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Short Communication

Combination of acyclic retinoid with branched-chain amino acids inhibits xenograft growth of human hepatoma cells in nude mice

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Aim: Combination chemoprevention is a promising strategy to improve the prognosis of hepatocellular carcinoma (HCC). A malfunction of retinoid X receptor-α (RXR-α) due to phosphorylation by Ras/mitogen-activated protein kinase is closely associated with liver carcinogenesis and acyclic retinoid (ACR) can prevent HCC development by inhibiting RXR-α phosphorylation. The present study examined the possible combined effects of ACR plus branched-chain amino acids (BCAA), which can also prevent the development of HCC in obese patients with liver cirrhosis, in human HCC xenografts in nude mice.

Methods: This study examined the effects of the combination of ACR plus BCAA on the growth of Huh7 human HCC xenografts in nude mice. The effects of the combination on the phosphorylation of RXR-α, extracellular signal-regulated kinase (ERK), Akt and insulin-like growth factor-1 receptor (IGF-1R) proteins, and on the expression levels of retinoic acid receptor-β (RAR-β) and $p21^{CIP1}$ mRNA, were also examined by western blot and real-time reverse transcription polymerase chain reaction analyses, respectively.

Results: The combined treatment with ACR plus BCAA significantly inhibited the growth of Huh7 xenografts. The combination of these agents caused a marked inhibition of the phosphorylation of RXR-α, ERK, Akt and IGF-1R proteins in the xenografts. In addition, the expression levels of RAR-β and $p21^{CIP1}$ mRNA significantly increased by these agents.

Conclusion: The combination of ACR and BCAA restores the function of RXR-α by inhibiting its phosphorylation and increasing the level of RAR-β, a heterodimeric partner for RXR-α, and thus suppresses the growth of HCC xenografts. Therefore, this combination might be an effective regimen for the treatment and, probably, chemoprevention of HCC.

Key words: acyclic retinoid, branched-chain amino acids, hepatocellular carcinoma, phosphorylated retinoid X receptor-α, retinoic acid receptor-β

INTRODUCTION

THE POOR PROGNOSIS for patients with hepatocellular carcinoma (HCC) has created an urgent need to develop more effective strategies for prevention of this malignancy. Retinoids, which have tumor-suppressive and chemopreventive properties in various organs, are considered to be promising agents for improving outcomes in individuals with HCC.[1,2] A clinical trial demonstrated that the administration of acyclic retinoid (ACR), a synthetic retinoid that targets retinoid X receptor-α (RXR-α), significantly reduced the incidence of post-therapeutic recurrence of HCC.[3] ACR inhibits growth in human HCC cells by inducing apoptosis and arrest of the cell cycle in G_0–G_1.[4,5] The inhibition of growth in cancer cells by ACR is also associated with induction of cellular levels of retinoic acid receptor-β (RAR-β), an important retinoid receptor for regulation of apoptosis, and the inhibition of RXR-α phosphorylation.[5–8] The latter effect is more significant because the accumulation of phosphorylated (i.e. inactivated) RXR-α (p-RXR-α) interferes with the function of normal RXR-α in a dominant-negative manner, and therefore plays a critical role in the development of HCC.[2,9,10]

In addition, ACR acts synergistically with various agents (e.g. β-interferon, OSI-461, trastuzumab, valproic

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acid and vitamin K_2) that target other signaling pathways in suppressing growth and inducing apoptosis in human HCC cells.[5,11–14] These findings have clinical significance for the treatment and chemoprevention of HCC because the combined use of two or more agents can diminish drug toxicity while exerting synergistic effects. Branched-chain amino acids (BCAA; leucine, isoleucine and valine), which improve protein malnutrition in patients with liver cirrhosis, are candidate partners in ACR-based combination chemoprevention because a recent clinical trial showed that oral supplementation with these agents reduced the risk of HCC in obese patients with chronic viral liver disease.[15] Treatment with BCAA also prevents the development of liver tumorigenesis in a rodent model, while also inhibiting the growth of HCC cells.[16–18] The purpose of this study is to investigate whether the combination of ACR plus BCAA significantly inhibits the growth of human HCC xenografts and to examine the possible mechanisms of this action.

