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**Abstract**

Characteristics of human hepatoma cell lines with the wild-type p53 were compared with those of human hepatoma cell lines with the mutant-type p53. The p21 protein located downstream of p53 was expreed in cell lines with the wild-type p53 but was not expreed in cell lines with the mutant-type p53. As to other tumor suppreor genes such as p16 and p27, there was no difference in their expreion between both types of cell lines. In addition, no marked difference was observed in the activities of CDK2 and CDK4 between cell lines with the wild-type and the mutant-type p53. Phosphorylated Rb protein was detected in all cell lines except the HLE line, indicating that this cell line may have a deletion of and/or a mutation of the Rb gene. These results indicate that abnormalities of tumor suppreor genes other than p53, p16, p27, and Rb may be involved in hepatocarcinogenesis. The population doubling time of the wild-type p53 cells was significantly longer than that of the mutant p53 cells. Neither type of cell line showed a specific chromosome distribution which would indicate karyotype instability. The cell lines expreing the wild-type p53 produced tumors at lower frequency than those with the mutant p53 gene. Although there was no significant difference in effects of TGF-$\beta$1, EGF, cholera toxin, and db-cAMP on cell growth between the two types of cells, all three cell lines with the wild-type p53 were resistant to cytotoxicity of TNF-$\alpha$, while two of the three with the mutant p53 were very sensitive to its cytotoxic effects.

**KEYWORDS:** hepatoma, p53, p21?p16?p27?Rb, TNF-?

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# Comparison of Cellular Characteristics Between Human Hepatoma Cell Lines with Wild-Type p53 and Those with Mutant-Type p53 Gene

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Characteristics of human hepatoma cell lines with the wild-type p53 were compared with those of human hepatoma cell lines with the mutant-type p53. The p21 protein located downstream of p53 was expressed in cell lines with the wild-type p53 but was not expressed in cell lines with the mutant-type p53. As to other tumor suppressor genes such as p16 and p27, there was no difference in their expression between both types of cell lines. In addition, no marked difference was observed in the activities of CDK2 and CDK4 between cell lines with the wild-type and the mutant-type p53. Phosphorylated Rb protein was detected in all cell lines except the HLE line, indicating that this cell line may have a deletion of and/or a mutation of the Rb gene. These results indicate that abnormalities of tumor suppressor genes other than p53, p16, p27, and Rb may be involved in hepatocarcinogenesis. The population doubling time of the wild-type p53 cells was significantly longer than that of the mutant p53 cells. Neither type of cell line showed a specific chromosome distribution which would indicate karyotype instability. The cell lines expressing the wild-type p53 produced tumors at lower frequency than those with the mutant p53 gene. Although there was no significant difference in effects of TGF-$\beta$1, EGF, cholera toxin, and db-cAMP on cell growth between the two types of cells, all three cell lines with the wild-type p53 were resistant to cytotoxicity of TNF-$\alpha$, while two of the three with the mutant p53 were very sensitive to its cytotoxic effects.

**Key words:** hepatoma, p53, p21, p16, p27, Rb, TNF-$\alpha$

The p53 tumor suppressor gene, which acts at the G1 checkpoint in the cell cycle, is a frequent target for mutation in many kinds of human cancers (for review, 1). Approximately 50 % of all human tumors are estimated to contain a mutation in p53 (for review, 2). Nevertheless, about 70 % of hepatocellular carcinomas (HCC) do not show any p53 abnormalities. These observations raise a question about the significance of p53 inactivation during the process of hepatocarcinogenesis. Recent studies show that tumor suppressor genes other than p53 play important roles in the tumorigenesis of a variety of human cancers. For instance, deletions and mutations in the p16 or p27 have been reported in the development of human cancers (for review, 3).

We previously determined the status of the p53 gene by FASAY assay on human liver tumor cell lines, among which three cell lines (JHH-1, HuH-6 and HepG2) had the wild-type p53 and the other three lines (HLE, HuH-7 and PLC/PRF/5) showed the mutant p53 (4). In this study, to learn the pathogenesis of human liver tumors, we compared genetic and biological differences between human hepatoma cell lines with the wild-type p53 and those with the mutant-type p53 gene.

