Table 4. Characteristics of HCC Patients Examined for Detection of p18^{INK4C}-Bound Cdk4 and Cdk6

Patient	Sex	Age	Virus	*Histological Background	†Histological Grade	‡TNM Stage	p18^{INK4C}-Bound Cdk4	p18^{INK4C}-Bound Cdk6
F.O.	M	70	C	F4	WD	I	D	D
S.K.	M	54	C	F4	WD	I	D	ND
M.T.	M	62	C	F4	WD	II	D	ND
T.K.	F	66	C	F4	WD	I	D	ND
Y.S.	M	56	B	F3	WD	I	D	ND
K.H.	F	65	C	F4	WD	II	D	ND
T.K.	M	58	C	F3	WD	I	D	ND
N.S.	M	74	C	F4	MD	II	D	ND
Y.K.	M	61	C	F4	MD	I	D	ND
R.K.	M	52	B	F3	MD	I	ND	ND
T.K.	F	71	C	F4	MD	II	D	ND
Y.K.	M	60	C	F4	MD	III	D	ND
F.E.	M	66	C	F4	MD	I	D	D
S.N.	F	64	C	F4	MD	II	D	ND
T.M.	M	58	B	F3	MD	II	D	ND
Y.M.	M	74	C	F4	MD	II	D	ND
K.N.	M	67	C	F4	MD	III	D	ND

Abbreviations: B, hepatitis B; C, hepatitis C; WD, well-differentiated; MD, moderately differentiated; D, detection; ND, no detection.
*The fibrosis stage of the tissue surrounding HCC was assessed according to Desmet's classification.
†Histological grading of HCC was determined using the criteria of the International Working Party.[17]
‡TNM stage was determined using the classification proposed by the International Union Against Cancer and the American Joint Committee on Cancer.

has also been observed in testicular cancer and oligodendroglia.[22,23] These previous reports are consistent with the results of reduced p18^{INK4C} expression in HCC found in this study. Roncalli et al.[12] reported that p18^{INK4C} was not methylated in any of the HCCs examined. Therefore, there is little possibility that the cause of loss of p18^{INK4C} expression is related to the promoter methylation of p18^{INK4C}. However, because there have been no other papers examining methylation of the p18^{INK4C} gene in HCC, more studies are needed to draw any conclusions. In addition, loss of p18^{INK4C} protein at the level of translation, or proteosome-mediated degradation, might be responsible for the down-regulation of p18^{INK4C} in HCC.

All tissue samples used in this study were either HCV- or hepatitis B virus (HBV)-positive. In this study, the expression of p18^{INK4C} in HCV- or HBV-induced CH was detected in all cases, whereas expression was not detected in a subset of HCV- or HBV-induced HCCs. These data suggest that the changes in p18^{INK4C} expression in the process of HCC from CH are not affected by hepatitis viral infection but that its changes are affected by the malignant process.

Identification of the grade of tumor malignancy would facilitate treatment selection for patients and provide important information for predicting their prognosis. In HCC, clinical–pathological prognostic factors, such as tumor size, the number of tumor nodules, capsule formation, capsule invasion, and vascular invasion, have been studied.[34] Cell cycle-related molecules, such as proliferating nuclear antigen, p53, p21^{CIP1}, p27^{KIP2}, and p73, were shown to be prognostic biomarkers in various types of human cancer including HCC.[35–39] To date, the relationship between p18^{INK4C} and prognosis in cancers has been reported in only one study on oligodendroglioma.[23] However, there have been no reports on the relationship between p18^{INK4C} expression and prognosis in human HCC. In the present study, survival analysis by the Kaplan-Meier method revealed that p18^{INK4C} expression was associated with the overall survival of patients with HCC. Of particular importance is the finding that the loss of p18^{INK4C} expression was significantly associated with short survival of patients with HCC. According to the multivariate analysis, p18^{INK4C} and tumor stage were independent prognostic factors for overall survival. These data suggest that loss of p18^{INK4C} in HCC might serve as an indicator of poor prognosis.

In the univariate analysis, p18^{INK4C} expression correlated with the differentiation status and the tumor stage of HCCs, whereas only tumor stage and p18^{INK4C} expression level, and not the differentiation status, were independent prognostic markers in the multivariate analysis. In recent reports, Hu et al.[40,41] showed that after multivariate analysis, the differentiation of HCC is not an independent prognostic marker for overall survival, although it was a prognostic marker for overall survival in the univariate analysis; this is consistent with the results obtained in the present study. Collectively, the differentiation of HCC is a prognostic marker dependent on other factors, such as TNM stage and p18^{INK4C} expression.

The p18^{INK4C} protein has been shown to interact with, and subsequently inactivate Cdk4. Half of the HCCs in

this study lacked detectable levels of p18^{INK4C} protein when assayed by immunohistochemistry. Regarding Cdk4, we found previously that increase in its activity was particularly important in the development of HCC in humans and in the Long-Evans Cinnamon rat, an animal HCC model.[1,2] In addition, in our previous report[2] we found that the kinase activity of Cdk4 was markedly increased in poorly differentiated HCC compared to that in well- and moderately differentiated HCC, suggesting that Cdk4 activation may be closely related to the histopathological grade of HCC. Therefore, to determine whether reduced p18^{INK4C} in HCC is related to increased cell proliferative activity, we studied the activities of the target proteins of p18^{INK4C}, Cdk4, and Cdk6 in HCCs with the same degree of differentiation (*i.e.*, in order to not reflect the differentiation in HCC) in p18^{INK4C}-positive and p18^{INK4C}-negative HCCs. Although Cdk6 activity in p18^{INK4C}-positive and p18^{INK4C}-negative HCCs was not significantly different between well- and moderately differentiated HCCs, Cdk4 activity was significantly higher in well- and moderately differentiated p18^{INK4C}-negative HCCs than in p18^{INK4C}-positive HCCs. On the other hand, p18^{INK4C}-positive HCCs were not detected in any cases of poorly differentiated HCCs used in this study. Thus, we could not study the relationship between p18^{INK4C} expression and the activities of Cdk4 and Cdk6 in poorly differentiated HCC. The kinase activity levels of Cdk4 and Cdk6 in poorly differentiated HCCs were 16.5 ± 3.8 and 2.8 ± 1.3, respectively (data not shown). Based on these data, Cdk4 activity level was also markedly increased in poorly differentiated HCCs of p18^{INK4C}-negative HCCs. These data suggest that the up-regulation of Cdk4 activity in p18^{INK4C}-negative HCCs might not be related to the increase of its protein but rather to the reduction of p18^{INK4C} relative to that in p18^{INK4C}-positive HCCs.

Why does the reduced p18^{INK4C} lead to the up-regulation of Cdk4 activity but not of Cdk6? We hypothesized that p18^{INK4C} might bind to Cdk4 rather than to Cdk6 in HCC tissues. To study the formation of these complexes (p18^{INK4C}/Cdk4 and p18^{INK4C}/Cdk6), we immunoprecipitated p18^{INK4C} from HCC lysates with p18^{INK4C} expression and performed Western blot analysis of Cdk4 and Cdk6 (Fig. 5G). Based on these data, p18^{INK4C} was shown to substantially bind to Cdk4 but not to Cdk6 in HCC. Therefore, it was suggested that, in HCC, reduced p18^{INK4C} contributed only to the up-regulation of Cdk4. The p18^{INK4C} protein has been shown to interact with, and subsequently inactivate, Cdk4.[5,6] Conversely, there have been no reports that activation of Cdk4 does not directly decrease p18^{INK4C} expression. Therefore, it is difficult to guess whether increased Cdk4 activity in a subset of HCC does decrease p18^{INK4C} expression.

To date, p18^{INK4C} has been reported to play an important role in the regulation of cell differentiation.[22,23] To investigate the possible involvement of this protein in the differentiation of HCCs, we studied the relationship between the level of p18^{INK4C} expression and the histological grade of HCC. Expression of p18^{INK4C} was reduced in poorly differentiated HCCs compared with levels in well- and moderately differentiated HCCs, supporting the finding in previous reports that p18^{INK4C} accumulates at high levels in terminally differentiated cells.[22,42,43] However, our studies have not yet clarified whether the loss of p18^{INK4C} in HCC promotes or inhibits differentiation. These data suggest that decreased expression of p18^{INK4C} may play a role in the regulation of tumor differentiation as well as in the malignant transformation leading to HCC. In the present study, p18^{INK4C} was expressed in the nuclei of hepatocytes in NL and CH, and p18^{INK4C} was also detected in the cytoplasm in liver cirrhosis. p18^{INK4C} has been shown to localize both in the nucleus and in the cytoplasm of cells.[23,44] However, the significance of the cytoplasmic expression of p18^{INK4C} in hepatocytes of livers with cirrhosis remains unclear.

In conclusion, loss of p18^{INK4C} expression may be important in the process of malignant transformation and appears to be closely related to histological differentiation of HCC. In addition, loss of p18^{INK4C} was shown to be associated with up-regulation of Cdk4 activity. Furthermore, expression of p18^{INK4C} may be an effective predictor of clinical behavior in HCC, and, therefore, a new prognostic marker for HCC.

References

1. Masaki T, Shiratori Y, Rengifo W, Igarashi K, Matsumoto K, Nishioka M, et al. Hepatocellular carcinoma cell cycle: study of Long-Evans cinnamon rat. HEPATOLOGY 2000;32:711–720.
2. Masaki T, Shiratori Y, Rengifo W, Igarashi K, Yamagata M, Kurokohchi K, et al. Cyclins and cyclin-dependent kinases: comparative study of hepatocellular carcinoma versus cirrhosis. HEPATOLOGY 2003;37:534–543.
3. Yamagata M, Masaki T, Okudaira T, Imai Y, Shina S, Shiratori Y, et al. Small hyperechoic nodules in chronic liver diseases include hepatocellular carcinomas with low cyclin D1 and Ki-67 expression. HEPATOLOGY 1999; 29:1722–1729.
4. Hunter T, Pines J. Cyclins and cancer II: cyclin D and CDK inhibitors come of age. Cell 1994;79:573–582.
5. Harper JW, Elledge SJ. Cdk inhibitors in development and cancer. Curr Opin Genet Dev 1996;6:56–64.
6. Hall M, Peters G. Genetic alterations of cyclins, cyclin dependent kinases, and Cdk inhibitors in human cancer. Adv Cancer Res 1996;68:67–108.
7. Nobori T, Miura K, Wu DJ, Lois A, Takabayashi K, Carson DA. Deletions of the cyclin-dependent kinase-4 inhibitor gene in multiple human cancers. Nature 1994;368:753–756.
8. Hui AM, Sakamoto M, Kanai Y, Ito Y, Gotoh M, Yokota J, et al. Inactivation of p16^{INK4} in hepatocellular carcinoma. HEPATOLOGY 1996;24: 575–579.

9. Kudoh K, Ichikawa Y, Yoshida S, Hirai M, Kikuchi Y, Nagata I, et al. Inactivation of p16/CDKN2 and p15/MTS2 is associated with prognosis and response to chemotherapy in ovarian cancer. Int J Cancer 2002;99: 579–582.
10. Yi J, Wang ZW, Cang H, Chen YY, Zhao R, Yu BM, et al. p16 gene methylation in colorectal cancers associated with Duke's staging. World J Gastroenterol 2001;7:722–775.
11. Gerdes B, Ramaswamy A, Ziegler A, Lang SA, Kersting M, Baumann R, et al. p16^{INK4A} is a prognostic marker in resected ductal pancreatic cancer: an analysis of p16^{INK4A}, p53, MDM2, and Rb. Ann Surg 2002;235:51–59.
12. Roncalli M, Bianchi P, Bruni B, Laghi L, Destro A, Di Gioia S, et al. Methylation framework of cell cycle gene inhibitors in cirrhosis and associated hepatocellular carcinoma. HEPATOLOGY 2002;36:427–432.
13. Koga H, Sakisaka S, Harada M, Takagi T, Hanada S, Taniguchi E, et al. Involvement of p21$^{WAF/Cip\ 1}$, p27^{KIP1}, and p18^{INK4C} in troglitazone-induced cell-cycle arrest in human cell lines. HEPATOLOGY 2002;33:1087–1097.
14. Bai F, Pei XH, Godfrey VL, Xiong Y. Haploinsufficiency of p18^{INK4C} sensitizes mice to carcinogen-induced tumorigenesis. Mol Cell Biol 2003; 23:1269–1277.
15. Desmet VJ, Gerber M, Hoofnagle JH, Manns M, Scheuer PJ. Classification of chronic hepatitis: diagnosis, grading and staging. HEPATOLOGY 1994;19:1513–1520.
16. International Union Against Cancer. TNM classification of malignant tumors. In: Sobin LH, Wittekind C, eds. 5th ed. New York: Wiley-Liss, 1997:74–77.
17. International Working Party. Terminology of nodular hepatocellular lesions. HEPATOLOGY 1995;22:983–993.
18. Kitagawa M, Higashi H, Jung HK, Suzuki-Takahashi I, Ikeda M, Tamai K, et al. The consensus motif for phosphorylation by cyclin D1-Cdk4 is different from that for phosphorylation by cyclin A/E-Cdk2. EMBO J 1996;15:7060–7069.
19. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 1976;72:248–254.
20. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 1970;227:680–685.
21. Towbin H, Staehelin T, Gordon J. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc Natl Acad Sci U S A 1979;76:4350–4354.
22. Bartkova J, Thullberg M, Rajpert-De Mayts E, Skakkebaek NE, Bartek J. Cell cycle regulators in testicular cancer: loss of p18^{INK4C} marks progression from carcinoma in situ to invasive germ cell tumours. Int J Cancer 2000;85:370–375.
23. Korshunov A, Golanov A. Immunohistochemical analysis of p18^{INK4C} and p14ARF protein expression in 117 oligodendrogliomas: correlation with tumor grade and clinical outcome. Arch Pathol Lab Med 2002;126:42–48.
24. Zariwala M, Liu E, Xiong Y. Mutational analysis of the p16 family cyclin-dependent kinase inhibitors p15^{INK4b} and p18^{INK4c} in tumor-derived cell lines and primary tumors. Oncogene 1996;12:451–455.
25. Franklin DS, Godfrey VL, Lee H, Kovalev GI, Schoonhoven R, Chen-Kiang S, et al. CDK inhibitors p18^{INK4C} and p27^{KIP1} mediate two separate pathways to collaboratively suppress pituitary tumorigenesis. Genes Dev 1998;12:1899–1911.
26. Franklin DS, Godfrey VL, O'Brien DA, Deng C, Xiong Y. Functional collaboration between different cyclin-dependent kinase inhibitors suppresses tumor growth with distinct tissue specificity. Mol Cell Biol 2000; 20:6147–6158.
27. Latres E, Malumbres M, Sotillo R, Martin J, Ortega S, Martin-Caballero J, et al. Limited overlapping roles of p15^{INK4b} and p18^{INK4C} cell cycle inhibitors in proliferation and tumorigenesis. EMBO J 2000;19:3496–3506.
28. Kovalev GI, Franklin DS, Coffield VM, Xiong Y, Su L. An important role of CDK inhibitor p18^{INK4C} in modulating antigen receptor-mediated T cell proliferation. J Immunol 2001;167:3285–3292.
29. Ruas M, Peters G. The p16INK4A/CDKN2A tumor suppressor and its relatives. Biochim Biophys Acta 1998;1378:115–177.
30. Wu CW, Chen GD, Fann CS, Lee AF, Chi CW, Liu JM, et al. Clinical implications of chromosomal abnormalities in gastric adenocarcinomas. Genes Chromosomes Cancer 2002;35:219–231.
31. Bieche I, Khodja A, Lidereau R. Deletion mapping of chromosomal region 1p32-pter in primary breast cancer. Genes Chromosomes Cancer 1999; 24:255–263.
32. Matsuzaki M, Nagase S, Abe T, Miura K, Shiiba K, Sunomura M, et al. Detailed deletion mapping on chromosome 1p32-p36 in human colorectal cancer: identification of three distinct regions of common allelic loss. Int J Oncol 1998;13:1229–1233.
33. Yasui K, Arii S, Zhao C, Imoto I, Ueda M, Nagai H, et al. TFDP1, GLU4A, and CDC16 identified as targets for amplification at 13q34 in hepatocellular carcinomas. HEPATOLOGY 2002;35:1476–1484.
34. Takenaka T, Kasahara N, Yamamoto K, Kajiyama K, Maeda T, Itasaka H, et al. Results of 280 liver resections for hepatocellular carcinoma. Arch Surg 1996;131:71–76.
35. Hsu HC, Wu TT, Wu MZ, Sheu JC, Lee CS, Chen DS. Tumor invasiveness and prognosis in resected hepatocellular carcinoma. Clinical and pathogenetic implications. Cancer 1988;61:2095–2099.
36. Kitamoto M, Nakanishi T, Kira S, Kawaguchi M, Nakashio R, Suemori S, et al. The assessment of proliferating cell nuclear antigen immunohistochemical staining in small hepatocellular carcinoma and its relationship to histologic characteristics and prognosis. Cancer 1993;72:1859–1865.
37. Tannapfel A, Grund D, Katalinic A, Uhlmann D, Kockerling F, Haugwitz U, et al. Decreased expression of p27 protein is associated with advanced tumor stage in hepatocellular carcinoma. Int J Cancer 2000;20:350–355.
38. Tannapfel A, Wasner M, Krause K, Geissler F, Katalinic A, Hauss J, et al. Expression of p73 and its relation to histopathology and prognosis in hepatocellular carcinoma. J Natl Cancer Inst 1999;91:1154–1158.
39. Mise K, Tashiro S, Yogita S, Wada D, Harada M, Fukuda Y, et al. Assessment of the biological malignancy of hepatocellular carcinoma: relationship to clinicopathological factors and prognosis. Clin Cancer Res 1998; 4:1475–1482.
40. Hu TH, Huang CC, Liu LF, Lin PR, Liu SY, Chang HW, et al. Expression of hepatoma-derived growth factor in hepatocellular carcinoma. Cancer 2003;98:1444–1456.
41. Hu TH, Huang CC, Lin PR, Chang HW, GerLP, Lin YW, et al. Expression and prognostic role of tumor suppressor gene PTEN/MMAC1/TEP1 in hepatocellular carcinoma. Cancer 2003;97:1929–1940.
42. Schrantz N, Beney GE, Auffredou MT, Bourgeade MF, Leca G, Vazquez A. The expression of p18^{INK4} and p27^{KIP1} cyclin-dependent kinase inhibitors is regulated differently during human B cell differentiation. J Immunol 2000;165:4346–4352.
43. Franklin DS, Xiong Y. Induction of p18^{INK4C} and its predominant association with CDK4 and CDK6 during myogenic differentiation. Mol Biol Cell 1996;7:1587–1599.
44. Bartkova J, Thullberg M, Slezak P, Jaramillo E, Rubio C, Thomassen LH, Bartek J. Aberrant expression of G1-phase cell cycle regulators in flat and exophytic adenomas of the human colon. Gastroenterology 2001;120: 1680–1688.

