Suppression of the neoplastic phenotype of aflatoxin B₁-induced rat hepatoma cells by the retinoblastoma tumor suppressor gene

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大坪浩一郎^{*2}, 田代 文夫^{*1}: レチノブラストーマ癌抑制遺伝子による
アフラトキシン B₁ 誘発ラット肝癌細胞の癌形質の抑制

Summary

The retinoblastoma tumor suppressor gene product, RB protein, is a negative regulator of cell growth and antagonizes c-Myc oncoprotein. We have previously reported that the c-myc gene expression is aberrantly elevated in rat hepatocellular carcinomas induced by aflatoxin B₁ (AFB₁). In this paper, to elucidate the significance of the overexpression of c-myc gene in AFB₁ hepatocarcinogenesis, we analyzed the effects of a human RB cDNA expression vector, pSV2•RB, on the growth and tumorigenicity of Kagura-2 (K2) cells, which have been established from AFB₁-induced rat hepatoma and overexpressing c-myc gene just as an original tumor. Even though any effects of exogenous expression of RB gene on the transcriptional levels of c-myc and glutathione-S-transferase P (GST-P) genes were not detected, pSV2•RB-transfected K2 cells significantly reduced the ability to form colonies in semi-solid medium, and their growth rate was markedly decreased in a lower concentration of serum. Moreover, tumors that formed by the transfectants in nude mice were much smaller, but were histologically indistinguishable from those of parental K2 cells. These results indicate that the enforced expression of RB gene suppresses the growth of K2 cells *in vitro* and *in vivo* without any effects on the expression level of c-myc mRNA.

Key words: aflatoxin B₁; アフラトキシン B₁, c-myc gene; c-myc 遺伝子, hepatocarcinogenesis; 肝発癌. RB gene; RB 遺伝子, tumorigenicity; 腫瘍形成能

Introduction

It is widely known that aflatoxin B₁ (AFB₁) is one of the environmental risk factors for human liver cancer especially in South-east Asia, China and sub-Saharan Africa¹⁾. Molecular epidemiological approaches show the high frequency of mutation in codon 249 of the tumor suppressor p53 gene in these endemic areas^{2,3)}. In rat hepatocellular carcinomas induced by AFB₁, overexpression of the c-myc gene⁴⁾ and point mutation of *ras* family genes^{5,6)} have been reported. However, detailed mechanism of AFB₁ hepatocarcinogenesis is still obscure.

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In this situation, we have established the hepatoma cell lines designated as Kagura-1 (K1) and Kagura-2 (K2) cells from AFB₁-induced rat hepatocellular carcinomas to clarify the detailed mechanism of AFB₁ hepatocarcinogenesis⁷⁾. We have demonstrated so far that K1 and K2 cells overexpress *c-myc* and 14-3-3 β genes⁸⁾ and hepatoma-specific tumor markers such as glutathione-S-transferase (GST-P) and γ -glutamyltranspeptidase⁷⁾, although mutations of suppressive oncogenes including *RB* and p53⁹⁾ and oncogenes such as *c-Ha-ras*, *c-Ki-ras* and *N-ras* (Habano *et al.*, unpublished observation) were not detected. On the other hand, these cells do not maintain liver-specific functions such as albumin and tyrosine aminotransferase syntheses⁷⁾. In addition, recently we have reported the possibility that glucocorticoid hormones promote AFB₁ hepatocarcinogenesis through the prevention of apoptosis induced by various cellular defence systems, including TGF- β 1 and perhaps TNF, whose actions are mediated at least in part by arachidonic acid metabolites¹⁰⁾.

Inactivating mutations of *RB* gene are found in a wide variety of tumor cells. Replacement of wild-type *RB* can suppress the tumorigenicity of some of these cells^{11,12)}, showing that RB protein negatively regulates cell growth. In contrast, as activation of *c-myc* gene expression promotes cell proliferation and blocks differentiation¹³⁾, c-Myc protein is a positive regulator of cell growth in the presence of growth factors. c-Myc and RB proteins localize in the nucleus as a transcription factor and a regulator of transcription factor, respectively¹⁴⁾. The unphosphorylated form of RB protein suppresses *c-myc* gene expression through an inactivation of transcription factor, E2F^{15,16)}. In addition, c-Myc protein directly binds to RB protein through its N-terminal region¹⁷⁾ and inhibits the ability of RB protein to arrest cell cycle at G1 phase¹⁴⁾. However, c-Myc protein does not inhibit the activity of another tumor suppressor, p53 protein¹⁴⁾. Thus, c-Myc and RB proteins specifically antagonize one another in the cell.