METHODS

Materials

AN ACYCLIC RETINOID (peretinoin) was supplied by Kowa Pharmaceutical (Tokyo, Japan). BCAA was obtained from Ajinomoto (Tokyo, Japan). The BCAA composition (2:1:1.2 = leucine : isoleucine : valine) was set at the clinical dose used for the treatment of decompensated liver cirrhosis in Japan.[15]

Experimental procedure

Thirty-two male BALB/c nude mice (5 weeks of age) were obtained from Charles River Japan (Tokyo, Japan). Xenograft tumors were made by the s.c. injection of Huh7 human HCC cells (Japanese Cancer Research Resources Bank, Tokyo, Japan) into the flanks of the mice at a concentration of 5×10^6 cells per 200 μL.[19] The mice were randomly divided into four groups (eight mice per group) 1 week after tumor cell injection. The mice in group 2 (ACR alone) were given the basal diet, CRF-1 (Oriental Yeast, Tokyo, Japan), containing 0.03% ACR with free access to feeding for 5 weeks. Group 3 (BCAA alone) was given the basal diet supplemented with 3.0% BCAA (w/w). The mice in group 4 (combination group) received a diet containing 0.03% ACR and 3.0% BCAA. Group 1 was given the basal diet and served as an untreated control. The tumor volume was calculated at the termination of the experiment using the formula: largest diameter $\times$ (smaller diameter)$^2 \times 0.5$.

Protein extraction and western blot analysis

Total protein was extracted from the xenografts of Huh7 cells and equivalent amounts of protein (20 mg/lane) were examined by a western blot analysis.[19] The primary antibodies for RXR-α (ΔN-197 and D-20), extracellular signal-regulated kinase (ERK), phosphorylated ERK (p-ERK), Akt, phosphorylated Akt (p-Akt), insulin-like growth factor-1 receptor (IGF-1R), phosphorylated IGF-1R (p-IGF-1R) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) have been previously described.[5,10,19,20] The ΔN-197 antibody is regarded as a specific antibody for the phosphorylated form of RXR-α protein.[8,10] The intensities of the blots were quantified with NIH Image software ver. 1.62.

RNA extraction and quantitative real-time reverse transcription polymerase chain reaction analysis

Total RNA was isolated from the xenografts of Huh7 cells using the RNAqueous-4PCR kit (Ambion Applied Biosystems, Austin, TX, USA). The cDNA was amplified from 0.2 μg of total RNA using SuperScript III First-Strand Synthesis System (Invitrogen, Carlsbad, CA, USA). The primers used for the amplification of RAR-β, $p21^{CIP1}$ and GAPDH-specific genes have been previously described.[6,19] A quantitative real-time RT–PCR analysis was performed in a LightCycler (Roche Diagnostics, Mannheim, Germany) with SYBR Premix Ex Taq (TaKaRa Bio, Shiga, Japan).[16] The gene expression levels were normalized to the GAPDH expression levels using a standard curve.

Statistical analysis

The data are expressed as the mean ± standard deviation. Statistical significance of the difference in mean values was assessed by one-way ANOVA, followed by Scheffé's *t*-test.

RESULTS

Combined treatment with ACR plus BCAA significantly inhibits growth of HCC xenografts

AS SHOWN IN Figure 1, neither treatment with 0.03% ACR alone nor 3.0% BCAA alone inhibited the growth of Huh7 xenografts. These findings suggest that such doses of ACR and BCAA are insufficient to suppress the tumor growth of HCC in the present study, although similar concentrations of these agents have had a significant effect on preventing the development

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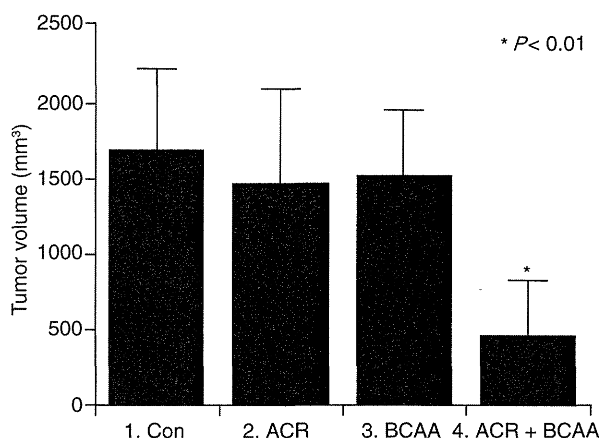