## Materials and Methods

***Cells and cultures.*** Human hepatocellular carcinoma (HCC) cell lines, JHH-1 (5), HLE (6), HuH-7 (7), PLC/PRF/5 (8), and hepatoblastoma (HB) cell lines, HuH-6 Clone 5 (9), HepG2 (10), were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 % fetal bovine serum (FBS) and 100 $\mu$g/ml kanamycin. The cultures were maintained at 37°C

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in a humidified atmosphere of 5% $CO_2$, and when cells reached confluence they were subcultured with 0.2% trypsin (Difco, Detroit, MI, USA) plus 0.02% EDTA solution in $Ca^{2+}$- and $Mg^{2+}$-free PBS.

***Immunohistochemical staining.*** Cells grown on cover slips were fixed with 95% ethanol for 60 min, and endogenous peroxidase activity was quenched for 20 min with 0.3% $H_2O_2$ solution in methanol. After washing cells twice with PBS, antigenic cross-reactivity was blocked with 1:40 diluted normal horse serum for 30 min. The cells were incubated for 2h with 1:100 diluted mouse primary antibody (PAB 1801, Oncogene Science, NY, USA) against p53, washed 3 times with PBS for 10 min, reacted for 30 min with horse anti-mouse IgG labelled with biotin (Vector Lab., Burlingame, CA, USA), and visualized with the streptavidin-peroxidase system according to the manufacturer's instructions (DAKO LSAB kit, Dako Co., Carpinteria, CA, USA).

***Western blot analysis.*** Logarithmically growing cells were lysed in a lysis buffer [50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 0.5% Nonidet P-40, 1 mM EDTA, 1 mM EGTA, 1 mM sodium fluoride, 0.1 mM sodium orthovanadate, 1 mM dithiothreitol, 200 $\mu$g/ml phenylmethylsulfonylfluoride, 2 $\mu$g/ml aprotinine and 2 $\mu$g/ml leupeptin]. After cell lysates were centrifuged at 12,000 $\times$ *g* for 30 min at 4°C, 40 $\mu$g protein was incubated at 65°C for 15 min with Laemmli sample buffer, fractionated by SDS polyacrylamide gel electrophoresis, and blotted on a nitrocellulose membrane filter. The filters were incubated for 1h with TBST [20 mM Tris-HCl (pH 7.2), 100 mM NaCl, 0.1% Tween 20] containing 5% dried milk, and then for 1.5h with 1:1000 dilutions of primary antibodies against p16, p21, p27, CDK2, CDK4, and cyclin D1 (Santa Cruz Biotechnology, Santa Cruz, CA, USA). After washing, the filters were incubated for 1h with 1:1000 diluted second horseradish peroxidase-conjugated sheep anti-rabbit IgG (MBL, Nagoya, Japan), and the reaction products were visualized using an enhanced chemiluminescence system (Amersham, Aylesbury, UK). Signal intensity was quantified using Image Quant version 3.3 (Molecular Dynamics). To isolate Rb protein, cell lysates were immunoprecipitated with rabbit IgG against synthetic pRb peptide p5. Then, the immunocomplexes were denatured, and subjected to 8% SDS polyacrylamide gel electrophoresis. The blotted Rb protein was detected with rabbit IgG against synthetic Rb peptide p3 (11).