In this paper, to elucidate the significance of the overexpression of *c-myc* gene in AFB₁ hepatocarcinogenesis, we analyzed the effect of exogeneously enforced expression of human *RB* cDNA on the neoplastic phenotype of K2 cells.

Materials and Methods

Cell culture K2 cells were cultivated in collagen-coated dishes with Dulbecco's modification of Eagle's medium (DMEM) supplemented 10% fetal calf serum (FCS). The cells were maintained at 37°C in a humidified atmosphere of 5% CO₂ in air⁷⁾.

Detection of *RB* and *p53* mRNAs by RT-PCR Total RNAs were extracted by a LiCl-urea method as described previously⁴⁾ and poly (A)⁺ mRNAs were prepared by the use of oligo(dT)-cellulose column¹⁸⁾. The first-stranded cDNA synthesis was carried out in a reaction volume of 40 μ l containing 1 μ g of poly (A)⁺ mRNA, 1 μ g of random hexamer primer and 8 μ l of solution comprising 250 mM Tris-HCl, pH 8.3, 15 mM MgCl₂, 375 mM KCl, and 50 mM dithiothreitol. After heating for 1 min at 95°C, the reaction was initiated by the addition of 20 μ l of dNTP mix (1 mM each of dATP, dCTP, dGTP and dTTP) and 300 U of MMLV reverse transcriptase. The reaction was allowed to proceed at 37°C for 40 min and finally terminated by boiling for 5 min. The synthesized cDNA was stored at -20°C until use.

PCR on the cDNA was carried out according to Brenner *et al*¹⁹⁾. The first-stranded cDNA

(4 μ l) was added to PCR mixture in a total volume of 100 μ l containing the following components: 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 3 mM MgCl₂, 200 μ M each dNTP, 0.5 μ M each of 5' and 3' primers as indicated below and 2.5 U of Vent DNA polymerase.

Rat *RB* gene (pocket domain B)²⁰⁾

5' primer: 5' -CAAGCAGCCTCAGCCTTCCA-3'

3' primer: 5' -AGGTATTTGGTGACAAGGTAG-3'

Rat p53 gene (exon III and IV)²¹⁾

5' primer: 5' -CCTCCACCTGGTACCGCT-3'

3' primer: 5' -CAATGCTCTTCTTTTGTGCG-3'

PCR was carried out using Astec thermal cycler programmed for 35 cycles. The initial template denaturation was conducted at 94°C for 5 min and was followed by the following: primer annealing at 54°C for 2 min, primer extension at 72°C for 3 min, and denaturation at 94°C for 1 min. At the end of the cycle, the reaction mixture was kept at 75°C for 5 min. The PCR product was extracted with chloroform and electrophoretically analyzed on a 3.5% polyacrylamide gel containing ethidium bromide.

Transfection of human RB cDNA expression vector Human *RB* cDNA expression vector, pSV2•RB, was kindly supplied by N. Tsuchida of Tokyo Medical and Dental University. pSV2•RB was constructed by ligating the full length *RB* cDNA prepared from p4.95BT clone²²⁾ into a mammalian expression vector, pSV2²³⁾, which contained the SV40 early promoter and splicing/poly (A) additional signals. Sparse culture of K2 cells in 60-mm culture dish were transfected with 9 μ g of pSV2•RB and 1 μ g of pSV2neo which contains a selection marker²⁴⁾ by the CaPO₄ method²⁵⁾. After 24 hr of the transfection, the cells were replated and cultivated in the presence of 1 mg/ml G418 (Wako). G418-resistant *neo*⁺ colonies developed were isolated. The *neo*⁺ cell lines that express the human *RB* cDNA were selected by Northern blot using ³²P-labeled 1.8-kbp *Bgl* II fragment of p4.95BT as a probe. Finally we obtained two *neo*⁺/human *RB*⁺ cell lines and designated as K2SR5 and K2SR8 cells. A *neo*⁺/*RB*⁻ cell line was also isolated as a control transfectant and designated as K2neo cells.

