RAPID COMMUNICATION Jpn. J. Cancer Res. 87, 1102–1105, November 1996

Expression of p21$^{waf-1/cip-1}$ Is Significantly Induced in the Livers of LEC Rats with Chronic Liver Injury

Norimasa Sawada,[1,3] Takashi Kojima,[2] Hiroyuki Obata,[1] Hiroshi Isomura,[1] Shin-ichi Atsumi,[1] Masakuni Sawaki,[1] Naoto Tsuzuki,[1] Hirotoshi Tobioka,[1] Yasuo Kokai,[1] Masaaki Satoh[1] and Michio Mori[1]

[1]*Department of Pathology and* [2]*Department of Pathology, Cancer Research Institute, Sapporo Medical University School of Medicine, Minami-1, Nishi-17, Chuo-ku, Sapporo 060*

It is reported that hepatocytes isolated from LEC rats with chronic liver injury show reduced growth activity in primary culture. To elucidate the molecular basis of this phenomenon, we examined expression of p21$^{waf-1/cip-1}$ and p27, cyclin-dependent kinase inhibitors, by northern blot analysis. The expression of p21$^{waf-1/cip-1}$ in the LEC rat liver was 3-fold higher than that of age-matched SD rat liver, while there was no significant difference in p27 expression level. Western blot analysis also revealed a significant increase in p21$^{waf-1/cip-1}$ in the nuclear matrix fraction of the LEC rat liver. Immunohistochemically, p21$^{waf-1/cip-1}$ was detected in the nuclei of normal LEC rat hepatocytes, but not in those of hepatocellular carcinoma cells, suggesting selective growth of neoplastic hepatocytes.

Key words: p53 — p21$^{waf-1/cip-1}$ — p27-LEC rat — Nuclear matrix — Hepatocarcinogenesis

The LEC rat is a mutant strain having a defect in copper-transporting ATPase,[1] resulting in an abnormal accumulation of copper in the liver. The rats spontaneously develop liver injury due to copper accumulation and, subsequently, liver cancer, though the mechanisms of development of cancer in the LEC rat liver have not been clarified.[2] We previously reported that a great majority of hepatocytes isolated from LEC rats with chronic liver injury do not respond to growth stimuli,[3] suggesting that selective growth of neoplastic cells is allowed in LEC rats with chronic hepatitis due to a reduction of the growth activity of normal hepatocytes in the liver. In the present study, we examined the expression of p53, p21$^{waf-1/cip-1}$ and p27 to elucidate the mechanisms involved in the decrease of growth activity of hepatocytes of LEC rats with chronic liver injury.

LEC rats were bred at the Animal Laboratory, Sapporo Medical University. Male Sprague-Dawley (SD) rats were purchased from Charles River Japan, Inc. (Kanagawa). These male rats were fed a basal diet (Oriental MF, Oriental Yeast Co., Tokyo) and water *ad libitum* for 30–40 weeks under scheduled lighting until they were killed. To obtain nuclear matrix protein and total RNAs, 5 LEC rats and 3 SD rats were killed. For immunohistochemistry, 3 LEC and 2 SD rats were killed, and three 18-month-old LEC rats were killed to obtain hepatocellular carcinoma tissues. Total RNAs were purified from rat liver tissues using the single-step thiocyanate-phenol-chloroform extraction method.[4] Ten micrograms of total RNAs was loaded in each lane. After electrophoresis, the RNAs were capillary-blotted onto nylon membrane and fixed by exposure to UV light. Mouse p53 cDNA was obtained from the Japanese Cancer Research Resources Bank.[5] Mouse p21 and mouse p27 were kindly provided by Dr. Hiroaki Kanda (Cancer Institute, Tokyo). The probes were labeled with ^{32}P using a multiprime DNA labeling kit. After hybridization, the membranes were exposed to Kodak Diagnostic film (Eastman Kodak, Rochester, NY) and analyzed using a Bio Imaging Analyzer BAS 2000 (Fujix, Tokyo).