Figure 1 Effects of the combination of acyclic retinoid (ACR) plus branched-chain amino acid (BCAA) on the growth of Huh7 xenografts. BALB/c nude mice were injected s.c. with 5×10^6 Huh7 cells. One week after the injection, the mice were divided into four groups and treated as follows for 5 weeks: group 1, untreated control group (Con); group 2, 0.03% ACR-treated group; group 3, 3.0% BCAA-treated group; group 4, 0.03% ACR and 3.0% BCAA-treated group. The tumor volumes in each group at the termination of experiment are represented. Bars, standard deviation. $*P < 0.01$, significant differences obtained by comparisons to groups 1, 2, and 3.

of HCC in clinical trials.[3,15] On the other hand, the simultaneous treatment of the mice with these concentrations of ACR plus BCAA produced a significant decrease in the growth of HCC xenografts; the tumor volume was inhibited by 73% in the combination treatment group in comparison to the control group ($P < 0.01$).

BCAA inhibits the phosphorylation of Akt and IGF-1R, and enhances the suppression of the RXR-α and ERK phosphorylation by ACR in HCC xenografts

Retinoid X receptor-α phosphorylation by Ras/mitogen-activated protein kinase is closely associated with the development of HCC, and thus might be a critical target for chemoprevention of HCC.[2,9] BCAA inhibits the activation of IGF-1R and Akt and this is associated with the cancer chemopreventive effects of this agent.[16,21] Therefore, the combined effects of ACR plus BCAA on the phosphorylation of RXR-α, ERK, Akt and IGF-1R proteins were investigated in Huh7 xenografts. The expression levels of p-RXR-α and p-ERK proteins, which decreased in the ACR alone-treated group in comparison to the control group (Fig. 2a, column 2), decreased to a greater extent when the mice were treated with the combination of ACR plus BCAA (Fig. 2a, column 4). The expression levels of p-Akt and p-IGF-1R proteins, which were decreased in the BCAA alone-treated group (Fig. 2a, column 3), were also further reduced by combined treatment with ACR plus BCAA (Fig. 2a, column 4).

Combined treatment of ACR plus BCAA induces the RAR-β and p21^{CIP1} mRNA in HCC xenografts

The combined effect of ACR plus BCAA on the induction of the RAR-β and p21^{CIP1} mRNA was next examined because, in addition to the inhibition of RXR-α phosphorylation, ACR is known to inhibit the growth of HCC cells by enhancing the expression of these molecules.[4,5,7] Semiquantitative RT–PCR analyses showed that treatment with both ACR alone and BCAA alone tended to increase the levels of RAR-β mRNA, but the differences were not significant (Fig. 2b, columns 2 and 3). On the other hand, when ACR was combined with BCAA, the expression levels of this mRNA were significantly enhanced in comparison to the control group (Fig. 2b, column 4). In addition, treatment with ACR alone and the combination of ACR plus BCAA significantly increased the expression of p21^{CIP1} mRNA (Fig. 2c, columns 2 and 4), a negative modulator of cell cycle progression,[22] in comparison to the control group.

DISCUSSION

COMBINATION CHEMOPREVENTION IS often advantageous because it provides the potential for additive or, in some instances, synergistic effects between specific agents. The present study clearly indicated that the combination of ACR plus BCAA, both of which exert chemopreventive properties on HCC development,[3,15] causes potent inhibition of growth in human HCC xenografts. The hypotheses that explain this beneficial effect are summarized in Figure 3.