Reprinted from GUT, August 2004, Vol 53, No 8, p 1167-1173

LIVER

Repeated adenoviral administration into the biliary tract can induce repeated expression of the original gene construct in rat livers without immunosuppressive strategies

K Tominaga, S Kuriyama, H Yoshiji, A Deguchi, Y Kita, F Funakoshi, T Masaki, K Kurokohchi, N Uchida, T Tsujimoto, H Fukui

Gut 2004;**53**:1167–1173. doi: 10.1136/gut.2003.013748

See end of article for authors' affiliations

Correspondence to: Professor S Kuriyama, Third Department of Internal Medicine, Kagawa University School of Medicine, 1750-1 Ikenobe, Miki-cho, Kita-gun, Kagawa 761-0793, Japan; skuriyam@kms.ac.jp

Accepted for publication 20 December 2003

Background: Systemic adenoviral readministration appears to be limited by immunogenicity.
Aims: We examined the feasibility of repeated adenovirus mediated gene transfer into the liver via the biliary tract.
Methods: Recombinant adenoviruses carrying a reporter *lacZ* gene were infused retrogradely into the common bile duct of rats. Transduction efficiency of the *lacZ* gene was estimated histochemically and quantitatively.
Results: Retrograde administration of recombinant adenoviruses into the common bile duct of rats resulted in efficient transgene expression in the liver, specifically in hepatocytes, but not in biliary epithelia. Transduction efficiency induced by intrabiliary adenoviral administration was not substantially different from that induced by intraportal adenoviral infusion. Transgene expression in the liver was however transient, and development of neutralising antibodies against adenovirus was observed in serum but not in bile. When adenoviruses were readministered into the common bile duct, successful re-expression of the transgene in the liver was achieved despite the existence of neutralising antibodies in serum. Interestingly, although proliferation of adenovirus specific T cells in response to adenoviral readministration was suppressed significantly by immunosuppressive FK506 treatment, levels of transgene expression in the liver achieved by intrabiliary adenoviral readministration were not significantly different between animals treated with and without FK506. Furthermore, third adenoviral administration into the common bile duct also induced successful transgene expression in the liver.
Conclusions: These results suggest that adenovirus mediated gene transfer into the liver may be repeatable without immunosuppressive strategies in clinical settings by means of endoscopic retrograde cholangiography.

Although adenoviral vectors are a prominent gene transfer vehicle, several factors significantly limit the utility of current early gene region 1 (E1) deleted adenoviral vectors. Transgene expression usually peters out in vivo within 2–3 weeks due, at least in part, to destruction of adenoviral vector transduced cells by host cellular immune responses directed against viral proteins and/or immunogenic transgene products.[1–3] Another important limitation of current adenoviral vectors is the difficulty in obtaining successful readministration using a vector of the same adenovirus serotype. Several studies have indicated that neutralising antibodies elicited by input viral particles substantially reduced the efficiency of vector readministration.[2–5] Humans are natural hosts of various serotypes of adenoviruses and the majority of humans possess neutralising antibodies against adenovirus serotypes 2 and 5.[6–9] Therefore, it should be noted that the first administration of adenoviral vectors to gene therapy patients who have been exposed to wild-type adenoviruses may correspond to re-administration of adenoviral vectors into animals. Although successful readministration of adenoviral vectors has been demonstrated in immunocompromised animals, studies using animals lacking a functional immune system do not address key issues pertaining to the use of adenoviral vectors in human clinical trials.

In the present study, using immunocompetent animals, we examined whether adenoviral administration into the liver via the biliary tract resulted in efficient transgene expression. Furthermore, we repeatedly infused adenoviruses retrogradely into the biliary tract with and without immunosuppressive FK506 treatment and examined whether repeated expression of the original gene construct was achievable in the liver.

METHODS

Adenoviral vector

Adex1CAlacZ adenovirus[10] was generously provided by Dr Izumu Saito (Institute of Medical Science, University of Tokyo, Tokyo, Japan). This adenoviral vector carries an adenovirus serotype 5 genome lacking the E1A, E1B, and E3 regions, and contains the *Escherichia coli* β-galactosidase gene, *lacZ* gene, as a reporter gene. The recombinant adenovirus was propagated and isolated in 293 cells, as described previously.[11] A single batch of high titre adenovirus stock (2×10^9 plaque forming units (pfu)/ml) was used throughout the subsequent experiments.

Adenoviral administration into the biliary tract

Ten week old female Sprague-Dawley rats were anaesthetised with ether and a midline abdominal incision was made. The intestines were displaced to expose the liver and common bile

Abbreviations: E1, early gene region 1; pfu, plaque forming units; ALT, alanine aminotransferase

duct. After clamping the distal site of the common bile duct to avoid antegrade outflow of the virus, a 30 gauge needle connected to a 1 ml syringe was inserted directly into the common bile duct. Adenovirus solutions (1×10^9 pfu/500 µl) were infused retrogradely into the biliary tract over one minute. On completion of the infusion, the needle was removed and pressure was gently applied over the puncture site of the common bile duct for five minutes. After removing the clamp from the common bile duct, the skin and fascia were closed.

Histochemical and quantitative estimations of transgene expression in the liver

LacZ gene expression in rat livers was evaluated histochemically by X-gal staining and quantitatively by a chemiluminescent assay, as described previously.[12-15] In all of the experiments performed in the present study, each group consisted of five animals.

Adenoviral readministration into the biliary tract

To examine the transduction efficiency in rat livers by adenoviral readministration, animals were infused with adenoviruses carrying the *lacZ* gene (1×10^9 pfu/500 µl) retrogradely into the common bile duct on day 0, as described above. Animals were then separated randomly into two groups. Animals in the former group received reinfusion of adenoviruses carrying the *lacZ* gene (1×10^9 pfu/500 µl) into the common bile duct on day 35, in the same way as in the first adenoviral infusion. Animals in the latter group were treated with FK506 around the time of adenoviral readministration. FK506 (5 mg/kg body weight in 100 µl of phosphate buffered saline/day) was injected intramuscularly every day from days 30 to 36. Adenoviruses carrying the *lacZ* gene (1×10^9 pfu/500 µl) were reinfused into the common bile duct on day 35, in the same way as in the first adenoviral infusion. Animals were sacrificed on days 37 and 42 (on days 2 and 7 after adenoviral readministration, respectively) and their livers were removed for analysis of *lacZ* gene expression, as described above.

Statistics

Results are expressed as means (SD). Standard descriptive statistics, Student's *t* test, and Welch's *t* test were used according to the distribution of experimental values. A p value of <0.05 was considered to indicate a significant difference between groups.

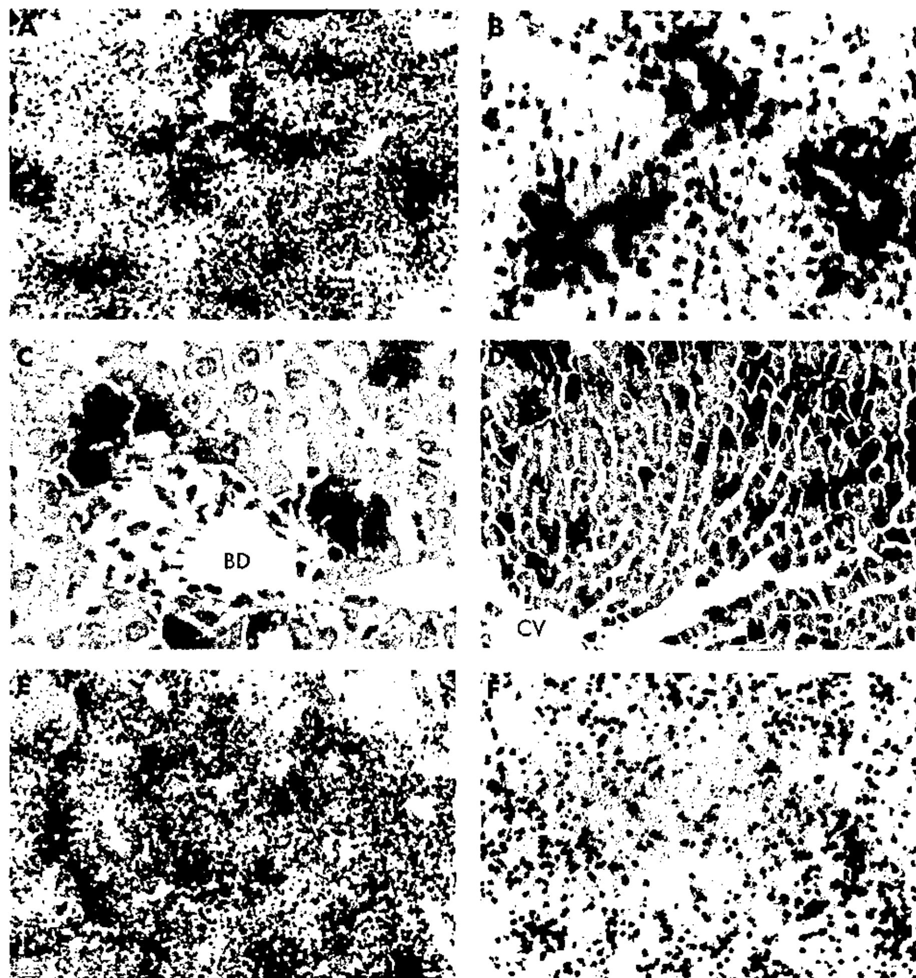

Figure 1 *LacZ* gene expression in rat livers induced by intrabiliary or intraportal administration of adenoviruses. Recombinant adenoviruses (1×10^9 pfu/500 µl) carrying the *lacZ* gene were infused into the common bile duct or into the portal vein of rats. Animals were killed on days 2 (A, B) and 7 (F) after intrabiliary adenoviral infusion, and on day 2 (E) after intraportal adenoviral administration. The livers were removed, sliced into 50 µm thick sections and subjected to X-gal staining. After X-gal staining, liver sections collected two days after intrabiliary adenoviral infusion were fixed in formaldehyde, embedded in paraffin, sliced into 4 µm thick sections, and counterstained with haematoxylin-eosin (C, D). BD, bile duct; CV, central vein. Each group consisted of five animals (a representative image is shown). (Original magnification A, E, F ×40; B ×100; C, D ×200.)

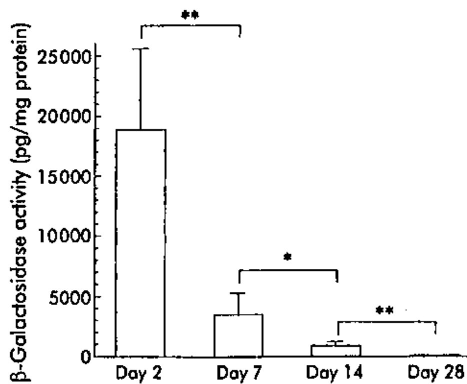

Figure 2 Quantitative estimation of *lacZ* gene expression in rat livers induced by retrograde adenoviral administration into the biliary tract. Recombinant adenoviruses (1×10^9 pfu/500 μl) carrying the *lacZ* gene were infused retrogradely into the common bile duct of rats. Animals were killed on days 2, 7, 14, and 28 after adenoviral infusion. Their livers were homogenised and β-galactosidase activity in the liver was estimated by the chemiluminescent reporter gene assay. Livers of rats that did not receive adenoviral administration were also subjected to the chemiluminescent assay to estimate background values of β-galactosidase activity in rat livers. Each bar represents the mean (SD) of five animals. $^*0.01<p<0.05$; $^{**}0.001<p<0.01$.

RESULTS

Transgene expression in the liver induced by adenoviral administration into the biliary tract

On day 2 after Adex1CAlacZ adenoviral infusion into the common bile duct, considerable areas in the liver were stained blue with X-gal staining (fig 1A). Although X-gal staining positive cells were seen predominantly at periportal areas, the so-called Rappaport's zone 1 (fig 1B), a considerable number of cells expressing the *lacZ* gene were observed in lobular and centrilobular areas, the so-called zones 2 and 3, respectively. Morphometric evaluation of liver sections using the public domain NIH Image program revealed that approximately 30% of cells in the liver expressed the *lacZ* gene. To identify cells positive for X-gal staining, liver sections after X-gal staining were fixed in 10% buffered formaldehyde, embedded in paraffin, sliced into 4 μm thick sections, and counterstained with haematoxylin-eosin. Interestingly, hepatocytes near the bile duct were positive for X-gal staining while biliary epithelia were negative for the staining (fig 1C). Furthermore, a number of hepatocytes in zones 2 and 3 were also positive for X-gal staining (fig 1D). Infiltration of inflammatory cells was seldom observed in the liver.