Nuclear matrices were prepared according to the method of Fey and He *et al.*[6,7] Briefly, fresh livers were homogenized in solution A containing 100 m*M* NaCl, 300 m*M* sucrose, 10 m*M* Pipes (pH 6.8), 3 m*M* $MgCl_2$, 1 m*M* EGTA, 0.5% Triton X-100, vanadyl ribonucleoside complex and protease inhibitors, followed by centrifugation at 2,000 rpm for 10 min and salt extraction with solution A containing 0.25 *M* ammonium sulfate at 4°C for 10 min. After centrifugation at 2,000 rpm for 10 min, the pellet was treated with DNase-I (100 μg/ml, Boehringer Mannheim, Mannheim, Germany) and RNase-A (25 μg/ml, Sigma, St. Louis, MO) at 25°C for 45 min. The remaining pellet was solubilized in solution B containing 8 *M* urea, 20 m*M* Pipes (pH 6.8), 1 m*M* EGTA, 0.1 m*M* $MgCl_2$, protease inhibitors and 1% 2-mercaptoethanol, and insoluble components were removed by ultracentrifugation at 250,000*g* for 60 min. After dialysis, insoluble materials were removed by ultracentrifugation at 150,000*g* for 45 min. The nuclear matrix fraction obtained was immediately precipitated with acetone and

[3] To whom all correspondence should be addressed.

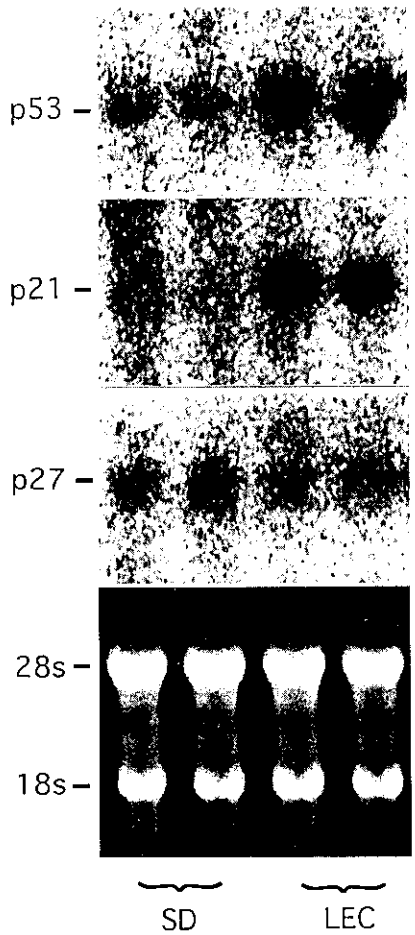

Fig. 1. Northern blot analysis of p53, p21 and p27 in 30- to 40-week-old SD and LEC rats.

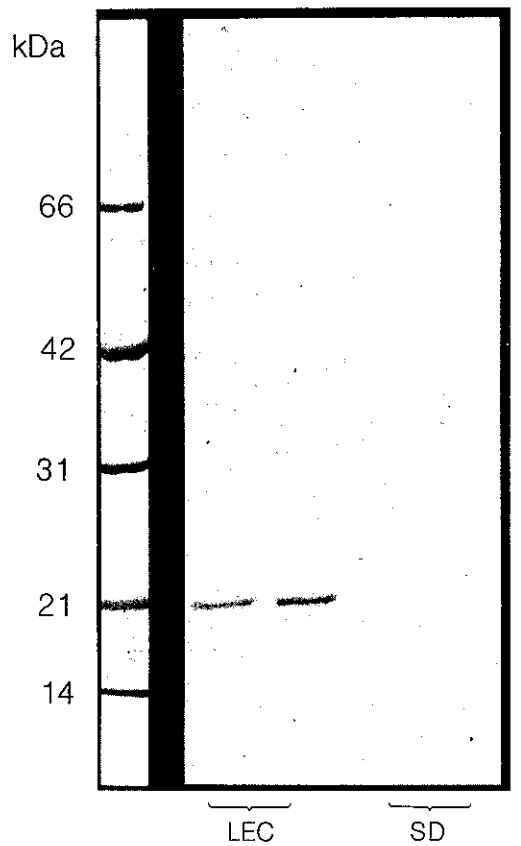

Fig. 2. Western blot analysis of p21 using nuclear matrix fraction obtained from 30- to 40-week-old SD and LEC rats.

stored at −80°C until use. Twenty micrograms of sample was applied per lane. After electrophoresis, the proteins were electrophoretically transferred onto a nitrocellulose membrane for immunoblotting using rabbit anti-p21 polyclonal antibody (1:100 dilution, Santa Cruz Biotechnology, Inc., Santa Cruz, CA).

For immunohistochemistry, the livers were perfused with buffered 10% formalin, embedded in paraffin and sectioned. After blocking with 4% skim milk in PBS, the sections were incubated with the rabbit p21 polyclonal antibody (1:50 dilution) overnight at 4°C. The sections were stained using the ABC method (ABC Elite kit, Vector Laboratory, Burlingame, CA).