***Kinase assay of CDK2 and CDK4.*** Cells were treated with a hypotonic buffer [50 mM Tris-HCl (pH 8.0), 1 mM EDTA, 1 mM EGTA, 1 mM sodium fluoride, 0.1 mM sodium orthovanadate, 200 $\mu$g/ml phenylmethylsulfonylfluoride, 2 $\mu$g/ml aprotinine and 2 $\mu$g/ml leupeptin], and centrifuged. Pellets were resuspended in a nuclear extraction buffer [50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 0.1% Tween-20, 1 mM EDTA, 1 mM EGTA, 1 mM sodium fluoride, 0.1 mM sodium orthovanadate, 200 $\mu$g/ml phenylmethylsulfonylfluoride, 2 $\mu$g/ml aprotinine and 2 $\mu$g/ml leupeptin]. Immunoprecipitation was carried out with 1 $\mu$g of the indicated antibodies, and the immunocomplexes were recovered with protein A-Sepharose. For Rb kinase assay, the immunocomplexes bound to protein A-beads were washed 3 times with the nuclear extraction buffer and twice with a kinase buffer [50 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 0.1 mM EDTA, 0.1 mM EGTA and 1 mM dithiothreitol]. Finally, pellets were resuspended in 30 $\mu$l of the kinase buffer supplemented with a substrate (0.4 U Rb fragment, Pharmacia, Uppsala, Sweden) and 10 $\mu$Ci of [$\gamma$-$^{32}$P] ATP, and incubated for 30 min at 30°C. The reaction was stopped by addition of 15 $\mu$l of 3 $\times$ Laemmli sample buffer. The reaction mixtures were then run on a 12% SDS polyacrylamide gel, and the phosphorylated substrates were visualized by autoradiography.

***Determination of population doubling time (PDT).*** Exponentially proliferating cells were trypsinized and the suspended cells were seeded onto a 24-well culture plate with 1 ml culture medium. On days 1 and 4, cells were detached with the trypsin-EDTA solution, stained with 1% crystal violet in 0.1 M citric acid, and counted with a hemocytometer. Since the cells grew logarithmically during this period of time (3 days), PDT was calculated as follows: PDT = 72 (h)/($\log_2$ number of cells on day 4 $-$ $\log_2$ number of cells on day 1). Each point was obtained from triplicate cultures.

***Effects of TGF-$\beta$, EGF, TNF-$\alpha$, cholera toxin and db-cAMP on cell growth.*** Cells were seeded onto a 24-well culture plate at concentrations of $10^4$ to $5 \times 10^4$ per well with DMEM supplemented with 10% FBS. The following day, the culture medium was replaced by 1 ml of serum-free culture medium consisting of DMEM/F12 (1:1), 0.1% human serum albumin, 10 $\mu$g/ml transferrin, 10 $\mu$g/ml insulin, $10^{-8}$ M selenious acid, 50 $\mu$g/ml kanamycin, and then the cells were treated for 3 days with TGF-$\beta$1 (R & D Systems, Minneapolis, MN, USA), EGF (Sigma Chemical Co., St. Louis, MO, USA), TNF-$\alpha$ (12), cholera toxin

(Wako Chemical Co., Osaka, Japan) or dibutyryl-cAMP (db-cAMP, Sigma) at various concentrations indicated in the Results. Cells were counted as described above. The cell survival rate was determined as follows: (number of cells treated/number of cells untreated) × 100 (%). Each point was obtained from triplicate cultures.

***Chromosome analysis.*** Chromosome preparation was performed using the conventional method.

***Assay of tumorigenicity.*** The tumorigenicity of cells was assayed by subcutaneous injection into athymic nude mice (C3H/He-nu/nu). Each mouse was given $10^7$ cells suspended in 0.1 ml of culture medium. Animals were observed for up to 3 months.

## Results and Discussion

The morphologies of the six human liver tumor cell lines grown as monolayers are shown in Fig. 1. All cell lines appear epithelial-like, but their features differ from one another. The cell size of each cell line increased in the