To compare the transduction efficiency in the liver induced by intrabiliary adenoviral administration with that induced by intraportal adenoviral infusion, Adex1CAlacZ adenoviruses were infused directly into the portal vein. On day 2 after adenoviral infusion, a considerable number of cells in the liver were stained blue with X-gal staining (fig 1E). Similar to the results for adenoviral administration into the common bile duct, X-gal staining positive cells were seen predominantly in zone 1. Morphometric evaluation of liver sections revealed that approximately 40% of cells in the liver expressed the *lacZ* gene. Although transduction efficiency induced by intraportal adenoviral infusion was slightly higher than that induced by intrabiliary adenoviral administration, the difference was not substantial.

We then examined duration of transgene expression in the liver caused by retrograde adenoviral administration into the biliary tract. X-gal staining positive areas were decreased significantly on day 7 after adenoviral infusion and approximately 5% of cells in the liver were stained blue with X-gal staining (fig 1F). X-gal staining positive cells in the liver were decreased markedly thereafter and there were few cells expressing the *lacZ* gene by day 28 (data not shown). As shown in fig 2, considerable levels of *lacZ* gene expression were observed in the liver on day 2 after adenoviral infusion, with mean level of β-galactosidase activity being approximately 19 000 pg/mg protein. Expression of the *lacZ* gene in the liver was decreased rapidly thereafter and mean levels of β-galactosidase activity on days 7, 14, and 28 were approximately 3500, 910, and 47 pg/mg protein, respectively. On day 28, β-galactosidase activity in the liver was not significantly different from that of naive untreated control rats.

Development of neutralising antibodies against adenovirus

Sera were collected from rats administered with adenoviruses retrogradely into the biliary tract and neutralising antibodies against adenovirus serotype 5 were examined, as previously described.[16] As rats are not natural hosts of adenovirus serotype 5, there were no detectable levels of neutralising antibodies against adenovirus in naive control rats. However, as shown in fig 3, development of neutralising antibodies against adenovirus was observed as early as day 2 after intrabiliary adenoviral infusion, with the mean titre of neutralising antibodies being ×120. Titres of neutralising antibodies against adenovirus were increased profoundly with the passage of time and mean titres were ×704, ×2816, and ×6656 on days 7, 14, and 28, respectively.

Adenoviral readministration into the biliary tract

We have already shown that adenoviral readministration into the portal vein or into the tail vein of rats without immunosuppressive strategies failed to induce significant transgene expression in the liver.[13, 14] Furthermore, we have also shown that a second adenoviral infusion into the tail vein of rats performed six or 12 months after the initial administration could not induce detectable levels of transgene expression in the liver.[17] Therefore, in the present study, we did not perform intravenous adenoviral readministration. Instead, to examine the feasibility of adenoviral readministration into the biliary tract, Adex1CAlacZ adenoviruses (1×10^9 pfu/500 μl) were readministered retrogradely into

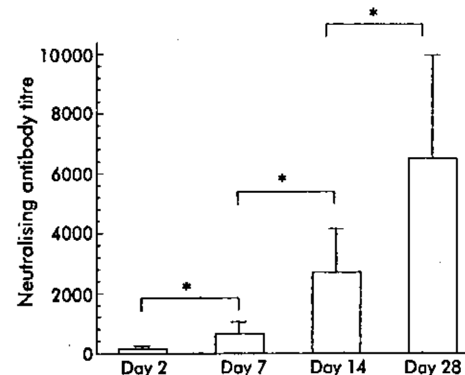

Figure 3 Development of neutralising antibodies against adenovirus elicited by retrograde adenoviral administration into the biliary tract. Serum samples were collected from rats infused with adenoviruses (1×10^9 pfu/500 μl) into the common bile duct when they were killed on days 2, 7, 14, and 28 after adenoviral infusion. Serum samples were decomplemented, serially diluted, and then analysed for neutralising antibodies against adenovirus serotype 5. The titre of neutralising antibody for each sample is expressed as the reciprocal dilution of serum that inhibited adenoviral infection by 50%. Each bar represents the mean (SD) of five animals. $^*0.01<p<0.05$.

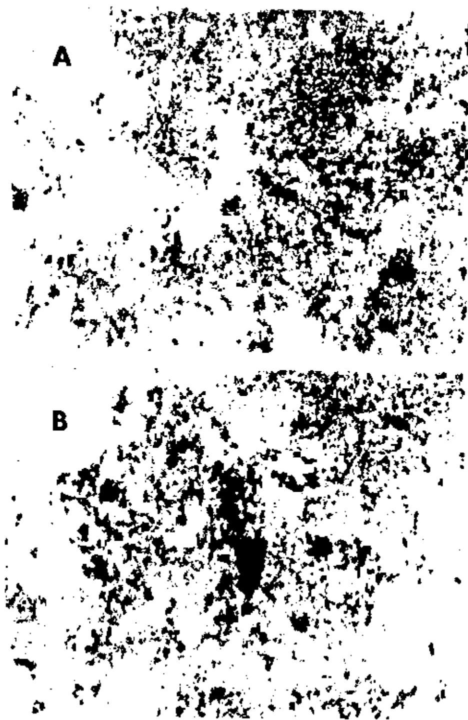

Figure 4 *LacZ* gene expression in rat livers induced by adenoviral readministration into the biliary tract. Recombinant adenoviruses (1×10^9 pfu/500 µl) carrying the *lacZ* gene were infused retrogradely into the common bile duct of rats. Animals were then separated randomly into two groups and treated with and without FK506. Animals in the FK506 treatment group were given intramuscular injections of FK506 (5 mg/kg body weight) from days 30 to 36. Animals in both groups were reinfused with adenoviruses (1×10^9 pfu/500 µl) retrogradely into the common bile duct on day 35. Animals were killed on day 37 and *lacZ* gene expression in the liver was examined by X-gal staining. Cells stained blue were observed in the liver of animals treated with (B) and without (A) FK506. A representative image is shown. (Original magnification ×40.)

the common bile duct on day 35 after the first administration. Furthermore, to examine the effect of an immunosuppressive strategy on intrabiliary adenoviral readministration, animals were separated randomly into two groups. Animals in the former group did not receive any immunosuppressive treatment and those in the latter group received intramuscular injections of FK506 daily from days 30 to 36 after the first adenoviral administration. Animals were killed on days 37 and 42 (on days 2 and 7, respectively, after adenoviral readministration) for analysis of transgene expression in the liver. As shown in fig 4A, X-gal staining of liver sections of animals without FK506 treatment revealed that there were a considerable number of cells expressing the *lacZ* gene in the liver. Although X-gal staining positive areas were seen predominantly in zone 1, a number of cells expressing the *lacZ* gene were observed in zones 2 and 3. As shown in fig 4B, X-gal staining positive cells were also observed in the liver of animals treated with FK506. The number of cells expressing the *lacZ* gene in the liver appeared to be similar between animals treated with and without FK506. Subsequent morphometric evaluation revealed that approximately 5% of cells in the liver expressed the *lacZ* gene, irrespective of FK506 treatment.

The results were estimated quantitatively by the subsequent chemiluminescent reporter gene assay. As shown in fig 5, levels of β-galactosidase activity in the liver of animals readministered with adenoviruses into the common bile duct with and without FK506 treatment were approximately 2200 and 1600 pg/mg protein, respectively, on day 2 after adenoviral readministration. Differences were not statistically significant between the groups. Levels of β-galactosidase activity in the liver induced by adenoviral readministration into the biliary tract were decreased significantly not only in animals without FK506 treatment but also in those with FK506 treatment, with levels of β-galactosidase activity being approximately 260 and 430 pg/mg protein, respectively, on day 7 after adenoviral readministration. Levels of transgene expression in the liver on day 7 after adenoviral readministration were not significantly different between the groups.

Humoral and cellular immune responses to adenoviral readministration into the biliary tract

To examine humoral responses to adenoviral readministration into the biliary tract, development of neutralising antibodies against adenovirus was examined. Adenoviruses were reinfused retrogradely into the common bile duct on day 35 after the first administration, and animals treated with and without FK506 around the time of adenoviral readministration were killed on days 37 and 42 (on days 2 and 7, respectively, after adenoviral readministration). The mean titre of neutralising antibodies against adenovirus reached approximately ×6656 on day 28 after the first adenoviral infusion into the common bile duct (see fig 3). Irrespective of FK506 treatment, neutralising antibodies against adenovirus were further increased in response to adenoviral readministration and titres were >×40 960 (the highest tested) not only on day 2 but also on day 7 after adenoviral readministration. In marked contrast, irrespective of FK506 treatment, titres of neutralising antibodies against adenovirus in bile were <×10 (the lowest tested) in all animals that were given adenoviral readministration into the common bile duct.

To examine cellular immune responses to adenoviral readministration into the biliary tract, activation of adenovirus specific splenic T cell proliferation was estimated, as previously described.[18] As shown in fig 6, splenic cells collected from naive control animals did not proliferate significantly with stimulation of heat inactivated adenoviruses. Splenic cells collected from animals on days 2 and 7

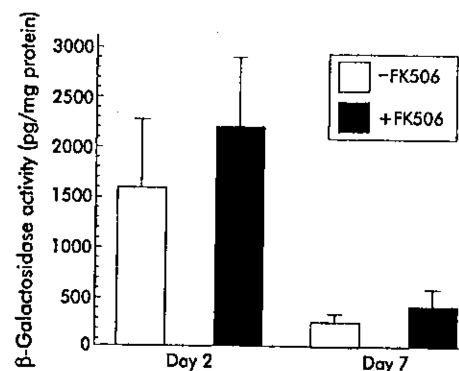

Figure 5 Quantitative estimation of *lacZ* gene expression in rat livers induced by adenoviral readministration into the biliary tract. Recombinant adenoviruses carrying the *lacZ* gene were infused retrogradely into the common bile duct of rats, as described in the legend of fig 4. Animals treated with and without FK506 around the time of intrabiliary adenoviral readministration were killed on days 2 and 7 after readministration. *LacZ* gene expression in the liver induced by adenoviral readministration was quantitatively estimated by the chemiluminescent assay. Each bar represents the mean (SD) of five animals. There were no significant differences in β-galactosidase activity between animals treated with and without FK506 not only on day 2 but also on day 7 after adenoviral readministration.

after adenoviral readministration also did not proliferate when they were not stimulated with heat inactivated adenoviruses. Conversely, when splenic cells collected from animals readministered with adenoviruses into the biliary tract were stimulated with heat inactivated adenoviruses, they exhibited marked proliferation. Levels of incorporation of [^{3}H] thymidine into splenic cells stimulated with heat inactivated adenoviruses were significantly higher in animals without FK506 treatment than in those with FK506 treatment not only on day 2 but also on day 7 after adenoviral readministration.

Third administration of adenovirus into the biliary tract

To confirm the feasibility of repeated adenovirus mediated gene transfer into the liver by way of the biliary tract, we performed the third adenoviral administration into the common bile duct. Adex1CAlacZ adenoviruses (1×10^9 pfu/500 µl) were infused retrogradely into the common bile duct of rats on days 0, 35, and 70 without FK506 treatment. Animals were killed on days 72 and 77 (on days 2 and 7, respectively, after the third administration) for analysis of transgene expression in the liver. As shown in fig 7, X-gal staining of liver sections of animals that were given the third adenoviral administration revealed that approximately 5% of cells in the liver, predominantly in zone 1, were positive for X-gal staining. Similar to the results of the first and the second adenoviral administrations into the biliary tract, X-gal staining positive cells were decreased significantly on day 7 after the third adenoviral administration (data not shown). The subsequent chemiluminescent assay showed that mean levels of β-galactosidase activity in the liver were approximately 1500 and 310 pg/mg protein on days 2 and 7, respectively, after the third adenoviral administration into the common bile duct. Duration of β-galactosidase expression in the liver after the third adenoviral administration was not substantially different from that after the first and second adenoviral administrations. Ratios of β-galactosidase activity on day 7 to those on day 2 after the first, second, and third adenoviral administrations were approximately 18%, 12%, and 21%, respectively.

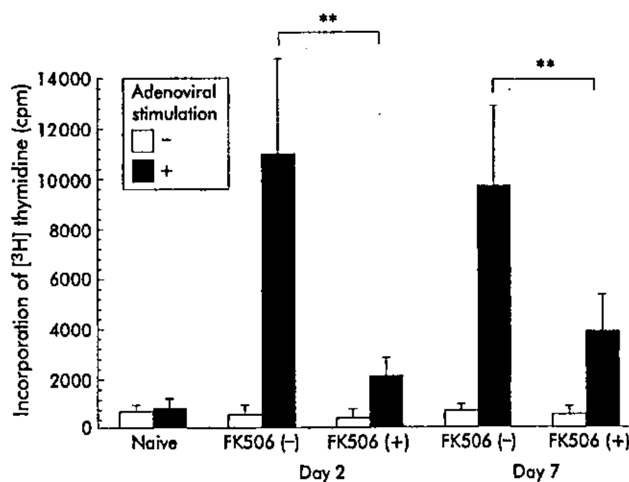

Figure 6 Cellular immune responses to adenoviral readministration into the biliary tract. To examine cellular immune responses to adenoviral readministration into the common bile duct, splenic cells were collected from rats and incubated with (+) or without (−) heat inactivated adenoviruses for five days and pulsed with [^{3}H] thymidine for the last 18 hours of incubation. Values are means (SD) of five animals. **$0.001<p<0.01$.

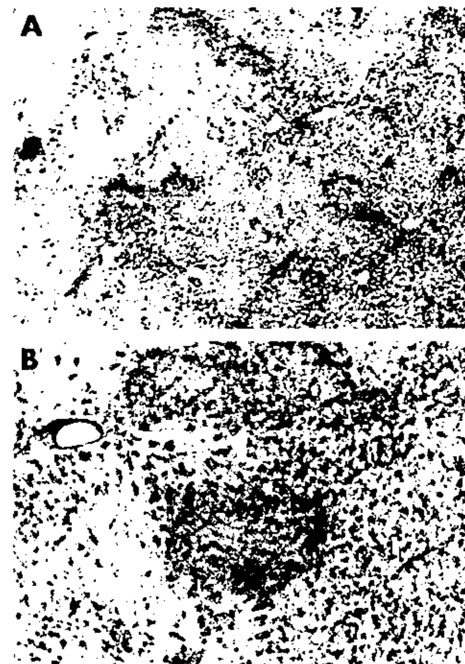

Figure 7 *LacZ* gene expression in rat livers induced by third adenoviral administration into the biliary tract. Recombinant adenoviruses (1×10^9 pfu/500 µl) carrying the *lacZ* gene were infused retrogradely into the common bile of rats on days 0, 35, and 70. Animals were killed on day 72 and *lacZ* gene expression in the liver was examined by X-gal staining. A representative image is shown. (Original magnification A ×40; B ×100.)

Serum alanine aminotransferase (ALT) levels in animals administered intrabiliary with adenoviruses

To examine the adverse reaction caused by intrabiliary adenoviral administration, serum samples were collected from rats two days after the first, second, and third adenoviral infusions into the common bile duct. Serum samples were also collected from rats two days after intraportal adenoviral administration, as well as from naive control animals. Serum ALT levels of naive animals were 42 (9) U/l/37°C and those of animals that received intraportal adenoviral administration were 66 (18) U/l/37°C. Serum ALT levels of rats that received intrabiliary adenoviral administration once, twice, and thrice were 51 (23), 64 (27), and 59 (15) U/l/37°C, respectively. Although values were slightly higher in animals that were given intraportal or intrabiliary adenoviral infusion than in naive controls, the differences were not statistically significant.

DISCUSSION

We have demonstrated here that retrograde administration of adenoviruses into the common bile duct could induce efficient transgene expression in rat livers. Although cells expressing the transgene were observed predominantly in zone 1, a considerable number of cells expressing the transgene were also observed in zones 2 and 3. Furthermore, it was shown that intrabiliary adenoviral administration resulted in transgene expression in hepatocytes but not in biliary epithelia. Transduction efficiency in the liver induced by adenoviral administration into the common bile duct was not substantially different from that induced by adenoviral administration into the portal vein.