Expression of p53, p21$^{waf-1/cip-1}$ and p27 in livers of LEC rats with chronic liver injury was examined by northern blot analysis. As shown in Fig. 1, expression of p53 and p21$^{waf-1/cip-1}$ in the LEC rat livers was higher than in age-matched SD rat livers. Densitometric analysis showed 2-fold and 3-fold increases in p53 and p21$^{waf-1/cip-1}$ expression, respectively. However, there was no significant difference in p27 expression between the LEC and age-matched SD rats. Western blot analysis also revealed an increase of p21$^{waf-1/cip-1}$ in the nuclear matrix fraction of the LEC rat liver, as shown in Fig. 2. In accordance with this, p21$^{waf-1/cip-1}$ was detected immunohistochemically in the nuclei of hepatocytes of LEC rats (Fig. 3). On the other hand, the nuclei of neoplastic nodule and hepatocellular carcinoma cells in 18-month-old LEC rat liver were virtually negative for p21$^{waf-1/cip-1}$ by immunohistochemistry (Fig. 4).

In the present study, we observed a significant increase in p53 and p21$^{waf-1/cip-1}$ expression in the livers of LEC rats with chronic liver injury, presumably resulting in reduced growth activity of the hepatocytes of the LEC rats. It has become clear that p53 expression is up-regulated upon DNA damage, p21$^{waf-1/cip-1}$ expression is induced, and then cell proliferation is arrested at the G1 phase of the cell cycle.[8–10] Since copper accumulation in the LEC rat liver generates an oxidized DNA-derived pro-

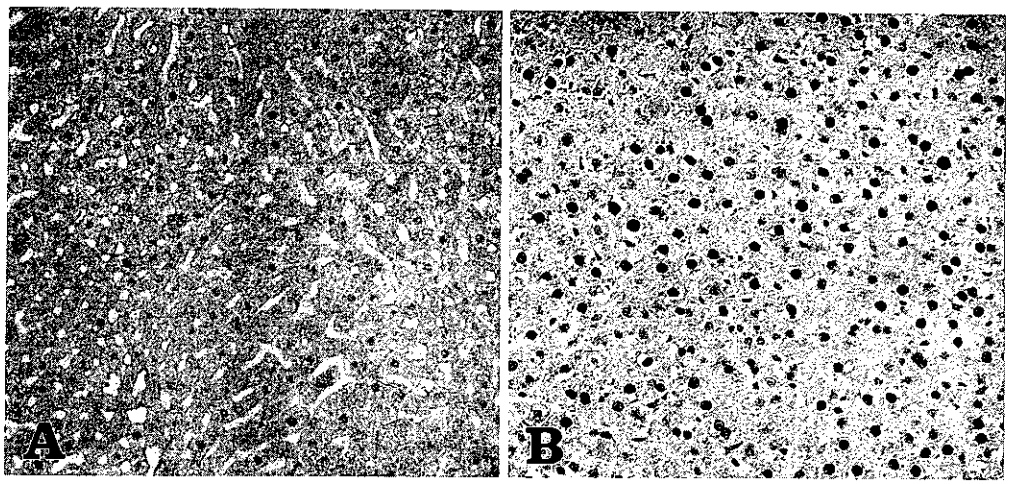

Fig. 3. Immunohistochemistry of p21 in livers of SD (A) and LEC (B) rats, counterstained with hematoxylin, ×96.

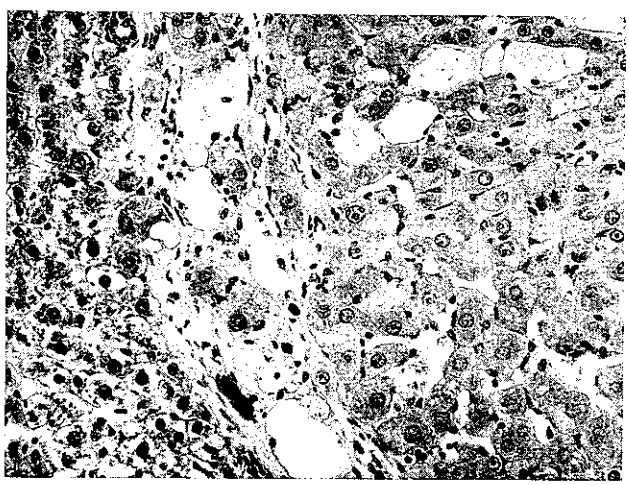

Fig. 4. Immunohistochemistry of p21 in a neoplastic nodule of the LEC rats, counterstained with hematoxylin, ×320.

duct,[11,12] it is most likely that expression of p53 and $p21^{waf\text{-}1/cip\text{-}1}$ is induced by DNA damage in the LEC rat liver.