Initially, it should be emphasized that the phosphorylation of RXR-α and ERK proteins was strongly inhibited by the combination of ACR plus BCAA. This study and prior ones[5,8,14,23] show that ACR alone inhibits the phosphorylation of these proteins, thus indicating that BCAA could enhance the effect of ACR in HCC xenografts. These findings seem to be significant because restoration of the function of RXR-α as a master regulator of nuclear receptors by targeting its phosphorylation might be an effective strategy for the prevention and treatment of HCC.[2] BCAA may support the effect of

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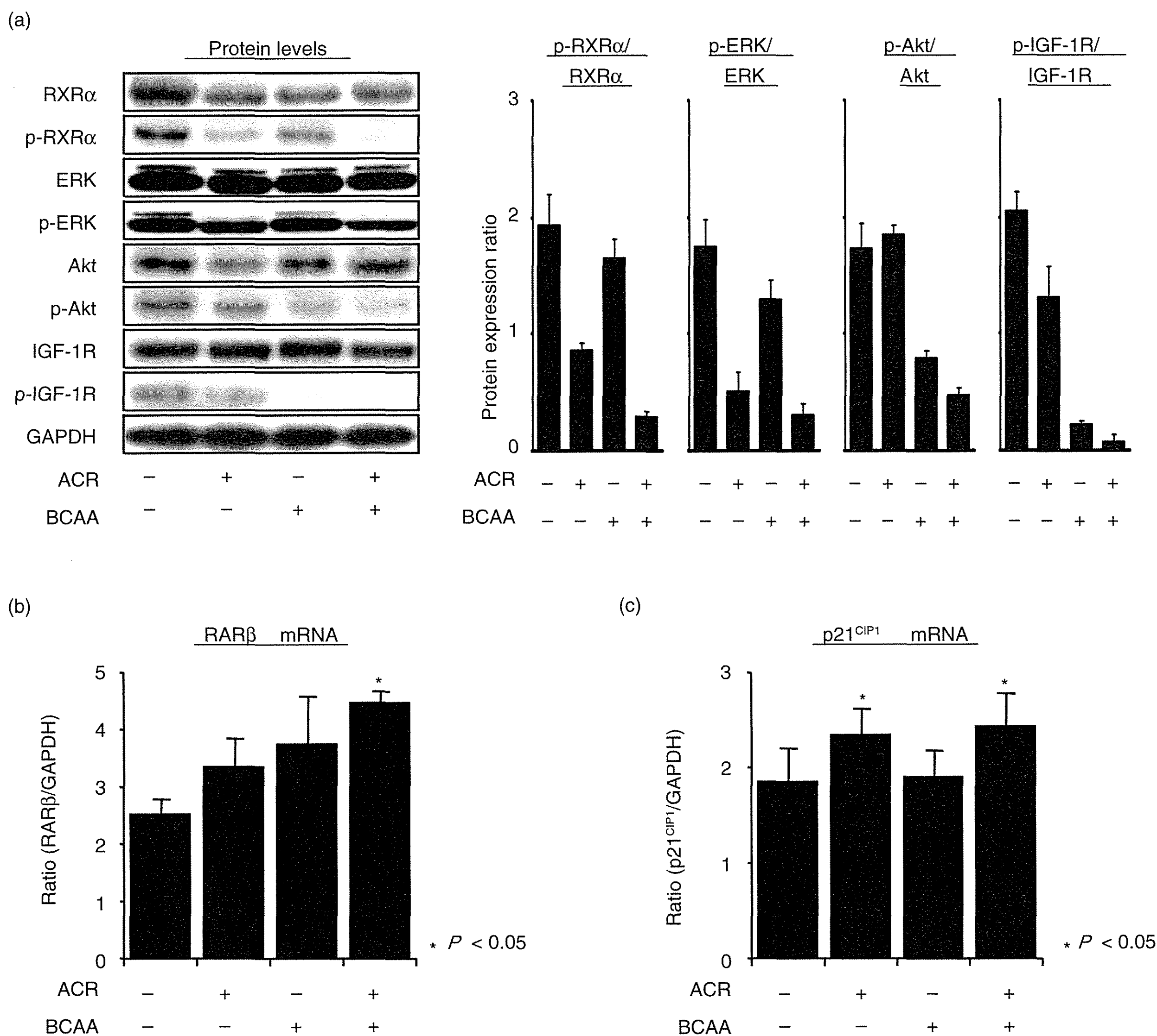