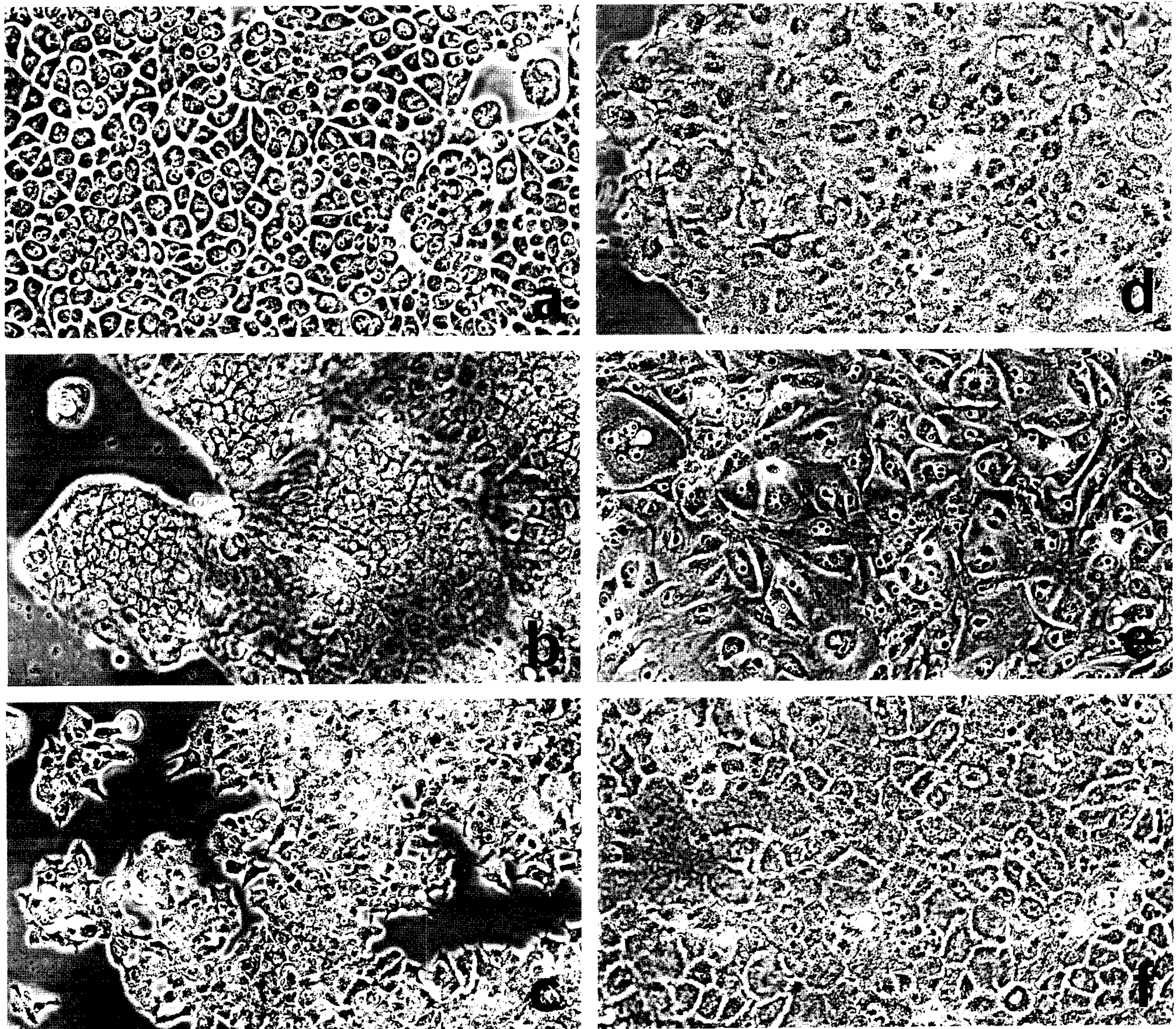

**Fig. 1** Phase contrast micrographs of six human liver tumor cell lines. **a**: JHH-1; **b**: HuH-6; **c**: HepG2; **d**: HuH-7; **e**: HLE; **f**: PLC/PRF/5. Magnification of all pictures: 280-fold.

following order: JHH-1 < HuH-6 = HepG2 < HuH-7 < PLC/PRF/5 < HLE. Small polygonal JHH-1 cells grow uniformly on the surface of culture dishes. Small cuboidal HuH-6 and HepG2 cells show an island-like growth pattern before they become confluent. HuH-7 cells exhibit a typical epithelial feature with a pavement-like cell arrangement and contain many fine granules in their cytoplasm. PLC/PRF/5 cells are similar to HuH-7 cells, but the former cells are larger and flatter than the latter cells.