In $p21^{waf\text{-}1/cip\text{-}1}$ transgenic mice, liver regeneration after partial hepatectomy is impaired,[13] strongly supporting our speculation that the elevated expression of $p21^{waf\text{-}1/cip\text{-}1}$ prevents LEC rat-hepatocyte proliferation in response to growth stimuli. In addition, hepatocytes of transgenic mice frequently have large polyploid nuclei,[13] similar to those of LEC rat hepatocytes.[14] Since $p21^{waf\text{-}1/cip\text{-}1}$ was detected in the nuclear matrix fraction, its accumulation might disturb the organization of the nuclear matrix.

We demonstrated that the nuclei of neoplastic hepatocytes were negative for $p21^{waf\text{-}1/cip\text{-}1}$ staining, while those of hepatocytes surrounding neoplastic lesions were positive for $p21^{waf\text{-}1/cip\text{-}1}$ staining. Furthermore, since the majority of preneoplastic hepatocytes in the LEC rat are resistant to copper accumulation,[15] preneoplastic and neoplastic cells have an advantage over normal hepatocytes and may selectively proliferate in response to the growth stimuli generated by chronic liver injury.[16] The difference in the responsiveness to growth stimuli between preneoplastic and normal hepatocytes, as we previously suggested using aged rats,[17,18] may eventually result in the appearance of neoplastic lesions in the LEC rat liver.

Recent reports[19,20] on the roles of TGF-β in promotion of hepatocarcinogenesis by phenobarbital during hepatocarcinogenesis suggest that phenobarbital permits a population of preneoplastic hepatocytes to proliferate selectively because of decreases in the expression of TGF-β receptors in the preneoplastic cells. Since it has been reported that TGF-β induces $p21^{waf\text{-}1/cip\text{-}1}$ expression,[21–23] $p21^{waf\text{-}1/cip\text{-}1}$ may generally play a role in preferential proliferation-dependent promotion during hepatocarcinogenesis.

We are grateful to the Japanese Cancer Research Resources Bank, for mouse p53 cDNA and Dr. Hiroaki Kanda (Cancer Institute, Tokyo), for mouse p21 and mouse p27 cDNAs. This work was supported by Grants-in-Aid from the Ministry of Health and Welfare and from the Ministry of Education, Science, Sports and Culture of Japan.

(Received June 21, 1996/Accepted August 13, 1996)