One of the major problems with systemic adenoviral administration is that the animal becomes resistant to a second therapeutic administration of adenoviruses.[2 4 19 20] As systemic readministration of adenoviruses appears to be limited, several studies have been performed to examine

whether local direct readministration of adenoviruses might be possible without immune suppression in immunocompetent animals. Bennett and colleagues[21] have shown that subretinal administration of an adenoviral vector resulted in minimal antiadenovirus antibody production relative to subcutaneous administration, and successful repeated administration was observed. Effective repeated dosing in this organ is probably a reflection of the immune privileged status of the anterior chamber of the eye.[22] Chen and colleagues[23] have also shown that successful readministration of an adenoviral vector to skeletal muscle was possible without using any immunosuppressive drugs. They reasoned that the concentration of neutralising antibodies against adenovirus in the muscle might be considerably lower than the concentration in serum and thus permitted effective readministration to the muscle under conditions that did not allow readministration to the liver via the intravenous route. Conversely, McClane and colleagues[24] have demonstrated that local delivery of an adenoviral vector into the pancreas induced systemic responses that prevented local direct readministration of the vector. However, the feasibility of adenoviral readministration into the biliary tract has not been examined extensively to date. In the present study, we demonstrated that although adenoviral readministration into the biliary tract induced both humoral and cellular immune responses in rats, it could induce re-expression of the original gene construct in rat livers without immunosuppressive strategies. We showed here that even after adenoviral readministration into the common bile duct, titres of neutralising antibodies against adenovirus in bile were less than ×10 (the lowest tested) while those in serum were more than ×40 960 (the highest tested). Therefore, the plausible explanation for this outcome is that because concentrations of neutralising antibodies against adenovirus in bile were not elevated substantially after adenoviral administration into the common bile duct, readministration directly into the biliary tract allowed adenoviruses to infect hepatocytes through the biliary system without encountering neutralising antibodies, resulting in re-expression of the same transgene in the liver. Furthermore, we showed that the third adenoviral administration into the biliary tract could induce similar transduction efficiency in the liver compared with the second adenoviral administration. Although adenoviral administration into the common bile duct resulted in mild elevation of serum ALT levels, the values were not significantly different from those of naive control animals. Furthermore, substantial histological damage was not caused in the liver by intrabiliary adenoviral administration. These results suggest that repeated gene transduction into the liver can be achieved safely by adenoviral administration into the biliary tract.

To inhibit the production of neutralising antibodies against adenovirus that prevent further readministration of vectors, immunosuppressive strategies have been undertaken. It has been demonstrated that treatment regimens with immunosuppressive drugs, such as cyclophosphamide, FK506, cyclosporin A, and deoxyspergualin, around the time of initial exposure to adenoviruses, permitted prolonged transgene expression, reduced inflammation, prevented the formation of neutralising antibodies, and permitted successful readministration of adenoviral vectors.[25–28] The practicality of these approaches is however questionable because the majority of prospective gene therapy patients have already been infected with adenovirus serotypes 2 and 5. Therefore, the first adenoviral administration to humans may correspond to readministration of adenoviruses to animals. We have shown that human sera with a relatively high titre (>×128) of antiadenovirus antibody completely inhibited adenovirus mediated gene transfer not only in vitro but also in vivo. Furthermore, human sera with the lowest positive titre (×4) of antiadenovirus antibody also substantially inhibited adenovirus mediated gene transduction.[29] It is therefore expected that humans may not be susceptible to immune downregulation because activation requirements tend to be reduced in primed lymphocyte populations.[30] Furthermore, when these immunosuppressive strategies are applied in clinical settings, there is a risk associated with systemic immunosuppression.

In the present study, we demonstrated that adenoviral readministration into the biliary tract induced significant humoral and cellular immune responses to adenoviruses. Nevertheless, when adenoviruses were readministered into the biliary tract without any immunosuppressive agents, successful re-expression of the original gene construct was achieved in the liver. We also examined the effect of an immunosuppressive strategy on adenoviral readministration into the biliary tract. To provide a closer approximation of the expected clinical setting, we gave the immunosuppressive agent FK506 around the time of the secondary adenoviral administration but not around the time of the first adenoviral administration. FK506 treatment around the time of adenoviral readministration into the common bile duct significantly suppressed cellular immune responses to adenoviral readministration. However, there were no significant differences in transgene expression in the liver induced by adenoviral readministration between animals treated with and without FK506 treatment. Our results support the feasibility of administering recombinant adenoviruses to the human biliary tract by a relatively non-invasive approach, namely endoscopic retrograde cholangiography. This widely practised procedure is relatively safe with rare complications. In this procedure, the common bile duct is cannulated during endoscopic visualisation of the papilla of Vater of the duodenum. In clinical practice, endoscopically placed biliary cannulas are used to safely deliver radio-opaque contrast agents to the biliary tract, and this approach should be effective for infusion of recombinant adenoviruses. It allows for retrograde infusion of adenoviruses while antegrade outflow can be limited by balloon catheterisation of the distal common bile duct. Furthermore, after termination of adenoviral infusion, excessive adenoviruses are delivered immediately into the duodenum and excreted in stool.

In conclusion, retrograde adenoviral administration into the biliary tract may be a clinically practical modality for inducing repeated expression of therapeutic genes in the liver. Although more investigations should be performed to establish useful gene therapy with adenoviruses, our results support the feasibility of adenovirus mediated gene transfer into the liver in the clinic.

ACKNOWLEDGEMENTS

This work was supported in part by a Grant-in-Aid for Scientific Research (B-14370185) from the Japanese Ministry of Education, Culture, Sports, Science, and Technology.

....

Authors' affiliations

K Tominaga, H Yoshiji, T Tsujimoto, H Fukui, Third Department of Internal Medicine, Nara Medical University, 840 Shijo-cho, Kashihara, Nara 634-8522, Japan
S Kuriyama, A Deguchi, Y Kita, F Funakoshi, T Masaki, K Kurokohchi, N Uchida, Third Department of Internal Medicine, Kagawa University School of Medicine, 1750-1 Ikenobe, Miki-cho, Kita-gun, Kagawa 761-0793, Japan

REFERENCES

1 Tripathy SK, Black HB, Goldwasser E, *et al.* Immune responses to transgene-encoded proteins limit the stability of gene expression after injection of replication-defective adenovirus vectors. *Nat Med* 1996;**2**:545–50.

2 **Yang Y**, Li Q, Ertl HCJ, *et al.* Cellular and humoral immune responses to viral antigens create barriers to lung-directed gene therapy with recombinant adenoviruses. *J Virol* 1995;**69**:2004–15.
3 **Kaplan JM**, St George JA, Pennington SE, *et al.* Humoral and cellular immune responses of nonhuman primates to long-term repeated lung exposure to Ad2/CFTR-2. *Gene Ther* 1996;**3**:117–27.
4 **Yei S**, Mittereder N, Tang K, *et al.* Adenovirus-mediated gene transfer for cystic fibrosis: quantitative evaluation of repeated in vivo vector administration to the lung. *Gene Ther* 1994;**1**:192–200.
5 **Dong J-Y**, Wang D, Van Ginkel FW, *et al.* Systematic analysis of repeated gene delivery into animal lungs with a recombinant adenovirus vector. *Hum Gene Ther* 1996;**7**:319–31.
6 **D'Ambrosio E**, Del Grosso N, Chicca A, *et al.* Neutralizing antibodies against 33 human adenoviruses in normal children in Rome. *J Hyg* 1982;**89**:155–61.
7 **Piedra PA**, Poveda GA, Ramsey B, *et al.* Incidence and prevalence of neutralizing antibodies to the common adenoviruses in children with cystic fibrosis: implication for gene therapy with adenovirus vectors. *Pediatrics* 1998;**101**:1013–9.
8 **Rosenecker J**, Harms K-H, Bertele RM, *et al.* Adenovirus infection in cystic fibrosis patients: implications for the use of adenoviral vectors for gene transfer. *Infection* 1996;**24**:5–8.
9 **Weinberg A**, Fink MC, Takimoto S, *et al.* Enzyme linked immunosorbent assay: determination of anti-adenovirus antibodies in an infant population. *Rev Inst Med Trop São Paulo* 1989;**31**:336–40.
10 **Nakamura Y**, Wakimoto H, Abe J, *et al.* Adoptive immunotherapy with murine tumor-specific T lymphocytes engineered to secrete interleukin 2. *Cancer Res* 1994;**54**:5757–60.
11 **Miyake S**, Makimura M, Kanegae Y, *et al.* Efficient generation of recombinant adenoviruses using adenovirus DNA-terminal protein complex and a cosmid bearing the full-length virus genome. *Proc Natl Acad Sci USA* 1996;**93**:1320–4.
12 **Kuriyama S**, Yoshikawa M, Ishizaka S, *et al.* A potential approach for gene therapy targeting hepatoma using a liver-specific promoter on a retroviral vector. *Cell Struct Funct* 1991;**16**:503–10.
13 **Kuriyama S**, Tominaga K, Kikukawa M, *et al.* Transient cyclophosphamide treatment before intraportal readministration of an adenoviral vector can induce re-expression of the original gene construct in rat liver. *Gene Ther* 1999;**6**:749–57.
14 **Kuriyama S**, Tominaga K, Mitoro A, *et al.* Immunomodulation with FK506 around the time of intravenous re-administration of an adenoviral vector facilitates gene transfer into primed rat liver. *Int J Cancer* 2000;**85**:839–44.
15 **Nakatani T**, Kuriyama S, Tominaga K, *et al.* Assessment of efficiency and safety of adenovirus mediated gene transfer into normal and damaged murine livers. *Gut* 2000;**47**:563–70.
16 **Yang Y**, Greenough K, Wilson JM. Transient immune blockade prevents formation of neutralizing antibody to recombinant adenovirus and allows repeated gene transfer to mouse liver. *Gene Ther* 1996;**3**:412–20.
17 **Tsujinoue H**, Kuriyama S, Tominaga K, *et al.* Intravenous readministration of an adenoviral vector performed long after the initial administration failed to induce re-expression of the original transgene in rats. *Int J Oncol* 2001;**18**:575–80.
18 **Kaplan JM**, Smith AE. Transient immunosuppression with deoxyspergualin improves longevity of transgene expression and ability to readminister adenoviral vector to the mouse lung. *Hum Gene Ther* 1997;**8**:1095–104.
19 **Kozarsky KF**, McKinley DR, Austin LL, *et al.* In vivo correction of low density lipoprotein receptor deficiency in the Watanabe Heritable hyperlipidemic rabbit with recombinant adenoviruses. *J Biol Chem* 1994;**269**:13695–702.
20 **Yang Y**, Trinchieri G, Wilson JM. Recombinant IL-12 prevents formation of blocking IgA antibodies to recombinant adenovirus and allows repeated gene therapy to mouse lung. *Nat Med* 1995;**1**:890–3.
21 **Bennett J**, Pakola S, Zeng Y, *et al.* Humoral response after administration of E1-deleted adenoviruses: immune privilege of the subretinal space. *Hum Gene Ther* 1996;**7**:1763–9.
22 **Green DR**, Ware CF. Fas-ligand: privilege and peril. *Proc Natl Acad Sci USA* 1997;**94**:5986–90.
23 **Chen P**, Kovesdi I, Bruder JT. Effective repeat administration with adenovirus vectors to the muscle. *Gene Ther* 2000;**7**:587–95.
24 **McClane SJ**, Chirmule N, Burke CV, *et al.* Characterization of the immune response after local delivery of recombinant adenovirus in murine pancreas and successful strategies for readministration. *Hum Gene Ther* 1997;**8**:2207–16.
25 **Vilquin J-T**, Guérette B, Kinoshita I, *et al.* FK506 immunosuppression to control the immune reactions triggered by first-generation adenovirus-mediated gene transfer. *Hum Gene Ther* 1995;**6**:1391–401.
26 **Jooss K**, Yang Y, Wilson JM. Cyclophosphamide diminishes inflammation and prolongs transgene expression following delivery of adenoviral vectors to mouse liver and lung. *Hum Gene Ther* 1996;**7**:1555–66.
27 **Smith TAG**, White BD, Gardner JM, *et al.* Transient immunosuppression permits successful repetitive intravenous administration of an adenovirus vector. *Gene Ther* 1996;**3**:496–502.
28 **Ilan Y**, Jona VK, Sengupta K, *et al.* Transient immunosuppression with FK506 permits long-term expression of therapeutic genes introduced into the liver using recombinant adenoviruses in the rat. *Hepatology* 1997;**26**:949–56.
29 **Kuriyama S**, Tominaga K, Kikukawa M, *et al.* Inhibitory effects of human sera on adenovirus-mediated gene transfer into rat liver. *Anticancer Res* 1998;**18**:2345–52.
30 **Weiss A**. T lymphocyte activation. In: Paul WE, eds. *Fundamental immunology*. New York: Raven Press, 1993:467–504.

INTERNATIONAL JOURNAL OF ONCOLOGY 25: 1089-1096, 2004

Enhanced expression of adaptor molecule p46 Shc in nuclei of hepatocellular carcinoma cells: Study of LEC rats

SHUHEI YOSHIDA[1], TSUTOMU MASAKI[1], HAN FENG[1], JIN YUJI[1], YOSHIAKI MIYAUCHI[1], TOSHIHARU FUNAKI[1], HITOSHI YOSHIJI[2], KOUZO MATSUMOTO[3], NAOHITO UCHIDA[1], SEISHIRO WATANABE[1], KAZUTAKA KUROKOHCHI[1] and SHIGEKI KURIYAMA[1]

[1]Third Department of Internal Medicine, Kagawa University School of Medicine, 1750-1 Ikenobe, Miki-cho, Kita-gun, Kagawa 761-0793; [2]Third Department of Internal Medicine, Nara Medical University, 840 Shijo-cho, Kashihara, Nara 634-8522; [3]Institute for Animal Experimentation, Tokushima University School of Medicine, 3-18-15 Kumamoto-cho, Tokushima 770-8503, Japan

Received April 13, 2004; Accepted June 14, 2004

Abstract. Shc protein is known to be related to cell proliferation and carcinogenesis. However, the involvement of Shc in hepatocellular carcinoma (HCC) remains unknown. In the present study, we report that p46 Shc is probably expressed in the nuclei of hepatocytes and/or cancer cells during the development of HCC in Long-Evans Cinnamon (LEC) rats. The expression and localization of Shc in various pathological liver tissues obtained from LEC rats were analyzed by immunohistochemical study and Western blotting. Furthermore, tyrosine phosphorylation of Shc in various pathological liver tissues of LEC rats was studied by immunoprecipitation using a monoclonal anti-phosphotyrosine antibody. Although p66 Shc was detected in none of the liver tissues, regardless of pathological status, the expression of p46 Shc and that of p52 Shc increased proportionately with the development of HCC in LEC rats. Furthermore, although p52 Shc was localized only in the cytoplasm of hepatocytes and/or cancer cells, p46 Shc was found to express in both the nuclei and the cytoplasm of hepatocytes and/or cancer cells in precancerous and cancerous tissues of LEC rat liver. Tyrosine phosphorylation of p46 Shc and p52 Shc was detected only in cancer cells, and p46 Shc in such cells was much more heavily phosphorylated than p52 Shc. These results suggest that enhanced expression of p46 Shc and p52 Shc, as well as p46 Shc tyrosine phosphorylation, was involved not only in the process from normal liver to chronic hepatitis, but also in the transition from chronic hepatitis into HCC in LEC rats. Furthermore, unlike p52 Shc, p46 Shc was detected not only in the cytoplasm but also in the nuclei of hepatocytes (especially in transformed hepatocytes), and p46 Shc expressed in the nuclei may be closely related to hepatocarcinogenesis in LEC rats.