We previously determined the p53 status of the six human hepatoma cell lines in this work by FASAY assay (4). To confirm those results, we employed immunohistochemistry to detect p53 protein to assess alterations in p53-gene expression. As shown in Table 1, all cell lines with the wild-type p53 showed less than 30 % positivity in p53 staining, while the cell lines with the mutant p53 showed more than 60 %. Thus, we concluded that the former three cell lines have the wild-type p53 and the latter three lines have the mutant p53. This judgment is based on the report of Mulder *et al.* (13), who classified nuclear immunoreactivity of p53 as negative if less than 30 % of nuclei stained positive and positive if more than 30 % nuclei stained positive. Although we used a p53 antibody (Ab 1801: Oncogene Science) which recognizes the p53 polypeptide from 39 to 79 and reacts with both wild-type and mutant-type p53, wild-type p53 protein is usually undetectable due to its short half life and low basal level. Thus, p53-positive nuclear staining suggests the accumulation of p53, implying alterations in p53-gene expression (13).

The p53 gene is a transcriptional regulatory factor with a cell-cycle checkpoint function (14). A gene named p21 whose induction is directly associated with wild-type p53 gene expression has been identified as an important mediator of p53-dependent tumor-growth suppression (15-17). Thus, we attempted to determine whether the wild and mutant types of p53 could function as transcriptional factors and induce p21 expression in these cell lines. As expected, Western blot analysis showed expression of p21 in all cell lines with the wild-type p53, but did not in those with the mutant-type p53 (Fig. 2). However, two other CDK inhibitors, p16 (18, 19) and p27 (20, 21),

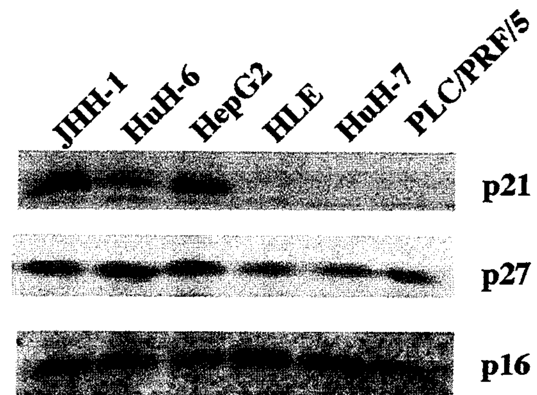


**Fig. 2** Western blotting of p21, p27 and p16 of liver tumor cell lines with the wild-type p53 (JHH-1, HuH-6 and HepG2) and those with the mutant-type p53 (HLE, HuH-7, PLC/PRF/5).

**Table 1** Comparison of cellular characteristics between human hepatoma cell lines with wild-type p53 and those with mutant p53

| | p53 stain[a] (%) | PDT (h) | Percent of control[b] | | | | | Tumorigenicity[e] |
|---|---|---|---|---|---|---|---|---|
| | | | TGF-$\beta$ (10 ng/ml) | EGF (100 ng/ml) | TNF-$\alpha$[c] (0.6 JRU) | Cholera toxin (100 ng/ml) | cAMP[d] (1 mM) | |
| JHH-1 | 24 | 52 | 50 | 75 | 100 | 111 | 37 | 0/2 |
| HuH-6* | 11 | 41 | 66 | 107 | 100 | 100 | 90 | 2/2 |
| HepG2* | 20 | 53 | 75 | 127 | 100 | 94 | 40 | 0/2 |
| HuH-7* | 84 | 34 | 29 | 80 | 43 | 125 | 72 | 1/2 |
| HLE* | 82 | 27 | 125 | 164 | 18 | 59 | 45 | 2/2 |
| PLC/PRF/5* | 60 | 33 | 75 | 110 | 100 | 113 | 86 | 2/2 |

*: The status of the p53 gene was also reported by Hsu *et al.* (Carcinogenesis 14: 987, 1993). HuH-6 and HepG2: wild, HuH-7: mutant (codon 220, 2A→G), HLE: mutant (codon 249, 3G→C), PLC/PRF/5: mutant (codon 249, 3G→T).