Immunohistochemistry Cells grown on collagen-coated coverslips were washed two times with Ca²⁺, Mg²⁺-free phosphate-buffered saline [PBS(-)], fixed with methanol (6 min at -20°C), rinsed three times with 0.1% Tween 20/PBS(-) and preincubated in 5% bovine serum albumin (BSA) 4% normal rabbit serum/0.1% Tween 20/PBS(-) for 2 hr at room temperature. The G3-349 anti-human RB monoclonal antibody (PharMingen) was diluted 1:50 in 3% BSA/1.5% normal rabbit serum/0.1% Tween 20/PBS(-) and was incubated with the cells overnight. The cells were then washed three times with 0.1% BSA/0.1% Tween 20/PBS(-) and incubated with a 1:1000 dilution of fluorescein isothiocyanate (FITC)-conjugated rabbit anti-mouse IgG antibody (Sigma). Finally, the cells were rinsed three times with 0.1% BSA/0.1% Tween 20/PBS(-) and mounted in 80% glycerol/PBS(-). The stained cells were examined under BH-2 fluorescent microscope (Olympus).

Tumorigenicity in nude mice Cells (5×10⁶) were suspended in 200 μ l of PBS(-) and implanted subcutaneously into the right and left flanks of 5-week-old athymic nude mice (JCLA *nu/nu*, Nihon Clea). After 70 days, cubic volumes (cm³) were estimated based on a three-

dimensional measurement of tumors formed in the both flanks. When necessary, the animals were killed and dissected. The tumor tissues were fixed in formaldehyde and stained with hematoxylin and eosin for histological examination.

Results

Expression levels of RB and p53 mRNAs in K2 cells Previously we have reported that inactivating mutations of tumor suppressor genes, *RB* and p53, were not detected in K1 and K2 cells⁹. However, the examination of expression levels of these genes are also important for the elucidation of tumorigenicity of these cells. Thus, we investigated the *RB* and p53 gene expression levels by RT-PCR. As shown in Fig. 1, the expression levels of these genes were similar to those of the normal rat liver, implying that the mutation and/or deregulation of *RB* and p53 genes are not largely involved in AFB₁ hepatocarcinogenesis in the rat.

Establishment of pSV2•RB-transfected K2 cells The function of *c-myc* gene, which is a positive regulator of cell growth, is suppressed by the product of *RB* gene through the direct binding to c-Myc protein^{14,17}. The transcription of *c-myc* gene is also suppressed by RB protein through the inactivation of E2F transcription factor^{15,16}. To elucidate the significance of the deregulated *c-myc* gene expression in AFB₁ hepatocarcinogenesis, human *RB* cDNA expression vector, pSV2•RB, was cotransfected with pSV2neo into K2 cells by the CaPO₄ method²⁵ and stable neo⁺/RB⁺ transfectants, K2SR5 and K2SR8, and a neo⁺/RB⁻ transfectant, K2neo, were obtained. As shown in Fig. 2, K2SR5 and K2SR8 cells, but not K2 and K2neo cells, expressed a 4.7-kb human *RB* mRNA. The expression level of exogenous *RB* mRNA in K2SR8 cells is 1.5-fold higher than that in K2SR5 cells.

These transfectants were also immunohistochemically stained by anti-human RB monoclonal antibody to determine the subcellular localization of RB protein. K2SR5 and K2SR8 cells showed positive nuclear RB protein staining, although cytoplasm was also stained in some cells (Fig. 3F and 3H). In contrast, the parental K2 and K2neo cells exhibited no nuclear staining under identical conditions (Fig. 3B and 3D). The results were compatible with other reports^{12,14}. Therefore the functional human RB protein might be synthesized in K2SR5 and K2SR8 cells.

Effect of pSV2•RB on the expression of c-myc and GST-P genes By the use of pSV2•RB transfectants, at first we analyzed the effect of *RB* gene on the transcription of the *c-myc* and tumor marker GST-P genes, because these genes are aberrantly expressed in K2 cells⁷. As shown in Fig. 4, any changes of these gene expressions were hardly detected, showing that RB protein could not affect on the transcriptional step of *c-myc* and GST-P genes. However, we did not rule out the possibility that RB protein might inhibit the function of c-Myc protein through the direct binding to c-Myc protein in the transfectants.