Correspondence to: Dr Shigeki Kuriyama, Third Department of Internal Medicine, Kagawa University School of Medicine, 1750-1 Ikenobe, Miki-cho, Kita-gun, Kagawa 761-0793, Japan
E-mail: skuriyam@med.kagawa-u.ac.jp

Key words: Src homology and collagen, hepatocellular carcinoma, phosphorylation, Long-Evans Cinnamon rats

Introduction

The inbred strain of Long-Evans Cinnamon (LEC) rats was established from the closed colony of Long Evans rats. These rats exhibit severe acute liver damage with jaundice spontaneously at the age of 4 to 5 months, leading to fulminant hepatic failure in more than 50% of individuals. The surviving rats usually develop chronic hepatitis and within a year develop cholangiofibroblast and hepatocellular carcinoma (HCC) (1). Because the natural history of the liver disease in LEC rats resembles that of human liver disease, in which HCC follows persistent chronic liver disease, LEC rats are regarded as one of the most useful animal models of HCC (2). Hitherto, little has been known about the mechanism that leads to the progression of HCC (3-7). Recently, it has been shown that the expression of adaptor proteins changes during the malignant transformation process of various cancers (8-13). Therefore, the deranged expression of Shc, one of the adaptor proteins, may also be the key to hepatocarcinogenesis in LEC rats.

The processes of malignant transformation and proliferation of HCC are controlled, at least in part, by various growth factor receptors (14-22). For instance, the process of hepatocarcinogenesis is known to involve extracellular signal molecules in addition to the following growth factors: fibroblast growth factor (FGF) (14,15), platelet-derived growth factor (PDGF) (15), epidermal growth factor (EGF) (16), hepatocyte growth factor (HGF) (17-20), and insulin growth factor (IGF) (21,22). Recently, the extracellular signals have been studied extensively in HCC, but little is known about the intracellular process of these extracellular signal molecules in HCC.

Many growth factors, cytokines, and adhesion molecules exert their effects by activating specific tyrosine kinases.

The activated receptor allows the docking of additional src homology 2 (SH2) and protein tyrosine binding (PTB) domain-containing adaptor molecules, which both coordinate and integrate intracellular signaling events. Src homology and collagen Shc is also an SH2-containing cytoplasmic adaptor protein that undergoes phosphorylation by receptors of the tyrosine kinase family, suggesting the possible role of Shc in the malignant process. The Shc locus is highly conserved throughout evolution, and this locus codes for three overlapping proteins (p46 Shc, p52 Shc, and p66 Shc) of 46, 52, and 66 kDa, respectively (23,24). p46 Shc and p52 Shc arise from the use of alternative translation initiation sites within the same transcript. In contrast, p66 Shc contains a unique N-terminal region and is generated as a result of an alternative splicing (12). Each isoform can serve as a substrate for activated cytoplasmic tyrosine kinases and/or receptor tyrosine kinases (RTKs) via either the PTB or SH2 domain, and these isoforms can then associate with other SH2 domains that contain signal molecules. Shc is involved in responses to stimuli that activate cell proliferation (25-31), differentiation (32), transformation, and invasion (6-13). p46 Shc and p52 Shc seem to drive these reactions forward. An alternative p66 Shc isoform seems to inhibit some of these processes (24-26). Thus, the roles of Shc isoforms in carcinogenesis are not straightforward and remain controversial (24,25,33,34).

Protein phosphorylation catalyzed by protein tyrosine and serine/threonine kinases has been recognized as a key regulatory event in a variety of cell functions, such as growth, differentiation, and malignant cell transformation. We previously demonstrated the importance of protein phosphorylation in signal transduction in relation to the proliferation of transformed hepatocytes (4,6,13,35-37). Shc is also a cytoplasmic adaptor protein that undergoes phosphorylation by various receptors of the tyrosine kinase family, including FGF (38), PDGF (39), EGF (23), HGF (40), and IGF (41) receptors. Therefore, it is important to study the role of Shc in HCC. However, to date, there have been no other studies on this topic. In the present study, we analyzed the levels of p46 Shc, p52 Shc, and p66 Shc as well as their localization in various liver diseases, such as normal liver, chronic hepatitis, and cancer. We found that p46 Shc was expressed in the nuclei of hepatocytes and/or cancer cells in the LEC rat liver, and that p46 Shc protein expressed in the nuclei of cancer cells and/or hepatocytes may be closely correlated with hepatocarcinogenesis in LEC rats. This is the first study to demonstrate that the main localization of p46 Shc is in the nuclei of cancer cells and highly proliferative hepatocytes of LEC rat livers.

Materials and methods

Animals. Inbred LEC rats were reared under specific pathogen-free conditions at the Institute for Animal Experimentation, University of Tokushima School of Medicine, and were coded as Tj (Tokushima, Japan).

Chemicals and antibodies. An avidin-biotin-peroxidase complex (ABC) kit was purchased from Funakoshi Chemical Co. (Tokyo, Japan). All other chemicals were from Sigma Chemical Co. (St. Louis, MO, USA) or Wako Pure Chemical Co. (Tokyo, Japan). Two anti-Shc antibodies used in this study were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). One is a monoclonal antibody PG-797, which was raised against a GST-tagged fusion protein sequence corresponding to the SH2 domain amino acids 366-473 of Shc of human and rat origin. The other is the polyclonal antibody C-20, which is affinity-purified rabbit polyclonal antibody raised against a peptide mapping at the carboxy terminus of Shc of human and rat origin. These anti-Shc antibodies are nonreactive with other SH2 domain-signaling intermediates. The Shc epitope recognized by PG-797 is different from that recognized by C-20. An anti-phosphotyrosine monoclonal antibody (PY20) was also purchased from Santa Cruz Biotechnology. PY20 (clone IgG2b) was produced *in vitro* by a mouse-mouse hybridoma, and it reacts with tyrosine phosphorylated proteins but not with proteins containing phosphoserine or phosphothreonine. An anti-proliferating cell nuclear antigen (anti-PCNA) was purchased from Dako (Glostrup, Denmark). All secondary antibodies were purchased from Amersham Life Sciences (Little Chalfont, UK). The optimal dilutions of antibodies for Western blot analysis in this study were as follows: monoclonal antibody PG-797, 1:300; polyclonal antibody C-20, 1:300; horseradish peroxidase (HRP)-anti-mouse IgG, 1:2000; HRP-anti-rabbit IgG, 1:2000. The optimal dilutions of antibodies for immunohistochemistry in this study were as follows: monoclonal antibody PG-797, 1:500; monoclonal antibody PC10, 1:700. The optimal amount of phosphotyrosine monoclonal antibody PY20 for immunoprecipitation was 4 μg for 1000 μg of protein.

Immunohistology. We prepared 2-μm-thick sections from formalin-fixed, paraffin-embedded tissue blocks. Sections of the liver specimens were immunohistologically stained by the ABC staining kit (Funakoshi Chemical, Tokyo, Japan), as described previously (4,13,35-37). To detect Shc, the sections were placed in 10 mM citrate-buffer (pH 6.0) and processed at 500 W and 95°C for 10 min in a microwave oven. The sections were deparaffinized in xylene, dehydrated in a graded series of alcohol solutions, and then mixed with a solution containing 0.5% hydrogen peroxide to block endogenous peroxidase activity. After washing with phosphate-buffered saline (PBS), sections were processed for immunostaining by the method described above. Primary incubation was performed overnight at 24°C with the monoclonal antibodies against Shc and PCNA. For signal amplification, the Renaissance TSA amplification kit (NEN Life Science Products, Boston, MA, USA) was used. Immunoreactive products were visualized by using diaminobenzidine, and the sections were counterstained with Mayer's hematoxylin. The specificity of immunostaining was examined by using nonimmune rabbit serum or PBS as a negative control for the primary antibody. The specificity of each immunostaining was further confirmed by the results of absorption testing. In brief, the primary antibody was mixed with an excess amount of peptide, which was used for immunization. The results were negative staining in every sample. These procedures confirmed the specificity of each immunostaining. Nuclear and cytoplasmic labeling indexes for Shc-positive cells (positive nuclei or cytoplasm/total counted)

were determined by evaluating at least 1000 hepatocytes or cancer cells at random in the microscopic field by each of two observers (T. Masaki and S. Yoshida). Nuclear labeling indexes for PCNA-positive cells (positive nuclei/total counted) were determined by the same method. The labeling index in the HCC liver tissues [tumor (T) tissues of 12-month-old LEC rats] was determined exclusively for cancer cells. In short, we counted only the cancer cells of T tissue of 12-month-old LEC rats.

Isolation and verification of a tumor's relative purity. At first, we distinguished the non-tumor (N) and T tissues of the liver tissues of 12-month-old LEC rats with the naked eye. A portion of each type of tissue was fixed with formalin and embedded in paraffin. Sections of the liver specimens were stained by hematoxylin and eosin, and were reviewed by two observers (T. Masaki and S. Yoshida). We confirmed that more than 80% of the cells in T tissues obtained in this way consisted of cancer cells, and that the remaining 20% were fibroblast and non-parenchymal cells. In brief, the T tissue samples used for preparation in this study were mostly cancer cells.

Tissue lysate. The tissue samples were frozen in dry ice within 20 min after collection. The samples were homogenized in a lysis buffer [50 mM N-2 hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES; pH 7.0), 250 mM NaCl, 0.1% Nonidet P-40, 100 mM NaF, 200 mM sodium orthovanadate, 0.5 mM phenylmethylsulfonyl fluoride, and 10 μg/ml aprotinin] and the lysates were centrifuged at 29,000 g for 20 min at 4˚C. The protein concentration of each sample was measured by the dye binding protein assay according to the method of Bradford (42).

Gel electrophoresis and Western blotting. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to the method of Laemmli (43), and Western blot was performed as described by Towbin *et al* (44), using optimal dilutions of primary antibodies and horseradish peroxidase-linked secondary antibodies. Immunoreactive proteins were visualized with an enhanced chemiluminescence detection system (Amersham) on a radiograph film. The exposure time with the Western blotting was 30 sec at room temperature for all samples.

Preparation of nuclear fractions in liver tissues. The nuclear protein was extracted as described in our previous report (5). All steps were carried out at 4˚C. Liver tissue samples obtained from LEC rats were homogenized in 5 volumes of 50 mM Tris-HCl (pH 7.4) containing 0.32 M sucrose, 1 mM ethylene glycol-bis (ß-aminoethyl ether)-N, N, N', N'-tetraacetic acid (EGTA), 3 mM benzamidine, 0.1 μg/ml of soybean trypsin inhibitor, 10 μg/ml of leupeptin, 25 mM KCl, and 5 mM $MgCl_2$. The homogenate was centrifuged at 600 g for 10 min. After collection, the pellet was homogenized in two volumes of 2.2 M sucrose containing 3.3 mM $CaCl_2$, 3 mM bezamidine, 0.1 μg/ml of soybean trypsin inhibitor, and 10 μg/ml of leupeptin. After centrifugation at 4,000 g for 60 min, the pellet was collected in the same buffer and subjected to SDS-PAGE and Western blot analyses.

Preparation of cytoplasmic fractions in liver tissues. All steps were carried out at 4˚C. Tissue samples obtained from LEC rat liver tissues were homogenized in two volumes of 50 mM Tris-buffer (pH 7.4) containing 1 mM EGTA, 3 mM benzamidine, 0.1 μg/ml soybean trypsin inhibitor, and 10 μg/ml leupeptin. The homogenate was centrifuged at 8,000 g for 15 min; then the supernatant was collected and centrifuged at 100,000 g for 60 min. The supernatant was collected in the same buffer and subjected to SDS-PAGE and Western blot analyses.

Immunoprecipitation. The protein concentration of the tissue samples was adjusted to an equivalent level (250 μg) and precleaned with protein-A sepharose Cl-4B. The samples were incubated with the polyclonal Shc antibody for 24 h at 4˚C and then incubated with 50 μl of protein-A sepharose Cl-4B (50% slurry). The samples were washed four times with the immunoprecipitation buffer [50 mM HEPES (pH 8.0), 150 mM NaCl, 2.5 mM EGTA, 1 mM dithiothreitol (DTT), 0.1% Tween-20] containing 10% glycerol, 0.1 mM PMSF, 10 mM ß-glycerophosphate, 1 mM NaF, and 0.1 mM HEPES (pH 8.0) containing 1 mM DTT. Immunoprecipitates were resolved by 12. 5% SDS-PAGE, and tyrosine-phosphorylated Shc was detected using the polyclonal Shc antibody.

Densitometry. The density of the immunoreactive band for Shc on Western blotting was analyzed by densitometric scanning (Quantity One version 4.3: Bio-Rad Laboratories, Hercules, CA, USA).

Statistical analysis. Data are expressed as means ± SD. The significance of differences between observations was determined by the Scheffe multiple comparison method. Statistical significance was set at $p<0.05$.

Results

Expression of Shc in liver tissues of LEC rats. The dominant histopathologic findings in liver tissues from the 2-, 6-, and 12-month-old LEC rats used in this study were normal liver (Fig. 1A, a), chronic hepatitis (Fig. 1A, b), and HCC (Fig. 1A, c), respectively. In addition, all N tissues of 12-month-old LEC rats displayed chronic hepatitis (Fig. 1A, d). Although normal liver tissues of 2-month-old LEC rats before the development of hepatitis were negative for Shc staining (Fig. 1A, e), staining for the Shc antibody in 6-month-old LEC rats was scattered among the nuclei of some hepatocytes (Fig. 1A, f, arrows). In 6-month-old LEC rats, the staining in the cytoplasm of hepatocytes was weak, whereas the staining in the nucleus was very strong. Immunoreactive Shc-positive cells were also detected in most cancer cells of the T tissues of 12-month-old LEC rat livers, especially in the nuclei of cancer cells. Shc protein was also stained in the cytoplasm of most cancer cells (Fig. 1A, g). In addition, Shc was localized in the nuclei and cytoplasm of some hepatocytes in the N tissues with hyperplastic nodules of 12-month-old LEC rats (Fig. 1A, h, arrow). The staining in the cytoplasm of hepatocytes was far weaker than that in the nuclei. To estimate the proliferative activity of hepatocytes and HCC cells, liver sections were stained with anti-PCNA antibody. The expression of PCNA