*a*: 500 cells were counted.

*b*: $\frac{\text{Number of cells treated with each agent}}{\text{Number of cells in control}} \times 100\ (\%)$

*c*: Conditioned medium of TNF-$\alpha$-gene transfected human fibroblasts (12)

*d*: 1mM theophylline was used with db-cAMP

*e*: $\frac{\text{Number of mice with tumors}}{\text{Number of mice injected}}$

were equally expressed in all the cell lines (Fig. 2).

Then we measured the activities of CDK2 and CDK4 by the kinase assay using an Rb fragment as a substrate. Our results showed that there was no marked difference in activities of CDK2 and CDK4 between the cell lines with the wild-type and those with the mutant-type p53 (data not shown). In other words, CDK2 and CDK4 were active in all cell lines, independent of the presence or absence of p21 CDK inhibitor. Consequently, Rb protein was phosphorylated at similar levels in all cell lines except HLE cells, which lost expression of the protein. These findings indicate that all cell lines can enter the S-phase regardless of the presence of the p53-p21 CDK inhibitor system.

All cell lines grew continuously without showing cellular aging (22). However, there was a marked difference in the growth pattern between the cell lines with the wild-type p53 and those with the mutant-type p53. The mean PDT of the former cell lines was about 49 h, while that of the latter cell lines was about 31 h (Fig. 3 and Table 1).

It has been recognized that the loss of normal p53 function ensures genomic instability (23). Thus, it was expected that the cells with the mutant p53 would show more varied chromosome distribution (ploidy change) than the cells with the wild-type p53. As shown in Table 2, the number of chromosomes was prone to increase in the cells with the mutant p53, but no significant difference in the distribution range of chromosome numbers was observed between the cell lines with the wild-type and those with the mutant-type p53.

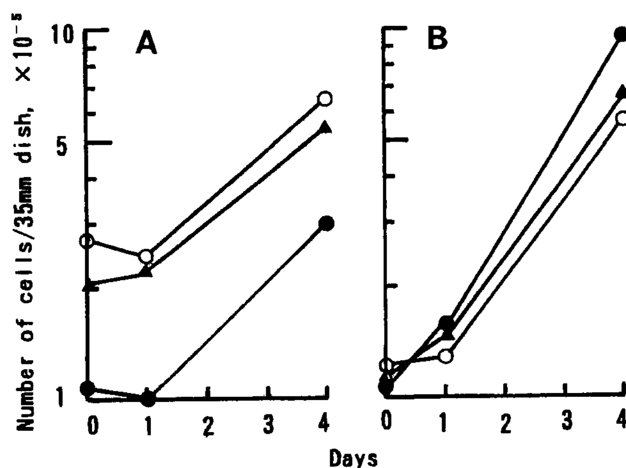


**Fig. 3** Comparison of cell growth. Between (**A**) the wild-type p53 expressing liver tumor cell lines (○: JHH-1; ●: HuH-6; ▲: HepG2) and (**B**) the mutant-type p53-expressing liver tumor cell lines (●: HLE; ▲: HuH-7; ○: PLC/PRF/5).

