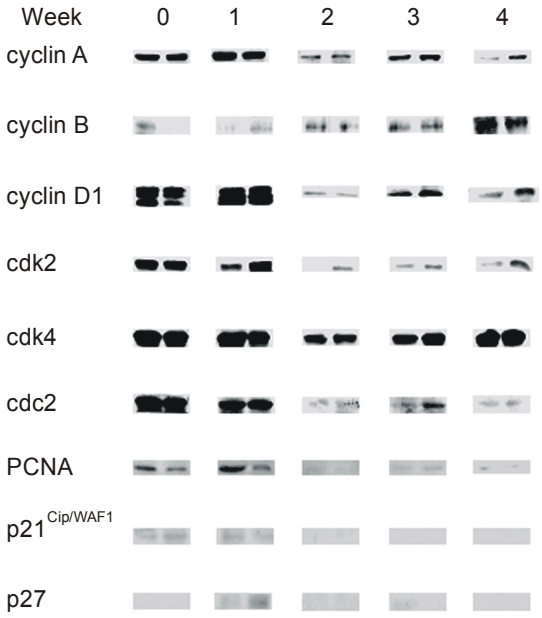

Figure 3. Western blots of cyclins, cyclin dependent kinases, PCNA and cyclin dependent kinase inhibitors. Each band demonstrates the responses of each proteins in hepatic stellate cells following DMN treatment using antibodies specific for each factor as described in Materials and Methods.

Cyclins (cyclin A, cyclin B, cyclin D1)

On day 7, cyclin A and cyclin D1 were 114% and 101% of the control group in expression, respectively. The increase of cyclin A showed a statistical significance compared to control group ($P < 0.001$) (Table 1 and Figure 4). This indicates cyclin A may give an important role in HSC proliferation during 1st week. Cyclin B was increased significantly on day 21 and day 28. This showed that late proliferation of HSCs seemed to be dependent on amplification of cyclin B level.

Cyclin dependent kinases (cdk2, cdk4, cdc2) and PCNA

All cdks of HSCs did not show increased expression throughout the phases. Only PCNA was induced 116% of the control level in HSCs at 1 week of DMN-treatment ($P < 0.001$) (Table 1 and Figure 4). This indicates that PCNA may also contribute to the HSC proliferation during 1st week.

Cyclin dependent kinase inhibitors ($p21^{Cip/WAF1}$ and p27)

P21 showed significant decrement to 88% of the control group ($P < 0.001$) and p27 had slight increment without any statistical difference at 1st week (Table 1 and Figure 4). This result indicates that decrease in p21 level contributed to proliferation of HSCs during 1 week after DMN treatment.

Table 1. Relative band densities of cyclins, cyclin dependent kinases, and PCNA. Each value demonstrates the mean ± standard-deviation of two or three samples in hepatic stellate cells following DMN treatment.

Week	0	1	2	3	4
cyclin A	0.28 ± 0.01	0.32 ± 0.01*	0.09 ± 0.08	0.25 ± 0.03	0.29 ± 0.07
cyclin B	0.15 ± 0.06	0.14 ± 0.02	0.17 ± 0.02	0.23 ± 0.03*	0.37 ± 0.04*
cyclin D1	0.24 ± 0.06	0.25 ± 0.13	0.09 ± 0.03	0.13 ± 0.02	0.19 ± 0.04
Cdk2	0.32 ± 0.00	0.25 ± 0.07	0.10 ± 0.05	0.16 ± 0.02	0.22 ± 0.06
Cdk4	0.22 ± 0.01	0.21 ± 0.08	0.11 ± 0.05	0.19 ± 0.02	0.23 ± 0.04
Cdc2	0.24 ± 0.02	0.19 ± 0.07	0.07 ± 0.01	0.11 ± 0.02	0.09 ± 0.01
PCNA	0.13 ± 0.05	0.18 ± 0.10*	0.03 ± 0.01	0.04 ± 0.01	0.03 ± 0.02
p21	0.20 ± 0.01	0.18 ± 0.02*	0.11 ± 0.02*	0.13 ± 0.01*	0.20 ± 0.03
p27	0.04 ± 0.01	0.04 ± 0.04	0.05 ± 0.01	0.05 ± 0.02	0.03 ± 0.00

*statistical difference between control and treated group, $P < 0.001$, non-parametric Man Whitney U test.