Phenotypic properties of pSV2•RB transfectants in culture *In vitro* properties including an anchorage-independent growth and continued proliferation despite growth-limiting serum concentration, have been correlated with the malignant phenotype *in vivo*. As shown in Table 1, the abilities of K2SR5 and K2SR8 cells to form colonies in soft agar were reduced to 68% and 61%, respectively, when compared with the parental K2 cells. On the other hand, the colony-forming ability of the plasmid-control K2neo cells was similar to that of K2 cells.

Fig. 1 Expression of tumor suppressor *RB* and *p53* genes in K2 cells. A) Detection of *RB* mRNA by RT-PCR. Pocket domain B region of *RB* gene was amplified for 35 cycles by RT-PCR. B) Detection of *p53* mRNA by RT-PCR. Exon III and IV regions of *p53* gene were amplified for 35 cycles by RT-PCR.

Fig. 2 Northern blot analysis of human *RB* cDNA expression in pSV2•RB-introduced K2 cells. A) Aliquots of 20 μ g total RNA extracted from the cells indicated were electrophoresed on 1.2% agarose-6% formaldehyde, transferred to nitrocellulose filter, and hybridized with the 32 P-labeled 1.8-kbp *Bgl* II fragment of human *RB* cDNA clone, p4.95BT, as a probe in the conditions described previously⁸⁾. B) Picture of ethidium bromide stained gel is indicated to allow comparison of total amount of RNA employed.

The effect of pSV2•RB on cell proliferation in monolayer culture was also examined at serum concentrations of 10% and 3%. In the presence of 10% FCS the growth abilities of K2SR5 and K2SR8 cells were reduced to 65% and 75% of the control K2 cells, respectively (Fig. 5A). Furthermore, the growth inhibition of K2SR5 and K2SR8 cells were more evident in 3% FCS and estimated to be 31% and 49% of the control, respectively (Fig. 5B). In contrast, the growth inability level of K2neo cells was very little in comparison with those of pSV2•RB transfectants. The results obtained from *in vitro* cell culture indicate that *RB* gene could suppress the anchorage-independent and -dependent growth of K2 cells.

Tumorigenicity in nude mice We next investigated the effect of pSV2•RB on the tumorigenicity of K2 cells. K2 and K2neo cells formed large, progressively growing solid tumors in all mice, when 5×10^6 cells were subcutaneously inoculated into the flanks of nude mice (Fig. 6A). At 70 days, the average volumes of tumors developed by K2 and K2neo cells were 10.33 cm³ and

Fig. 3 Immunohistochemical detection of nuclear human RB protein expressed in pSV2•RB transfectants. The cells were fixed and incubated with a monoclonal anti-human RB antibody (1 : 50, PharMingen). After washing, the cells were incubated with a FITC-conjugated rabbit anti-mouse IgG antibody (1 : 1000, Sigma). The photographs were taken under phase-contrast optics (A, C, E and G) and under fluorescent optics (B, D, F and H). (A and B) K2, (C and D) K2neo, (E and F) K2SR5, (G and H) K2SR8 cells.

Fig. 4 Effect of pSV2•RB on the expression of *c-myc* and GST-P genes. Aliquots of 20 μ g total RNA extracted from the cells indicated were electrophoresed and transferred to nitrocellulose filters. The filters were hybridized with the 32 P-labeled 0.8-kbp *Eco* RI-*Hind* III fragment of rat *c-myc* gene clone, pSmBH²⁶) (A) and 0.7-kbp *Eco* RI-*Sal* I fragment of rat GST-P cDNA clone, pGP5²⁷) (B). Picture of ethidium bromide stained gel is indicated to allow comparison of total amount of RNA employed (C).

11.68 cm³, respectively (Fig. 6B). Even though solid tumors were developed in all mice, K2SR5 and K2SR8 cells produced much smaller tumors in comparison with those of K2 and K2neo cells (Fig. 6A). The average volumes of tumors developed K2SR5 and K2SR8 cells were 5.07 cm³ and 4.09 cm³, respectively (Fig. 6B).

As shown in Fig. 7, histological examination of tumors indicated that all tumors possessed more malignant features, such as mitoses and nuclear atypia, than the primary tumor, which was well-differentiated hepatocellular carcinoma⁷). There was no histological difference among tumors developed by K2, K2neo, K2SR5 and K2SR8 cells.