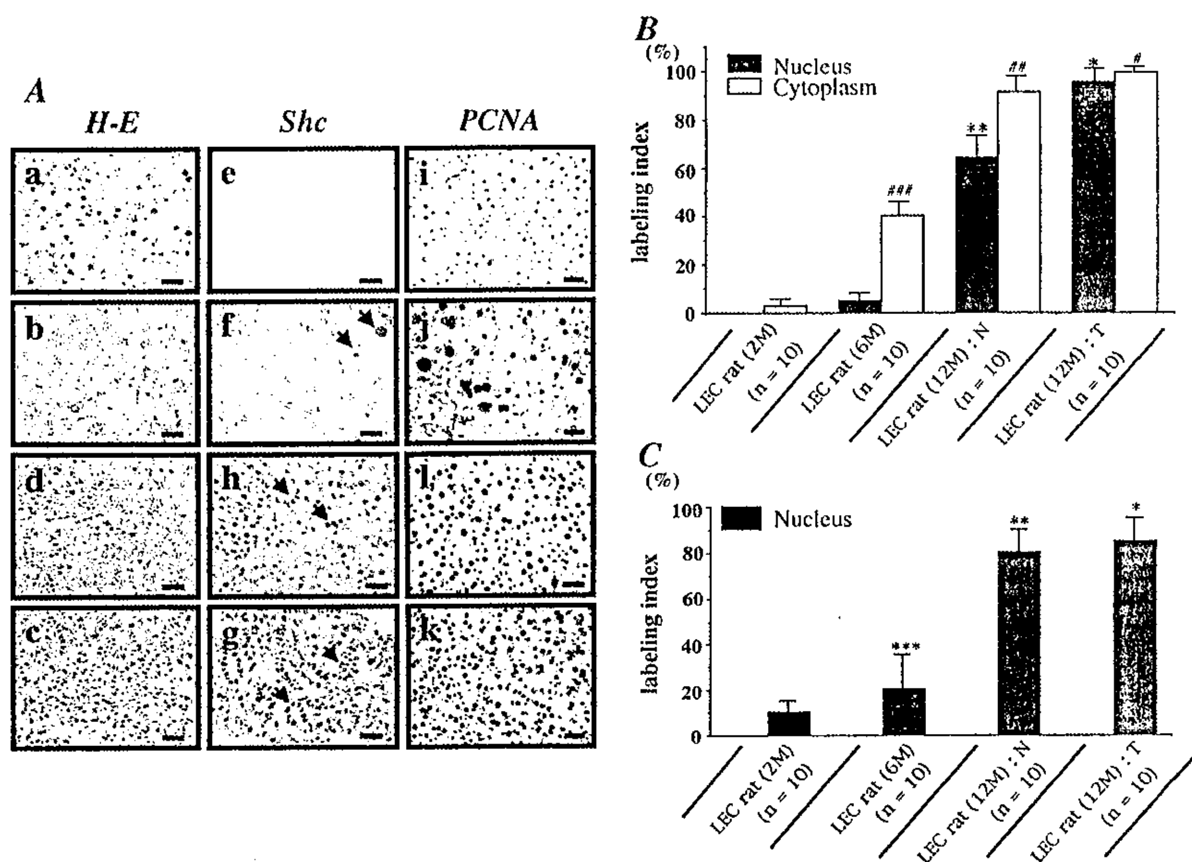

Figure 1. Immunohistochemical analyses of Shc and PCNA expression in LEC rat livers. (A), Liver sections obtained from LEC rats were for hematoxylin-eosin, Shc, and PCNA staining. (a-d), Histology of 2-, 6-, and 12-month-old LEC rat liver tissues by hematoxylin-eosin staining. (a), 2-month-old LEC rat. (b), 6-month-old LEC rat. (c), T tissue of a 12-month-old LEC rat liver. (d), N tissue of a 12-month-old LEC rat liver. The dominant histopathological findings in 2-, 6-, and 12-month-old LEC rat liver tissues were those of normal liver, chronic hepatitis, and hepatocellular carcinoma, respectively. The change in the hepatocytes with nuclear enlargement in N tissues of 12-month-old LEC rats is a characteristic histopathological feature of chronic hepatitis. (e-l), Immunohistochemistry of Shc (e-h) and PCNA (i-l) in the liver tissues of LEC rats. Photomicrographs of sections that reacted with the anti-Shc antibody or the anti-PCNA antibody in the liver tissues of 2- (e and i) and 6-month-old (f and j) LEC rats, and in the T (g and k) and N (h and l) portions of 12-month-old LEC rats, respectively. The immunostaining with the anti-Shc antibody was intense in the nuclei of hepatocytes and/or cancer cells in 6- or 12-month-old LEC rat livers (arrows in f, g, and h). The Shc staining in the cytoplasm of hepatocytes and cancer cells was weak, whereas the nucleic staining was strong in 6- and 12-month-old LEC rat livers. Shc was not stained at all in the liver tissues of 2-month-old LEC rats. Note that Shc staining was most intense in the nuclei of cancer cells of 12-month-old LEC rat livers. Although the expression of PCNA increased also proportionately with the development of HCC in LEC rats (i-l), the staining of PCNA did not change between N and T tissues of 12-month-old LEC rats. The sections (e and i), (f and j), (g and k), and (h and l) are adjacent to those shown in (a), (b), (c), and (d), respectively. All sections in (e-h) were counterstained with Mayer's hematoxylin. Bars, 50 µm. (B), Nuclear and cytoplasm labeling index for Shc in the liver tissues of 2-, 6-, and 12-month-old LEC rats. Values represent the mean ± SD ($^{*}p<0.001$ and $^{\#}p<0.05$ versus 2- or 6-month-old LEC rat livers and N tissue of 12-month-old LEC rats. $^{**}p<0.001$ and $^{\#\#}p<0.001$ versus 2- or 6-month-old LEC rat livers. $^{\#\#\#}p<0.001$ versus 2-month-old LEC rats) (C), Nuclear labeling index for PCNA in the liver tissues of 2-, 6-, and 12-month-old LEC rats. Values represent the mean ± SD ($^{*}p<0.05$ and $^{**}p<0.001$ versus 2-or 6-month old LEC rats. $^{***}p<0.05$ versus 2-month-old LEC rat. Note that there were no significant differences between N and T tissues in 12-month-old LEC rats.

increased proportionately with the development of HCC in LEC rats (Fig. 1A, i-l). Negative staining was demonstrated by using control mouse IgG (data not shown).

Labeling indexes of Shc-positive hepatocytes and/or cancer cells in the LEC rat liver. As shown in Fig. 1B, the labeling indexes of Shc-positive nuclei and cytoplasm of hepatocytes or cancer cells in the LEC rat liver tissues increased with age, and reached a peak (at 95.2±3.0% for the nuclei and at 98.1±1.7% for the cytoplasm) in T tissues of the 12-month-old LEC rats ($^{*}p<0.001$ and $^{\#}p<0.05$ as compared with the 2- or 6-month-old LEC rat liver as well as the N liver tissues of 12-month-old LEC rats). Although the labeling indexes of PCNA in the liver tissues of LEC rats also increased with age, there were no significant differences between N and T tissues in 12-month-old LEC rats (Fig. 1C).

Western blotting of Shc in the whole lysate of LEC rats. The expression of p52 Shc (Fig. 2A, arrowhead) and that of p46 Shc (Fig. 2A, arrow) were markedly increased in the T tissues of 12-month-old LEC rats compared with the liver tissues of 2- or 6-month-old LEC rats and the N tissues of 12-month-old LEC rats. The expression of p66 Shc was not detected in any LEC rat liver tissues. The amount of α-tublin (an internal control for protein loading) was almost the same in each lane in SDS-PAGE (Fig. 2B).

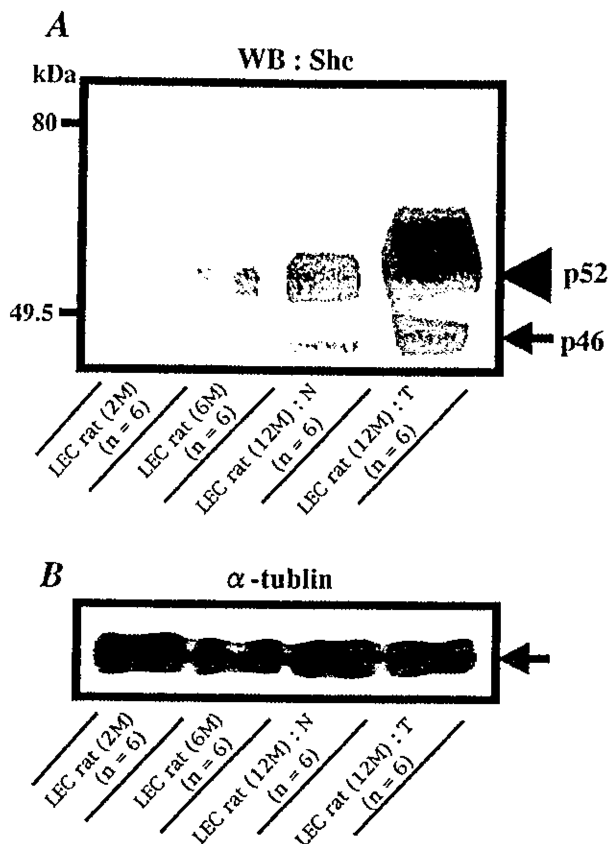

Figure 2. Expression of Shc in the whole liver lysate of 2- or 6-month-old LEC rats, and non-tumor (N) and tumor (T) portions of 12-month-old LEC rats. (A), 50 μg of the whole total lysate extracted from the liver was subjected to Western blot analysis using the Shc monoclonal antibody (PG-797). Note that the levels of p52 Shc and p46 Shc were markedly increased in the T tissue of 12-month-old LEC rats. (B), Western blotting of α-tublin in the whole tissue lysate of 2-, 6-, and 12-month-old LEC rat livers.

Western blotting of Shc in nuclear and cytoplasmic fractions of LEC rat liver tissues. To identify the Shc isoform expressed in the nuclei and cytoplasm of hepatocytes and cancer cells in LEC rats, we studied the expression of Shc isoforms in nuclear and cytoplasmic fractions of liver tissues by Western blot analyses. The expression of Shc was detected as having a molecular size of 46 kDa, corresponding to p46 Shc in the nuclear fractions of T tissues and N tissues of 12-month-old LEC rats (Fig. 3A). In contrast, p46 Shc expression was not detected in the nuclear fractions in any liver tissues of 2- or 6-month-old LEC rats. Neither p52 Shc nor p66 Shc was expressed in the nuclear fractions of any of the samples (n=6). The density of the immunoreactive 46 kDa bands obtained by Western blotting was analyzed by densitometric scanning. p46 Shc expression was elevated markedly in T tissues of 12-month-old LEC rats compared with the liver tissues of 2- or 6-month-old LEC rats or with N tissues of 12-month-old LEC rats (Fig. 3B). In cytoplasmic fractions, the expressions of p52 Shc and p46 Shc emerged from the liver tissues of 6-month-old LEC rats and 12-month-old LEC rats, respectively. Subsequent densitometric analyses revealed that the expressions of p46 Shc and p52 Shc in cytoplasmic fractions increased significantly with the age of the LEC rats, and both were the highest in T tissues (Fig. 3D). The p66 Shc was detected in neither the cytoplasmic fractions nor the nuclear fractions in liver tissues of 2-, 6-, or 12-month-old LEC rats (Fig. 3A and C).

Tyrosine phosphorylation of Shc. Tyrosine-phosphorylated Shc was examined in the whole lysate fraction of liver tissues from 2-, 6-, or 12-month-old LEC rats. No tyrosine phosphorylation of p46 Shc or p52 Shc was seen in 2- or 6-month-old LEC rats, while that of both p46 Shc and p52 Shc was detected in T tissues of 12-month-old LEC rats (Fig. 4). In T tissues, the protein level of p52 Shc was higher than that of p46 Shc (Fig. 2A). Nevertheless, the amount of tyrosine-phosphorylated p46 Shc was greater than that of tyrosine-phosphorylated p52 Shc in T tissues.

Discussion

The Ras-MAPK cascade is one of the most thoroughly investigated proliferative signal transduction pathways activated by growth factors (45). Recent reports have identified adaptor proteins such as Shc and Grb2, which couple with activated receptor tyrosine kinases to transmit signals to downstream effectors (23). In particular, Shc is believed to be involved in cellular signal transduction in inflammation (46), differentiation (32), proliferation (25-31), and carcinogenesis (8-13). However, no reports have focused on the role of Shc in the process of hepatocarcinogenesis. In this study we therefore examined the expression of Shc during hepatocarcinogenesis in LEC rats, an HCC experimental animal model (1-7). In this study, we showed that p46 Shc and p52 Shc increased proportionately with the development of HCC in LEC rats. Although p52 Shc was localized only in the cytoplasm of hepatocytes and/or cancer cells of LEC rat livers, p46 Shc expression was found in the nucleus and cytoplasm of hepatocytes and/or cancer cells in 12-month-old LEC rat livers. The tyrosine phosphorylation of p46 Shc and that of p52 Shc were detected only in cancer cells of 12-month-old LEC rat liver. Between the two, p46 Shc was more heavily tyrosine-phosphorylated than p52 Shc, although the protein level of p52 Shc was higher than that of p46 Shc.

In Western blot analysis of tissues from normal adult mice, p46 Shc was expressed in the pancreas, spleen, and stomach but not in the brain, spinal cord, heart, intestine, kidney, lung, or liver (47). Through Western blot and immunohistochemical analyses, we also found no expression of p46 Shc in normal liver tissues of 2-month-old LEC rats in this study, although such expression was observed in liver tissues of 12-month-old LEC rats, especially in cancer cells. On the other hand, p52 Shc has been detected in various liver disease including HCC. The present study also found p52 Shc expression in normal livers of 2-month-old LEC rats, and this expression was increased with the progression from normal liver or chronic hepatitis to HCC. These observations suggest that the induction of p46 Shc and p52 Shc expression is associated with the development of HCC. In addition, because the p46/p52 Shc gene has been mapped to chromosome 1q21 (48), the increased expression of p46 Shc and p52 Shc in HCC may be consistent with the previous results (49-51), indicating that the amplification of this chromosome

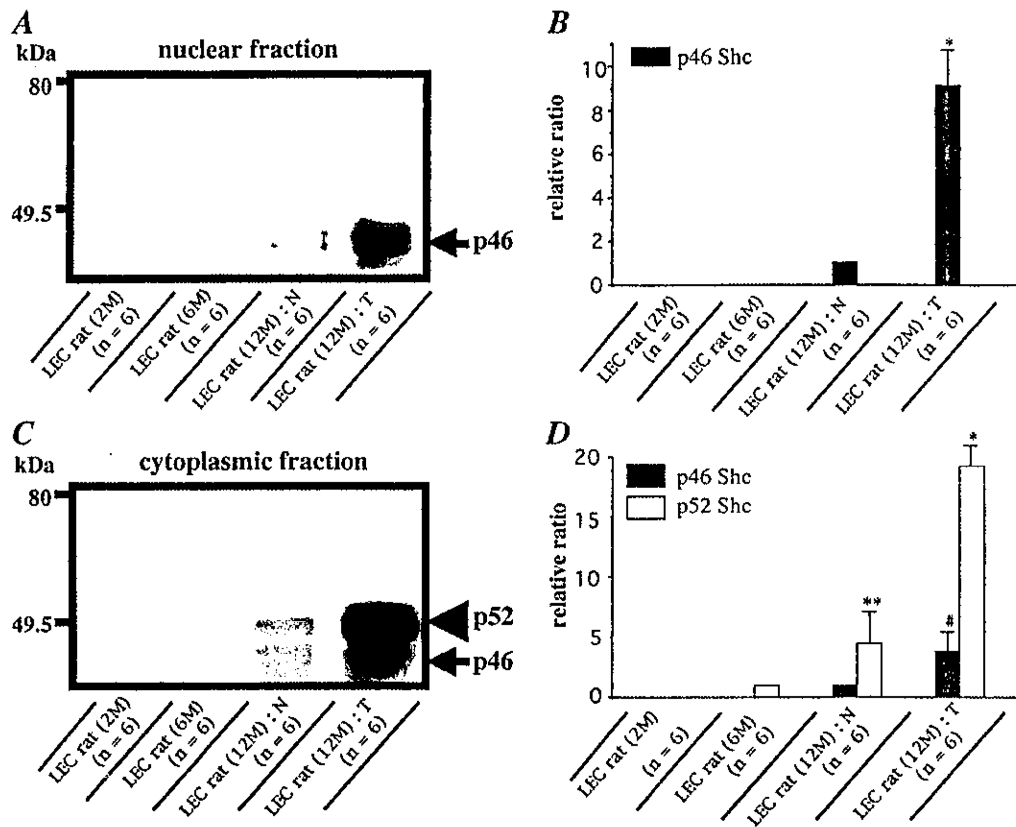

Figure 3. Expression of Shc in the nuclear and cytoplasmic fractions of LEC rat liver tissues. (A), Western blotting of Shc in the nuclear fractions of LEC rat liver tissues. In nuclear fractions, p46 Shc was not detected in liver tissues of 2- or 6-month-old LEC rats, but was detected in N and T tissues of 12-month-old LEC rat livers. Other Shc isoforms, i.e., p52 Shc and p66 Shc, were not detected in the nuclear fractions of liver tissues. Note that p46 Shc had the highest in T portion of the liver tissues of 12-month-old LEC rats. (B), Densitometric analysis of Western blotting of p46 Shc in 2-, 6-, and 12-month-old LEC rat livers. Levels of p46 Shc in nuclear fraction relative to the levels in N portion of liver tissues of 12-month-old LEC rats as the reference level (=1). Values represent the mean ± SD (*p<0.001 versus 2-, 6-month-old LEC rat livers, and N tissues in 12-month-old LEC rats). (C), Western blot analysis of Shc in the cytoplasmic fractions of the liver tissues of LEC rats. The major isoform of Shc in the cytoplasmic fractions of LEC rat livers was p52 Shc. Both p52 and p46 in cytoplasmic fractions increased proportionately with the development of HCC in LEC rats. (D), Densitometric analysis of Western blotting of p46 Shc and p52 Shc in 2-, 6-, and 12-month-old LEC rat livers. Levels of p46 Shc in cytoplasmic fraction are relative to the levels in N tissues of 12-month-old LEC rats as the reference level (=1), while levels of p52 Shc in cytoplasmic fraction are relative to the levels in liver tissues of 6-month-old LEC rats as the reference level (=1). Values represent the mean ± SD (*p<0.001 and #p<0.05 versus 2- or 6-month-old LEC rat livers and N tissues of 12-month-old LEC rats. **p<0.005 versus 2- and 6-month-old LEC rat livers).