**Table 2** Comparison of chromosome distribution between human hepatoma cell lines with wild-type p53 and those with mutant p53

| Chromosome number | Number of metaphages counted | | | | | |
|---|---|---|---|---|---|---|
| | Cell lines | | | | | |
| | JHH-1 | HuH-6 | HepG2 | HuH-7 | HLE | PLC |
| < 40 | | | 2 | | | |
| 40-50 | | 4 | 13 | | 8 | |
| 51-60 | | 6 | 21 | 1 | 18 | |
| 61-70 | 2 | 4 | | | 10 | 3 |
| 71-80 | 32 | 18 | | | 1 | 2 |
| 81-90 | 14 | 18 | | | | 21 |
| 91-100 | 1 | | 7 | 1 | | 16 |
| 100 < | 1 | | 8 | 48 | 13 | 8 |

As shown in Table 1, HuH-7 cells were the most sensitive to the growth-inhibitory effects of TGF-$\beta$1. The other 4 cell lines, JHH-1, HuH-6, HepG2 and PLC/PRF/5, were relatively sensitive to the growth-inhibitory effects. However, no significant difference was observed in TGF-$\beta$-induced growth inhibition between the wild-type and mutant-type p53-expressing cell lines. Interestingly, growth of HLE cells was enhanced by TGF-$\beta$1. This growth promoting effect of TGF-$\beta$1 may be due to depletion of Rb protein in HLE cells. These results are supported by the findings that TGF-$\beta$-induced growth inhibition requires the Rb protein (24). These observations could help to explain why TGF-$\beta$ is often found to enhance tumorigenicity *in vivo* and why inactivation of the Rb gene leads to tumorigenesis.

No significant difference in response to EGF, cholera toxin and db-cAMP was observed between the wild-type and the mutant-type p53 cell lines, although the effects of each agent differed in each cell line. For instance, EGF stimulated the growth of HLE and HepG2 cells but inhibited that of JHH-1 and HuH-7 cells. Cholera toxin, which increases cellular db-cAMP, did not have any influence on cell growth of the hepatoma cell lines except the HLE cell line, the growth of which was inhibited by the agent. db-cAMP inhibited the growth of all cell lines, although its inhibitory effects varied among the cell lines. Among them, the JHH-1 and HLE cells were markedly growth-inhibited by db-cAMP.

Two of the three mutant-type p53 cell lines were very sensitive to the cytotoxic effects of TNF-$\alpha$. On the other hand, no cell lines with the wild-type p53 showed growth