Discussion

In this study, to gain an insight into the significance of overexpression of *c-myc* gene in AFB₁

Fig. 5 Effect of pSV2•RB on the growth of K2 cells. A) Cells were seeded at 2×10^4 cells per 35-mm dish (day 0) and cultivated in DMEM containing 10% FCS for various times. B) Cells were seeded at 5×10^4 cells per 35-mm dish and cultivated in DMEM containing 3% FCS. Each value is the average of duplicate of culture dishes. ○, K2; ●, K2neo; △, K2SR5; ▲, K2SR8 cells.

hepatocarcinogenesis, we investigated the functional effects of a human *RB* cDNA expression construct, pSV2•RB, transfected into AFB₁-induced rat hepatoma K2 cells, whose *c-myc* expression is deregulated. In pSV2•RB transfectants, human *RB* cDNA was transcribed as a 4.7-kb mRNA and its translated product, RB protein, was mainly localized in the nucleus. Therefore, the observed phenotypic changes in the transfectants might be due to the result of exogenous expression of *RB* cDNA.

Several important effects of *RB* expression on K2 growth properties were observed. pSV2•RB transfectants reduced to form colonies in semi-solid medium and showed a strong inhibition of their growth, when cultivated at a low serum concentration. We also found that the exogenous expression of *RB* gene caused the cells to form substantially smaller tumors. However, pSV2•RB transfectants still expressed the *c-myc* and GST-P genes and possessed a tumorigenicity. In addition, histologically there was no difference among tumors induced by K2,

Fig. 6 Effect of pSV2•RB on the tumorigenicity of K2 cells. Cells were injected into the right and left flanks of nude mice at the concentration of 5×10^6 cells per flank. After 70 days, photographs of tumors formed were taken (A), and the average of total cubic volume of tumors formed in the right and left flanks of 2 or 3 mice was estimated (B). The values indicated in the figure present the mice with tumor per mice injected. 1) K2, 2) K2neo, 3) K2SR5, 4) K2SR8 cells.

K2neo, and pSV2•RB transfectants. These findings are more consistent with a model that by RB protein acts as a growth suppressor rather than by induction of terminal differentiation¹². Our results also indicate that the enforced expression level of *RB* gene might not be sufficient to

Fig. 7 Histological observation of tumors formed by pSV2-RB-introduced K2 cells in nude mice. After 70 days of the injections of K2 (A), K2neo (B), K2SR5 (C) and K2SR8 cells (D), the tumors formed were fixed in formaldehyde, and stained hematoxylin and eosin for histological examination. Magnification, $\times 100$.

Table 1 Effect of pSV2•RB on the colony formation of K2 cells in semi-solid medium. Cells were seeded in 0.3% agar at 4×10^3 cells per 35-mm dish and cultivated for 3 weeks as described previously²⁴. Then colonies formed were stained with 0.25% 1-*p*-iodophenyl-*p*-nitrophenyl-5-phenyl tetrazolium chloride for 24 hr and counted. Each value is the average $\pm$ SE of triplicate culture dishes.

Cell line	Colonies/dish
K 2	49.3 $\pm$ 0.9
K 2 neo	43.3 $\pm$ 3.1
K 2 SR 5	33.7 $\pm$ 2.2
K 2 SR 8	30.0 $\pm$ 2.7

suppress the tumorigenicity completely and the histological malignant phenotype of K2 cells, because the introduction of wild type *RB* gene into the *RB* gene deficient various human tumor cells markedly suppressed the tumorigenicity of these cells^{11,12}.

RB protein is a substrate of the cdc2 kinase and phosphorylation of RB oscillates during the cell cycle; Unphosphorylated forms appear as cells enter S phase and persist during the G2 and M phases. The injection of excess unphosphorylated RB into cells arrests G1 phase and blocks their entrance into S phase. G1 arrest by RB protein can be abrogated by c-Myc protein, but not EJ-Ras, c-Fos, or c-Jun protein¹⁴. Whereas c-Myc protein can not inhibit the arrest of cell cycle by p53¹⁴, showing that c-Myc and RB proteins specifically antagonize one another in the cell.

Taking into these facts and our present data, it seems likely that the exogeneous expression of *RB* gene could prevent AFB₁ hepatocarcinogenesis.

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