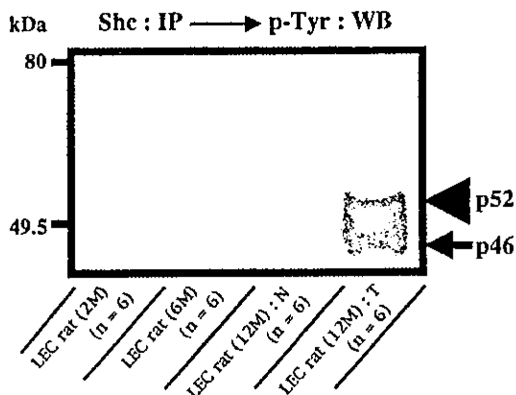

Figure 4. Expression of tyrosine phosphorylated Shc in the whole liver lysate of 2- or 6-month-old LEC rats, and N and T portions of 12-month-old LEC rats. The tyrosine phosphorylation of p46 Shc and p52 Shc was not detected in liver tissues of 2- or 6-month-old LEC rats, or in N tissues of 12-month-old LEC rats, but the tyrosine phosphorylation of both p46 and p52 Shc isoforms was detected in T tissues of 12-month-old LEC rats. Note that the tyrosine phosphorylation of p46 Shc was detected more strongly than that of p52 Shc in T tissues of 12-month-old LEC rats, although the protein level of p52 Shc was higher than that of p46 Shc (Fig. 2A).

region was detected in several cancers including human HCC.

Shc is a well-known cytoplasmic signal transducer involved in the transmission of mitogenic signals from various receptor tyrosine kinases to Ras (33). Therefore, it is believed that the expression of Shc is localized in the cell cytoplasm (52). The major location of p52 Shc in the present study also was in the hepatocellular cytoplasm, but not in the hepatocellular nuclei. On the other hand, p46 Shc expression was detected not only in the cytoplasm but also in the nuclei of hepatocytes and cancer cells in 12-month-old LEC rat livers. These data suggest that p46 Shc, expressed in nuclei of hepatocytes and cancer cells, may play a role in the malignant transformation of LEC rat liver. Cattaneo and Pelicci (53) have shown that although the expression of p46 Shc was not detected in normal astrocytes, it was observed in glial brain tumors that showed highly proliferative activity. Although the localization of p46 Shc expressed in cancer cells remains unknown following these reports, these results support our findings that p46 Shc is overexpressed in cancer cells of 12-month-old LEC rat livers.

Until this report, the presence of p46 Shc in the nuclei of cells had not been demonstrated. Although this study demonstrated the presence of p46 Shc in the nuclei of cancer cells of LEC rat livers, the mechanism for the existence of p46 Shc in the nuclei remains unclear. In general, it is known that many nuclear proteins have their own nuclear localization signals (NLSs); it appears possible that a protein without its own NLS could enter the nucleus via cotransport with a protein that does have an NLS. Therefore, we tried to detect NLSs in p46 Shc using PSORTS II (http://psort.nibb.ac.jp/), a computer program created to predict protein localization sites in cells (54). We have found that there are two NLSs in the amino acid sequence of human p46 Shc. One NLS is RRRK, corresponding to the 52-55 amino acid sequence of p46 Shc, and the other is RRKP, corresponding to the 53-56 amino acid sequence. These NLSs in p46 Shc may be one of the causes of p46 Shc expression in the nuclei of cancer cells and hepatocytes in LEC rat livers. However, these NLSs are involved in the common amino acids sequences in p46 Shc, p52 Shc, and p66 Shc. Therefore, certain extra N-terminal amino acid sequences in p52 Shc and p66 Shc may interfere with the NLSs and prevent the translocation of p52 Shc and p66 Shc to the nuclei of cancer cells and hepatocytes in LEC rats.

PCNA is closely related to DNA synthesis, which occurs during the late G1 to early G2 phases in the cell cycle (55). Therefore, PCNA-positive hepatocytes and cancer cells represent proliferating hepatocytes and/or cancer cells. Although the PCNA levels increased with the development of HCC in LEC rats, the difference in the expression of p46 Shc detected in the nuclei between hepatocytes in N tissues and cancer cells in T tissues was higher than it was in PCNA. These observations suggest that p46 Shc expression in the nucleus is a specific event that occurs in cancer cells and not always in proliferating normal hepatocytes.

In the present study, the tyrosine phosphorylation of p46 Shc and that of p52 Shc were detected only in cancer cells, and p46 Shc was tyrosine-phosphorylated more strongly than p52 Shc. These data suggest that the tyrosine phosphorylation of p46 Shc may play a role in the process of hepatocarcinogenesis. It is well known that various growth factor receptors are activated in HCC (15-22). Therefore, the tyrosine phosphorylation of p46 Shc may be caused by the upregulation of various growth factor receptor kinases during the malignant process in LEC rats. In fact, Nakayama *et al* (19) have reported the elevation of HGFR (c-Met) mRNA levels in HCC of LEC rats. In addition, the tyrosine phosphorylation of p46 Shc has been shown to occur in various cancer cell lines including those of stomach, pancreas, and thyroid melanoma, as well as in HCC cell lines (11). Collectively, these observations suggest that the tyrosine phosphorylation of p46 Shc may play a role in the malignant transformation of a broad range of cell types, including HCC.

In conclusion, the enhanced expression and tyrosine phosphorylation of p46 Shc and p52 Shc may be closely related to hepatocarcinogenesis in LEC rats. Furthermore, p46 Shc expressed in the nuclei of transformed hepatocytes may be one of the several factors that contribute to the malignant transformation and progression of HCC. Therefore, hepatocarcinogenesis in LEC rats may be a novel model for studying the functions of Shc in malignant transformation.

Acknowledgments

This study was supported in part by a Grant-in-Aid for Scientific Research (B-14370185 and C-15590654) from the Japanese Ministry of Education, Culture, Sports, Science and Technology.

References

1. Li Y, Togashi Y, Sato S, Emoto T, Kang JH, Takeichi N, Kobayashi H, Kojima Y, Une Y and Uchino J: Spontaneous hepatic copper accumulation in Long-Evans Cinnamon rats with hereditary hepatitis. J Clin Invest 87: 1858-1861, 1991.
2. Okuda K: A rat strain that spontaneously develops severe hepatic necrosis and later hepatocellular carcinoma. Hepatology 15: 948-963, 1992.
3. Kang JH, Togashi Y, Kasai H, Hosokawa M and Takeichi N: Prevention of spontaneous hepatocellular carcinoma in Long-Evans Cinnamon rats with hereditary hepatitis by the administration of D-penicillamine. Hepatology 18: 614-620, 1993.
4. Masaki T, Shiratori Y, Rengifo W, Igarashi K, Matsumoto K, Nishioka M, Hatanaka Y and Omata M: Hepatocellular carcinoma cell cycle: study of Long-Evans Cinnamon rats. Hepatology 32: 711-720, 2000.
5. Masaki T, Tokuda M, Shiratori Y, Shirai M, Matsumoto K, Nishioka M and Omata M: A possible novel src-related tyrosine kinase in cancer cells of LEC rats that develop hepatocellular carcinoma. J Hepatol 32: 92-99, 2000.
6. Masaki T, Okada M, Shiratori Y, Rengifo W, Matsumoto K, Maeda S, Kato N, Kanai F, Komatsu Y, Nishioka M and Omata M: pp60^{c-src} activation in hepatocellular carcinoma of humans and LEC rats. Hepatology 27: 1257-1264, 1998.
7. Kita Y, Masaki T, Funakoshi F, Yoshida S, Tanaka M, Kurokohchi K, Uchida N, Watanabe S and Kuriyama S: Expression of G1 phase-related cell cycle molecules in naturally developing hepatocellular carcinoma of Long-Evans Cinnamon rats. Int J Oncol 24: 1205-1211, 2004.
8. Davol PA, Bagdasaryan R, Elfenbein GJ, Marizel AL and Frackelton AR Jr: Shc proteins are strong, independent prognostic markers for both node-negative and node-positive primary breast cancer. Cancer Res 63: 6772-6783, 2000.
9. Stevenson LE and Frackelton AR Jr: Constitutively tyrosine phosphorylated p52 Shc in breast cancer cells: correlation with ErB2 and p66 Shc expression. Breast Cancer Res Treat 49: 119-128, 1998.
10. Saucier C, Papavasiliou V, Palazzo A, Naujokas MA, Kremer R and Park M: Use of signal specific receptor tyrosine kinase oncoproteins reveals that pathways downstream from Grb 2 or Shc are sufficient for cell transformation band metastasis. Oncogene 21: 1800-1811, 2002.
11. Pelicci G, Lanfrancone L, Salcini AE, Romano A, Male S, Grazia Borrello M, Segatto O, Di Fiore PP and Pelicci PG: Constitutive phosphorylation of Shc proteins in human tumors. Oncogene 11: 899-907, 1995.
12. Salcini AE, McGlade J, Pelicci G, Nicoletti I, Pawson T and Pelicci PG: Formation of Shc-Grb2 complexes is necessary to induce neoplastic transformation by overexpression of Shc proteins. Oncogene 9: 2827-2836, 1994.
13. Masaki T, Okada M, Tokuda M, Shiratori Y, Hatase O, Shirai M, Nishioka M and Omata M: Reduced C-terminal src kinase (Csk) activities in human hepatocellular carcinoma. Hepatology 29: 379-384, 1999.
14. Yoshiji H, Kuriyama S, Yoshiji J, Ikenaka Y, Noguchi R, Hicklin DJ, Huber J, Nakatani T, Tsujinoue H, Yanase K, Imazu H and Fukui H: Synergistic effect of basic fibroblast growth factor and vascular endothelial growth factor in murine hepatocellular carcinoma. Hepatology 35: 834-842, 2002.
15. Tsou AP, Wu KM, Tsen TY, Chi CW, Chiu JH, Lui WY, Hu CP, Chang C, Chou CK and Tsai SF: Parallel hybridization analysis of multiple protein kinase genes: identification of gene expression patterns characteristic of human hepatocellular carcinoma. Genomics 50: 331-340, 1998.

16. Harada K Shiota G and Kawasaki H: Transforming growth factor-alpha and epidermal growth factor receptor in chronic liver disease and hepatocellular carcinoma. Liver 19: 318-324, 1999.
17. Okano J, Shiota G and Kawasaki H: Expression of hepatocyte growth factor (HGF) and HGF receptor (c-met) proteins in liver diseases: an immunohistochemical study. Liver 19: 151-159, 1999.
18. Ueki T, Fujimoto J, Suzuki T, Yamamoto H and Okamoto E: Expression of hepatocyte growth factor and its receptor c-met proto-oncogene in hepatocellular carcinoma. Hepatology 25: 862-866, 1997.
19. Nakayama N, Kashiwazaki H, Kobayashi N, Hamada JI, Ogiso Y, Itakura Y, Matsumoto K, Nakamura T and Koike T: Hepatocytes growth factor and c-met expression in Long-Evans Cinnamon rats with spontaneous hepatitis and hepatoma. Hepatology 24: 596-602, 1996.
20. Boix L, Rosa JL, Ventura F, Castells A, Bruix J, Rodes J and Bartrons R: c-met mRNA overexpression in human hepatocellular carcinoma. Hepatology 19: 88-91, 1994.
21. Fan ZR, Yang DH, Cui J, Qin HR and Huang CC: Expression of insulin like growth factor II and its receptor in hepatocellular carcinogenesis. World J Gastroenterol 7: 285-288, 2001.
22. Kim SO, Park JG and Lee YI: Increased expression of the insulin-like growth factor I (IGF-I) receptor gene in hepatocellular carcinoma cell lines: implications of IGF-1 receptor gene activation by hepatitis B virus X gene product. Cancer Res 56: 3831-3836, 1996.
23. Pelicci G, Lanfrancone L, Grignani F, McGlade J, Cavallo F, Forni G, Nicoletti I, Grignani F, Pawson T and Pelicci PG: A novel transforming protein (SHC) with an SH2 domain is implicated in mitogenic signal transduction. Cell 70: 93-104, 1992.
24. Migliaccio E, Mele S, Salcini AE, Pelicci G, Lai KM, Superti-Furga G, Pawson T, Di Fiore PP, Lanfrancone L and Pelicci PG: Opposite effects of the p52shc/p46shc and p66shc splicing isoforms on the EGF receptor-MAP kinase-fos signalling pathway. EMBO J 16: 706-716, 1997.
25. Lanfrancone L, Pelicci G, Brizzi MF, Aronica MG, Casciari C, Giuli S, Pegoraro L, Pawson T, Pelicci PG and Arouica MG: Overexpression of Shc proteins potentiates the proliferative response to the granulocyte-macrophage colony-stimulating factor and recruitment of Grb2/SoS and Grb2/p140 complexes to the beta receptor subunit. Oncogene 10: 907-917, 1995.
26. Marshall MP: Ras target proteins in eukaryotic cells. FASEB J 9: 1311-1318, 1995.
27. Gotoh N, Toyoda M and Shibuya M: Tyrosine phosphorylation sites at amino acids 239 and 240 of Shc are involved in epidermal growth factor-induced mitogenic signaling that is distinct from Ras/mitogen-activated protein kinase activation. Mol Cell Biol 17: 1824-1831, 1997.
28. Faraldo MM, Deugnier MA, Thiery JP, Glukhova MA, Thiery JP and Glukhova MA: Growth defects induced by perturbation of beta1-integrin function in the mammary gland epithelium result from a lack of MAPK activation via the Shc and Akt pathways. EMBO Rep 2: 431-437, 2001.
29. Sayeski PP and Ali MS: The critical role of c-Src and the Shc/Grb2/ERK2 signaling pathway in angiotensin II-dependent VSMC proliferation. Exp Cell Res 287: 339-349, 2003.
30. Fiorucci S, Bufalari A, Distrutti A, Lanfrancone L, Servoli A, Sarpi L, Federici B, Bartoli A, Morelli A and Moggi L: Bombesin-induced pancreatic regeneration in pigs is mediated by p46Shc/p52Shc and p42/p44 mitogen-activated protein kinase upregulation. Scand J Gastroenterol 33: 1310-1320, 1998.
31. Yuji J, Masaki T, Yoshida S, Kita Y, Feng H, Uchida N, Yoshiji H, Kitanaka A, Watanabe S, Kurokohchi K and Kuriyama S: Identification of p46 Shc expressed in the nuclei of hepatocytes with high proliferating activity: Study of regenerating rat liver. Int J Mol Med 13: 721-728, 2004.
32. Laurino C and Cordera R: Role of IRS-1 and SHC activation in 3T3-L1 fibroblasts differentiation. Growth Horm Res 8: 363-367, 1998.
33. Bonfini L, Migliaccio E, Pelicci G, Lanfrancone L and Pelicci PG: Not all Shc's roads lead to Ras. Trends Biochem Sci 21: 257-261, 1996.
34. Okada S, Kao AW, Ceresa BP, Blaikie P, Margolis B and Pessin JE: The 66-kDa Shc isoform is a negative regulator of the epidermal growth factor-stimulated mitogen-activated protein kinase pathway. J Biol Chem 272: 28042-28049, 1997.
35. Masaki T, Tokuda M, Ohnishi M, Watanabe S, Fujimura T, Miyamoto K, Itano T, Matsui H, Arima K, Shirai M, Maeba T, Sogawa K, Konishi R, Taniguchi K, Hatanaka Y, Hatase O and Nishioka M: Enhanced expression of the protein kinase substrate annexin I in human hepatocellular carcinoma. Hepatology 24: 72-81, 1996.
36. Masaki T, Shiratori Y, Rengifo W, Igarashi K, Yamagata M, Kurokohchi K, Uchida N, Miyauchi Y, Yoshiji H, Watanabe S, Omata M and Kuriyama S: Cyclins and cyclin-dependent kinases: comparative study of hepatocellular carcinoma versus liver cirrhosis. Hepatology 37: 534-543, 2003.
37. Masaki T, Tokuda M, Fujimura T, Ohnishi M, Tai Y, Miyamoto K, Itano T, Matsui H, Watanabe S, Sogawa K, Yamada T, Konishi R, Nishoka M and Hatase O: Involvement of annexin I and annexin II in hepatocyte proliferation: can annexins I and II be markers for proliferative hepatocytes? Hepatology 20: 425-435, 1994.
38. Vainikka S, Joukov V, Wennstrom S, Bergman M, Pelicci PG and Alitalo K: Signal transduction by fibroblast growth factor receptor-4 (FGFR-4). Comparison with FGFR-1. J Biol Chem 269: 18320-18326, 1994.
39. Yokote K, Mori S, Hansen K, McGlade J, Pawson T, Heldin CH and Claesson-Welsh L: Direct interaction between Shc and the platelet-derived growth factor beta-receptor. J Biol Chem 269: 15337-15343, 1994.
40. Furge KA, Zhang YW and Vande Woude GF: Met receptor tyrosine kinase: enhanced signaling through adapter proteins. Oncogene 19: 5582-5589, 2000.
41. Pronk GJ, McGlade J, Pelicci G, Pawson T and Bos JL: Insulin-induced phosphorylation of the 46- and 52-kDa Shc protein. J Biol Chem 268: 5748-5753, 1993.
42. Bradford MM: A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72: 248-254, 1976.
43. Laemmli UK: Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227: 680-685, 1970.
44. Towbin H, Stadhelin T and Gordon J: Electrophoretic transfer of proteins from polyacrylamide gel to nitrocellulose sheets: procedure and some applications. Proc Natl Acad Sci USA 76: 4350-4354, 1979.
45. Stork PJ and Schmitt JM: Crosstalk between cAMP and MAP kinase signaling in the regulation of cell proliferation. Trends Cell Biol 12: 258-266, 2002.
46. Bates ME, Busse WW and Bertics PJ: Interleukin 5 signals through Shc and Grb 2 in human eosinophils. Am J Respir Cell Mol Biol 18: 75-83, 1998.
47. O'Bryan JP, Songyang Z, Cantley L, Der CJ and Pawson T: A mammalian adaptor protein with conserved Src homology 2 and phosphotyrosine-binding domains is related to Shc and is specifically expressed in the brain. Proc Natl Acad Sci USA 93: 2729-2734, 1996.
48. Huebner K, Kastury K, Druck T, Salcini AE, Lanfrancone L, Pelicci G, Lowenstein E, Li W, Park SH, Cannizzaro L, Pelicci PG and Schlessinger J: Chromosome locations of genes encoding human signal transduction adaptor proteins, Nck (NCK), Shc (SHC), and Grb 2 (GRB 2). Genomics 22: 281-287, 1994.
49. Yasui K, Arii S, Zhao C, Imoto I, Ueda M, Nagai H, Emi M and Inazawa J: TFDP1, CUL4A, and CDC16 identified as targets for amplification at 13q34 in hepatocellular carcinomas. Hepatology 35: 1476-1484, 2002.
50. Wong N, Chan A, Lee SW, Lam E, To KF, Lai PB, Li XN, Liew CT and Johnson PJ: Positional mapping for amplified DNA sequences on 1q21-q22 in hepatocellular carcinoma indicates candidate genes over-expression. J Hepatol 38: 298-306, 2003.
51. Wang Y, Wu MC, Sham JS, Zhang W, Wu WQ and Guan XY: Prognostic significance of c-myc and AIB1 amplification in hepatocellular carcinoma. A broad survey using high-throughput tissue microarray. Cancer 95: 2346-2352, 2002.
52. Clark SF, Martin S, Carozzi AJ, Hill MM and James DE: Intracellular localization of phosphatidylinositide 3-kinase and insulin receptor substrate-1 in adipocytes: potential involvement of a membrane skeleton. J Cell Biol 140: 1211-1225, 1998.
53. Cattaneo E and Pelicci PG: Emerging roles for SH2/PTB-containing Shc adaptor proteins in the developing mammalian brain. Trends Neurosci 21: 476-481, 1998.
54. Nakai K: Protein sorting signals and prediction of subcellular localization. Adv Protein Chem 54: 277-344, 2000.
55. Fairman MP: DNA polymerase delta/PCNA: actions and interactions. J Cell Sci 95: 1-4, 1990.