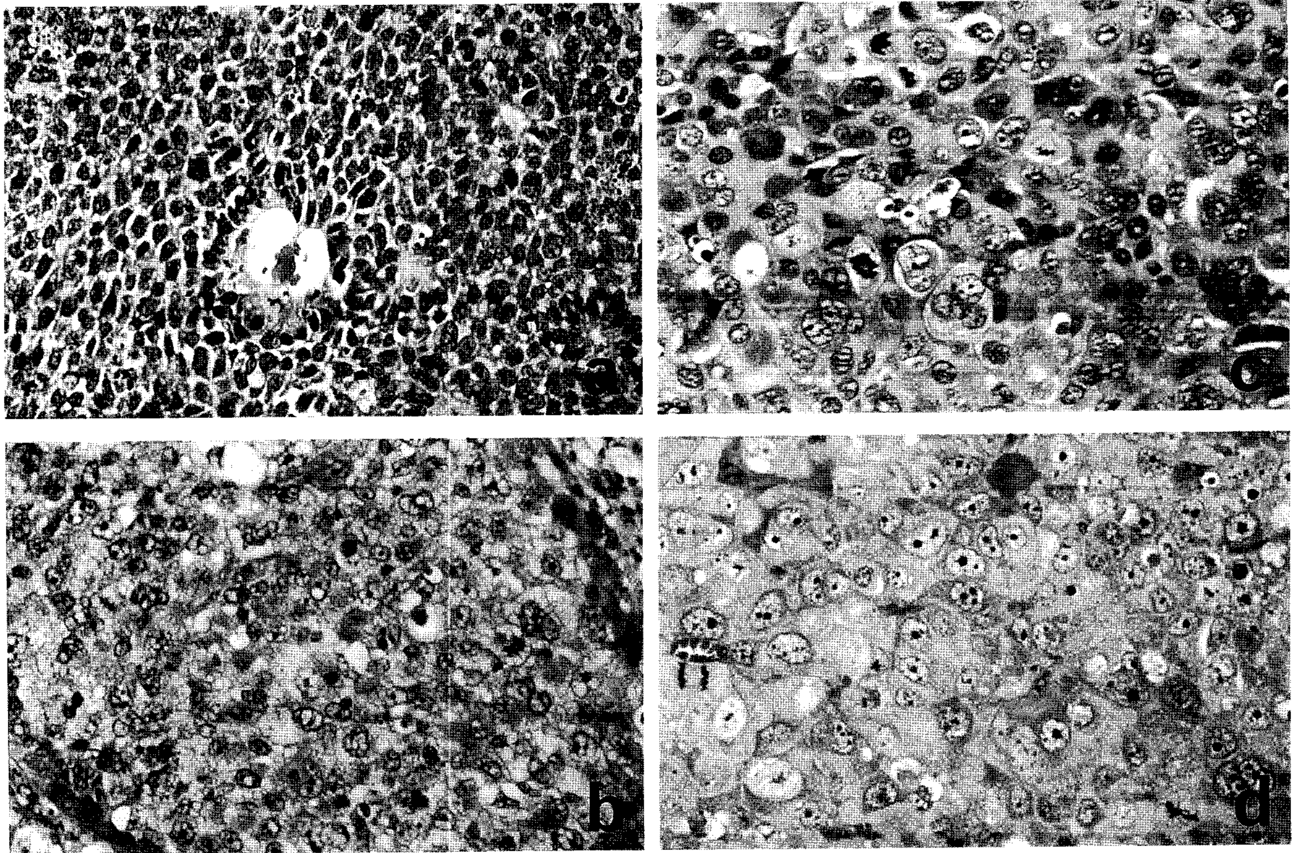

**Fig. 4** Histology of tumors produced in nude mice. **a**: HuH-6; **b**: HLE; **c**: HuH-7; **d**: PLC/PRF/5. HuH-6 cells were derived from hepatoblastoma, while HLE, HuH-7 and PLC/PRF/5 were derived from hepatocellular carcinoma. Magnification of all pictures: 560-fold.

inhibition by treatment with TNF-$\alpha$. It has been reported that the cytotoxic effects of TNF-$\alpha$ are partly due to reactive oxygen intermediates (ROIs) such as superoxide and hydroxy radicals produced by TNF-$\alpha$ (25). Several studies have indicated that the wild-type p53 induces G1 arrest after DNA damage to allow cells to repair damaged DNA (26, 27). In contrast, the mutant p53 abrogates the induction of G1 arrest and the cell cycle proceeds without optimal repair of the damaged DNA. Thus, proliferation of the cells harboring the mutant p53 appeared to be more impaired than that of normal cells after two to three cell divisions (28). We did not study the G1 check point of the cells treated with TNF-$\alpha$ in the present experiment.

Table 1 shows the tumorigenicity of each cell line. Two of the three cell lines with the wild-type p53 did not produce tumors in nude mice, while all the cell lines with the mutant-type p53 produced tumors. These findings indicate that mutations of the p53 genes cannot always be linked to hepatocarcinogenesis, but rather to transplantability of the cells into nude mice. Histology of the tumors did not correlate to the p53 status. Fig. 4 shows that the mutant-type p53 expressing-cell lines, HuH-7, HLE, and PLC/PRF/5, produced poorly, moderately, and poorly differentiated hepatomas, respectively.

The hepatitis B virus-encoded X antigen (HBxAg) binds to and inactivates the wild-type p53. If this is the case, p53 mutation is not necessary for hepatocarcinogenesis. In fact, Greenblatt *et al.* (29) described a low frequency of p53 mutations in HBxAg-positive HCC. They found the wild-type p53 sequence in 13 of 16 HBxAg-positive cases. In contrast, in the present experiment, no cell line with the wild-type p53 gene had HBxAG. Only PLC/PRF/5 cells that had a mutation of p53 harbored HBxAg. Although their test samples are different from ours, the relationship between p53 status and HB infection remains to be determined.

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