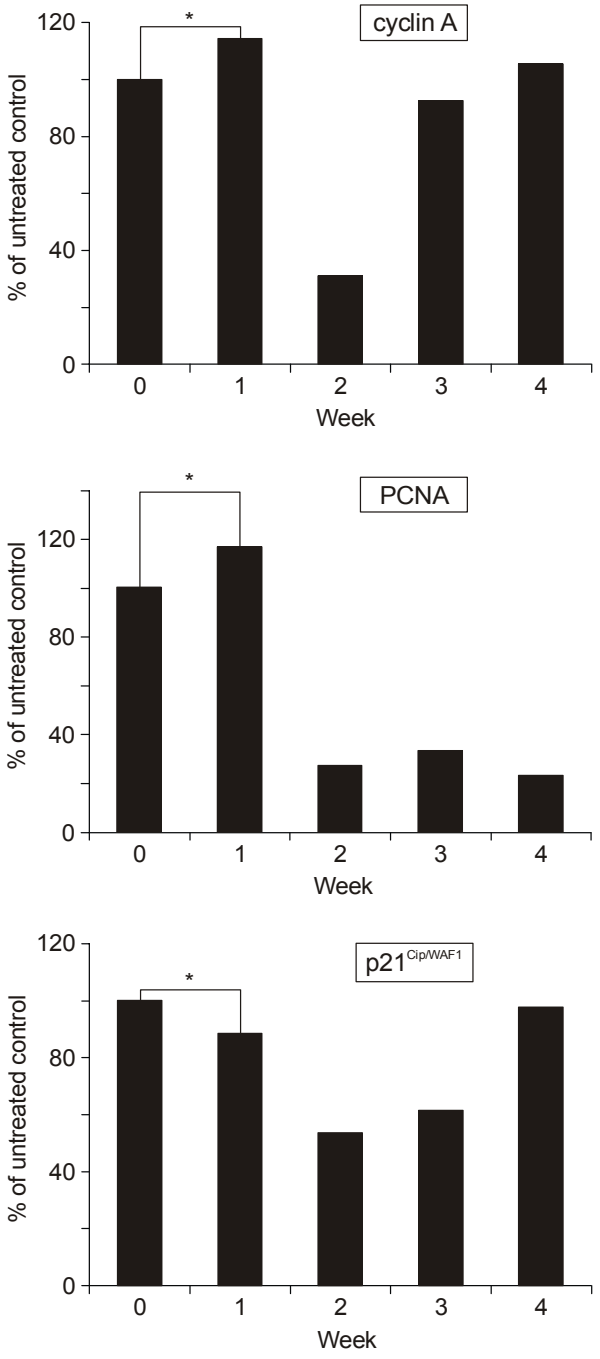

Figure 4. Percent of cyclin A, PCNA and $p21^{CIP/WAF1}$ expression in hepatic stellate cells compared to untreated control during DMN-induced fibrosis ($*P < 0.001$).

Discussion

Hepatic fibrosis induced by short-term treatment with DMN appears as a reproducible animal model and leads to rapid formation of septa with micronodular cirrhosis after 3th weeks of treatment (Jenkins *et al.*, 1985; Jezequel *et al.*, 1987; Jezequel *et al.*, 1989). As mentioned in the previous report, we could observe the increased level of collagen and α-smooth muscle positive cells at 4th week after DMN treatment by immunohistochemistry (data not shown). This result proved DMN could induce liver fibrosis at least until 4th week. In this protocol, the present study is concerned with the detection of cell cycle regulatory protein profile during early phase of DMN induced injury, which is related to the proliferation of HSCs.

To investigate the cell cycle proteins, we isolated HSCs and performed flow cytometry and immunoblotting assay. This experiment revealed that the ratios of S-phase of HSCs peaked at the early phase of DMN-treatment (1st week). This result was similar to previous studies (Paolucci *et al.*, 1990; Mancini *et al.*, 1992). These results suggest that proliferation of HSCs may be mostly active at 1st week after DMN-induced tissue damage and then these proliferated HSCs may transform into matrix producing myofibroblast-like cells, making liver fibrosis until 4th week.

During activation of HSCs, their number is increased in response to polypeptide growth factors, most of which signal through receptor tyrosine kinases. Platelet-derived growth factor (PDGF) is known to have most potent activity among these proliferative factors in hepatic fibrosis. This step is associated with both increased autocrine PDGF and up-regulation of PDGF receptor (Chen *et al.*, 1999). Activated PDGF receptor recruits the signaling molecule Ras, followed by activation of the ERK/mitogen-activated protein kinase pathway. Additionally, activation of phosphoinositol 3-kinase is necessary for both mitogenesis and chemotaxis (Marra *et al.*, 1997). The proliferative response to PDGF also requires a sustained intake of extracellular (Ca^{2+}) and increased intracellular pH (Chen and Davis, 1999). In human HSCs, activation of the ERK pathway followed by an increased expression of c-fos in response to PDGF (Marra *et al.*, 1995; 1996). However, the study concerning down stream molecules including cell cycle regulating proteins seems rare. To find the expression of cell cycle regulatory proteins that participate in proliferation of HSCs, we studied the expression of cyclins, cdks, PCNA, cdk-inhibitors by immunoblotting. The increment of cyclin A, and PCNA was found at 1st week in HSCs, which may indicate these proteins may contribute to active proliferation of HSCs at 1st week after DMN-treatment. Cyclin D1 increased slightly in amount on 7th day compared to control. Recent study reported that proteins and mRNAs for cyclin D1 started to increase 48 h after culture (Kawada *et al.*, 1999). These results suggested that cyclin D1 might play a role for stellate cell proliferation.

The expression of cyclin B slightly elevated until 3rd week of DMN treatment and markedly rose at 4th

week, which suggest it may participate in delayed proliferation of HSCs at 4th week. However, expression of cdks decreased or remained unchanged in the course of DMN-induced fibrosis. These results indicate that proliferation of HSCs seem not to depend on up-regulation of cdks, although cdk inhibitors $p21^{Cip/WAF1}$ and p27 showed no increment.

Recently, the association between PDGF and cyclins is reported *in vitro* studies with fibroblasts (Winston *et al.,*1996; Furstoss *et al.*, 2002). According to those results, PDGF seems to overcome inhibitory effect of p27 and increase the expression of cyclin A and cyclin E (Winston *et al.,*1996). Also, cyclin A and cyclin E seem to be induced by PDGF through Src during late G1 to S phase transition (Furstoss *et al.*, 2002). These data suggest that HSCs proliferation may be closely related to the expression of cyclin A through PDGF mediated-Src signal pathway. This hypothesis should be proven by further researches.

In conclusion, the result of our study demonstrated the cell cycle protein profile in the proliferation of HSCs during DMN-induced rat liver fibrosis. According to our experiment, the increase of cyclin A, PCNA, and the decrease of $p21^{Cip/WAF1}$ level seems to be closely related to the proliferation of HSCs during early phase of DMN induced liver fibrosis. After that, HSCs remained quiescent in proliferation. However, cyclin B seems to contribute to the proliferation of HSCs during late 3rd and 4th week of DMN treatment.

Acknowledgement

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