Angiotensin-I Converting Enzyme Inhibitors as Potential Anti-Angiogenic Agents for Cancer Therapy

Hitoshi Yoshiji*[,1], Shigeki Kuriyama[2], Ryuichi Noguchi[1] and Hiroshi Fukui[1]

[1] *Third Department of Internal Medicine, Nara Medical University, Nara, Japan*

[2] *Third Department of Internal Medicine, School of Medicine, Kagawa University, Kagawa, Japan*

Abstract: Angiotensin-I converting enzyme inhibitors (ACE-Is) are commonly used as safe antihypertensive agents, and it has recently been suggested that they decrease the risk of cancer development. Recent studies have revealed that the renin-angiotensin system (RAS) is involved in the development of many types of tumor. Angiotensin-II (AT-II) has many biological effects, including neo-vascularization, which plays a pivotal role in tumor development. AT-II induces a potent angiogenic factor, namely the vascular endothelial growth factor (VEGF). Some studies have proven that several ACE-Is are potent inhibitors of experimental tumor development and angiogenesis at clinically comparable doses. VEGF expression in tumors is also significantly suppressed by ACE-Is. When used in combination with the conventional anti-cancer drugs, ACE-Is exert more potent anti-tumor activities as compared with either single agent, in addition to suppression of the intra-tumoral angiogenesis. Furthermore, ACE-Is reportedly not only suppress tumor growth but also attenuate the carcinogenesis process in which angiogenesis is involved. Since ACE-Is are already in widespread clinical use without any serious adverse effects, they may represent a potential new strategy for cancer therapy and chemoprevention.

Keywords: Angiotensin-II, ACE inhibitor, angiogenesis, cancer, renin-angiotensin system, VEGF.

INTRODUCTION

Angiogenesis is a complex and critical process essential for supporting the growth of solid tumors [1, 2]. Any tumor mass in excess of a few cubic millimeters totally depends on the formation of a vascular network that provides the growing tumor with oxygen and essential nutrients. Therapies that aim at destroying the tumor vasculature can achieve rapid regression of the experimental tumors, and it has been shown that tumor cell apoptosis is significantly increased by treatment with anti-angiogenic agents [3-6]. It has been proven that anti-angiogenic therapy results in less drug resistance than conventional chemotherapy. With regard to conventional chemotherapy, drug resistance is encountered in about 30% of all cancer patients. The tumor cells readily acquire drug resistance because of their genetic instability, heterogeneity, and high mutation rate, whereas the endothelial cells (ECs) are genetically stable and acquire much less drug resistance. Recently, striking experimental results have been reported [7, 8]. One of those studies revealed that successive cycles of therapy using conventional chemotherapeutic agents led to acquired drug resistance as a result of selection for drug-resistant tumor cells. To the contrary, repeated cycles of anti-angiogenic therapy are followed by prolonged tumor dormancy without need for therapy. Thus, once a genuine anti-angiogenic therapy proves effective in a clinical trial, it may become a major anticancer therapy. Accordingly, anti-angiogenic therapy is under investigation around the world, including the use of gene therapy, anti-angiogenic recombinant proteins, monoclonal antibodies, and various small molecule drugs [9-11]. Although some of these agents are now undergoing Phases I, II and III clinical trials at certain institutes, no agent has become widely available at this time in the clinical practice. Some of the clinically available compounds, such as thalidomide and penicillamine, have been shown to possess an anti-angiogenic activity, and are currently under clinical trials [5, 12]. Avastin is a monoclonal antibody against vascular endothelial growth factor (VEGF) that has been approved for use in the treatment of colorectal cancer in the United States. Long-term application, however, of these agents sometimes leads to severe side-effects, such as bone marrow suppression and thromboembolism. Because of the nature of anti-angiogenesis therapy, long-term administration is required to examine its overall toxicity. Furthermore, it is difficult to simply compare the therapeutic effect of anti-angiogenesis therapy with that of conventional chemotherapy. Anti-angiogenesis agents have a cytostatic effect on cancer cells, whereas conventional chemotherapy directly injures cancer cells. These difficulties in assessing the anti-tumor activity of such cytostatic drugs clinically have also been discussed previously [9, 11, 12]. It appears that some time is required before the anti-angiogenic compounds under current trials can be applied widely in clinical practice. An alternative approach may be to find a clinically available compound that also shows an anti-angiogenic activity until these new drugs become widely available.

Recent studies have revealed that the clinically used anti-hypertensive agents; namely, angiotensin-I converting enzyme inhibitors (ACE-Is), exert a strong anti-angiogenic activity and suppress experimental tumor growth even at clinically comparable low doses [13, 14]. In this review, we summarize the interaction between the rennin-angiotensin system (RAS) and angiogenesis, and the feasibility of

*Address correspondence to this author at the Third Department of Internal Medicine, Nara Medical University, 840-Shijo-cho, Kashihara, Nara 634-8521, Japan; Tel: 81-744-22-3051 (ext 3415); Fax: 81-744-24-7122; E-mail: yoshijih@naramed-u.ac.jp

1568-0096/04 $45.00+.00 © 2004 Bentham Science Publishers Ltd.

utilizing ACE-Is as anti-angiogenic agents in clinical practice.

VASCULAR ENDOTHELIAL GROWTH FACTOR AND CANCER

To date, many positive and negative angiogenic factors have been identified [2, 15, 16]. Among the positive factors, the vascular endothelial growth factor (VEGF), originally identified as a vascular permeability factor (VPF) is the most infringing factor regarding tumor angiogenesis. VEGF is a specific mitogen for vascular ECs *in vitro* and can be an angiogenic factor for neovascularization *in vivo* [17-19]. In contrast to basic fibroblast growth factor (bFGF), which is also a representative positive angiogenic factor, VEGF has a typical signal peptide composed of 26 amino acids. Accordingly, it has been shown to be secreted abundantly in several human tumors and experimental animal models. An increase of VEGF expression in human surgical specimens has been shown to correlate with its aggressive tumor behavior and poor prognosis. In experimental animal models, overexpression of VEGF enhanced tumor growth, angiogenesis and dissemination whereas suppression of VEGF inhibited tumor growth in many tumor types [17-19].

Hepatocellular carcinoma (HCC) is one of the most common malignancies in the world with an estimated incidence greater than one million cases per year [20, 21]. One of the notable features of HCC in clinical practice is hypervascularity. As such, several studies have shown that the VEGF expression was up regulated in the tumor lesion of HCC more than in the non-cancerous lesion [22-27]. To elucidate the *in vivo* role of VEGF in tumor development and the mechanisms of VEGF-induced angiogenesis, we performed experimental studies using the HCC experimental models.

The tetracycline-controlled transactivator (tTA)-responsive promoter (Tet system) was originally a prokaryotic inducible promoter system which has been adapted for use in mammalian cells [28]. The Tet system is a novel drug-regulated gene expression system since it can manipulate gene expression using tetracycline. The gene expression of interest can be changed in a "switch on/off" manner in the Tet system, whereas conventional gene expression systems only exhibit the constitutive gene levels by either overexpression or suppression. The original two-plasmid-based Tet system, however, has limited applications for various reasons. First, in the original system, two separate plasmids must be introduced independently, and it cannot be used effectively *in vivo* except in transgenic animals. Second, it has been shown that the original system has some gene expression even when the system is switched off. The modified version of the Tet system that we used has been developed more recently [29]. In this modified system, the two components of the Tet system have been organized within one retroviral vector in the opposite direction, resulting in a decrease in the basal gene expression level. This "Retro-Tet" system allows overexpression of the gene under study in the absence of tetracycline and decreases the basal expression in the presence of tetracycline. With this Retro-Tet system, we have reported that overexpression of VEGF showed a marked increase in tumor development accompanied by augmentation of neo-vascularization. The degree of tumor enlargement corresponded to the level of the VEGF gene expression. On the other hand, suppression of VEGF led to a decrease in the tumor growth at the established tumor size, whether relatively small or large [30]. These results suggested that VEGF expression tightly regulated HCC development.

VEGF binds to and mediates its biological activity through two tyrosine kinases; namely, *fms*-like tyrosine kinase (flt-1: VEGFR-1) and the kinase insert domain-containing receptor/murine homologue, fetal liver kinase-1 (KDR/Flk-1: VEGFR-2), both of which are type III tyrosine kinase receptors, and have been identified as high affinity VEGF receptors [18, 31-33]. It has been suggested that VEGFR-1 and VEGFR-2 serve different roles in angiogenesis and signal transduction pathways, and that VEGFR-2 is a major regulator of angiogenesis, both *in vitro* and *in vivo*. Dominant negative VEGFR-2 and some other methods, which also inhibit the VEGF-receptor interaction, were found to substantially reduce the growth and angiogenesis of tumors [34-36]. The importance of VEGFR-2 in tumor angiogenesis suggests that blockade of this receptor would be a useful therapeutic strategy for inhibiting neo-vascularization and tumor growth. To examine the role of VEGF-VEGFR-2 interaction in tumor development, we combined the VEGFR-2-specific neutralizing monoclonal antibody (VEGFR-2mAb) and the Retro-Tet system and elucidated the role of VEGFR-2 in the VEGF-induced tumor development and angiogenesis in a murine HCC experimental model [37]. In a xenograft study, tumor augmentation induced by VEGF-overexpression was almost abolished by VEGFR-2mAb treatment, with concomitant inhibition of angiogenesis, VEGFR-2 auto-phosphorylation, without interference with VEGFR-1 activation. This inhibitory effect was achieved even on established tumors regardless of tumor size. VEGFR-2mAb treatment also significantly increased apoptosis in the tumor. We also directly injected HCC cells under the capsule of the liver, and found that VEGFR-2mAb also inhibited HCC development in the liver. These results suggest that VEGFR-2 is a major regulator of the VEGF-mediated HCC development and angiogenesis, not only at the initial stages, but also after the tumor has fully developed.

Recent studies have revealed that VEGFR-1 has a dual function in angiogenesis; i.e. acting in a positive or negative manner under different conditions [38]. In physiological angiogenesis, such as embryonic development, VEGFR-1 exerts a negative regulatory function, probably *via* its strong VEGF-trapping activity [39]. It has been revealed that VEGFR-1 can act as a potent positive regulator of VEGF under pathological conditions, such as tumor angiogenesis [40]. The other VEGF homologue; namely, the placental growth factor (PLGF), which binds to VEGFR-1 but not VEGFR-2, in transgenic mice, inhibited pathological angiogenesis [38]. Lung carcinoma cells overexpressing PLGF grew much faster in wild-type mice than in the VEGFR-1 tyrosine-kinase domain-deficient knockout mice [41]. Furthermore, it has been reported that VEGFR-1 is an important mediator of tumor angiogenesis, arthritis, and arteriosclerosis *via* bone marrow-derived hematopoietic stem-cell recruitment, and mobilization [42]. VEGFR-1 also plays an important role in lung metastasis through induction of matrix metalloproteinase (MMP)-9 [41]. On treatment with