REFERENCES

1) Ono, T., Takada, S. and Yoshida, M. C. The *WD* gene for Wilson's disease links to the hepatitis of LEC rats. *Jpn. J. Cancer Res.*, **85**, 771–774 (1994).
2) Mori, M., Hattori, A., Sawaki, M., Tsuzuki, N., Sawada, N., Oyamada, M., Sugawara, N. and Enomoto, K. The LEC rat: a model for human hepatitis, liver cancer, and much more. *Am. J. Pathol.*, **144**, 200–204 (1994).
3) Sakamoto, H., Sawada, N., Kamimura, Y., Enomoto, K. and Mori, M. High sensitivity of LEC rats with chronic hepatitis to hepatocarcinogenesis: decreases in unscheduled and replicative DNA synthesis of the hepatocytes. *Jpn. J. Cancer Res.*, **84**, 966–971 (1993).
4) Chomczynski, P. and Sacchi, N. Single-step method of RNA isolation by acid guanidium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.*, **162**, 156–159 (1987).
5) Zakut-Houri, R., Oren, M., Bienz, B., Lavie, V., Hazum, S. and Givol, D. A single gene and a pseudogene for the cellular tumour antigen p53. *Nature*, **306**, 594–597 (1983).
6) Fey, E. G. and Penman, S. Nuclear matrix proteins reflect cell type of origin in cultured human cells. *Proc. Natl. Acad. Sci. USA*, **85**, 121–125 (1990).
7) He, D., Nickerson, J. A. and Penman, S. Core filaments of the nuclear matrix. *J. Cell Biol.*, **110**, 569–580 (1990).
8) Xiong, Y., Hannon, G. J., Zhang, D., Casso, R., Kobayashi, R. and Beach, D. p21 is a universal inhibitor of cyclin kinases. *Nature*, **366**, 701–704 (1993).
9) Dulic, V., Kaufman, W., Wilson, S., Tlsty, T., Lees, E., Harper, J., Elledge, S. and Reed, S. p53-dependent inhibition of cyclin-dependent kinase activities in human fibroblasts during radiation-induced G1 arrest. *Cell*, **76**, 1013–1023 (1994).
10) El-Deiry, W. S., Harper, J. W., O'Connor, P. M., Velculescu, V. E., Canman, C. E., Jackman, J. and Pietenpol, J. A. WAF1/CIP1 is induced in p53-mediated G1 arrest and apoptosis. *Cancer Res.*, **54**, 1169–1174 (1994).
11) Suzuki, K., Miyazawa, N., Nakata, T., Seo, H. G., Sugiyama, T. and Taniguchi, N. High copper and iron levels and expression of Mn-superoxide dismutase in mutant rats displaying hereditary hepatitis and hepatoma (LEC rats). *Carcinogenesis*, **14**, 1881–1884 (1993).
12) Yamamoto, F., Kasai, H., Togashi, Y., Takeichi, N., Hori, T. and Nishimura, S. Elevated level of 8-hydroxydeoxyguanosine in DNA of liver, kidney, and brain of Long-Evans cinnamon rats. *Jpn. J. Cancer Res.*, **84**, 508–511 (1993).
13) Wu, H., Wade, M., Krall, L., Grisham, J., Xiong, Y. and Dyke, T. V. Targeted *in vivo* expression of the cyclin-dependent kinase inhibitor p21 halts hepatocyte cell-cycle progression, postnatal liver development, and regeneration. *Gene Dev.*, **10**, 245–260 (1996).
14) Fujimoto, Y., Oyamada, M., Hattori, A., Takahashi, H., Sawaki, M., Dempo, K., Mori, M. and Nagao, M. Accumulation of abnormally high ploid nuclei in the liver of LEC rats developing spontaneous hepatitis. *Jpn. J. Cancer Res.*, **80**, 45–50 (1989).
15) Sawaki, M., Enomoto, K., Hattori, A., Tsuzuki, N., Sugawara, N. and Mori, M. Role of copper accumulation and metallothionein induction in spontaneous liver cancer development in LEC rats. *Carcinogenesis*, **15**, 1833–1837 (1994).
16) Nakayama, N., Kashiwazaki, H., Kobayashi, N., Hamada, J., Matsumoto, K., Nakamura, T. and Takeichi, N. Differing distribution of hepatocyte growth factor-positive cells in the liver of LEC rats with acute hepatitis, chronic hepatitis and hepatoma. *Jpn. J. Cancer Res.*, **86**, 5–9 (1995).
17) Ogawa, K., Mukai, H. and Mori, M. Effect of aging on proliferative activity of normal and carcinogen-altered hepatocytes in rat liver after a two-thirds partial hepatectomy. *Gann*, **76**, 779–784 (1981).
18) Sawada, N. and Ishikawa, T. Reduction of potential for replicative but not unscheduled DNA synthesis in hepatocytes isolated from aged as compared to young rats. *Cancer Res.*, **48**, 1618–1622 (1988).
19) Reisenbichler, H., Chari, R. S., Boyer, I. J. and Jirtle, R. L. Transforming growth factor-beta receptors type I, II and III in phenobarbital-promoted rat liver tumors. *Carcinogenesis*, **15**, 2763–2767 (1994).
20) Mansbach, J. M., Mills, J. J., Boyer, I. J., De Souza, A. T., Hankins, G. R. and Jirtle, R. L. Phenobarbital selectively promotes initiated cells with reduced TGFβ receptor level. *Carcinogenesis*, **17**, 171–174 (1996).
21) Elbendary, A., Berchuck, A., Davis, P., Havrilesky, L., Bast, R. C., Inglehart, J. D. and Marks, J. R. Transforming growth factor beta 1 can induce CIP/WAF1 expression independent of the p53 pathway in ovarian cancer cells. *Cell Growth Differ.*, **5**, 1301–1307 (1994).
22) Datto, M. B., Li, Y., Panus, J. F., Howe, D. J., Xiong, Y. and Wang, X.-F. Transforming growth factor β induces the cyclin-dependent kinase inhibitor p21 through a p53-independent mechanism. *Proc. Natl. Acad. Sci. USA*, **92**, 5545–5549 (1995).
23) Li, C. Y., Suardet, L. and Little, J. B. Potential role of Waf1/Cip1/p21 as a mediator of TGF-β cytoinhibitory effect. *J. Biol. Chem.*, **270**, 4971–4974 (1995).