Figure 2 Effects of the combination of acyclic retinoid (ACR) plus branched-chain amino acid (BCAA) on phosphorylation of retinoid X receptor-α (RXR-α), extracellular signal-regulated kinase (ERK), Akt and insulin-like growth factor-1 receptor (IGF-1R) proteins and expression levels of retinoic acid receptor-β (RAR-β) and $p21^{CIP1}$ mRNA in Huh7 xenografts. The xenografts were excised from each animal at the termination of the experiment and tumor extracts were examined by a western blot analysis using the respective antibodies (a) or were examined by a quantitative real-time reverse transcription polymerase chain reaction analysis using RAR-β (b) and $p21^{CIP1}$ (c) specific primers. (a) Western blot analysis for glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was performed using a single membrane and equal protein loading was verified by the detection of this protein. Repeat western blots yielded similar results. Lanes, protein samples from each group (left). The intensities of blots were quantitated by densitometry (right). (b,c) The expression levels of *RAR-β* (b) and $p21^{CIP1}$ (c) genes were normalized to GAPDH expression. Bars, standard deviations of triplicate assays. $*P < 0.05$, significant differences obtained by comparison to the control group (group 1). p-, phosphorylated.

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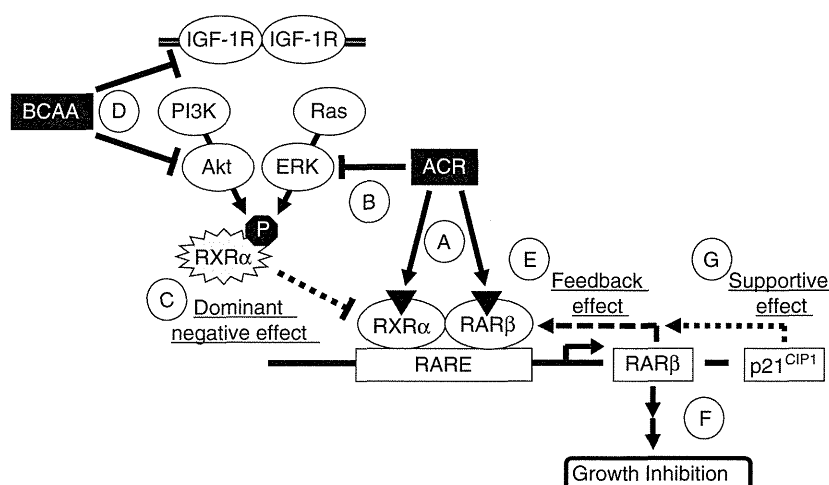

Figure 3 Hypothetical schematic representation of the effect of the combination of acyclic retinoid (ACR) plus branched-chain amino acid (BCAA) on growth inhibition in hepatocellular carcinoma (HCC) xenografts. ACR can bind to both retinoic acid receptor (RAR) and retinoid X receptor (RXR) as a ligand (a), and activate the retinoic acid responsive element (RARE) promoter activity, thus increasing the levels of both RAR-β and $p21^{CIP1}$ because the promoter region of these molecules contains RARE. In parallel, ACR inactivates the Ras/mitogen-activated protein kinase signaling pathways (b). This signaling pathway phosphorylates RXR-α, and thus impairs the function of this receptor in a dominant-negative manner (c). On the other hand, BCAA inhibits the activation of IGF-1R and its downstream Akt, which is also involved in RXR-α phosphorylation (d). Cooperative inhibition of RXR-α phosphorylation by ACR plus BCAA might restore the function of this receptor and subsequently increase RAR-β expression. This induction of RAR-β and its activation by the ligand ACR might produce a positive feedback effect on the expression of RAR-β itself (e), thus enhancing inhibition of growth in HCC cells (f). Induction of $p21^{CIP1}$ might support this positive feedback effect (g). For additional details see the "Discussion" section. ERK, extracellular signal-regulated kinase; IGF-1R, insulin-like growth factor-1 receptor.

ACR, at least in part, by inhibiting the activation of IGF-1R and its downstream Akt, because some types of receptor tyrosine kinases (RTK), including IGF-1R, might phosphorylate RXR-α through the phosphorylation of ERK and Akt. ACR and BCAA reduce the development of HCC and suppress the growth of cancer cells by inhibiting the activation of specific RTK, including IGF-1R and epidermal growth factor receptor (EGFR).[6,16,24] BCAA also suppresses insulin-induced hepatic tumor cell proliferation by inhibiting ERK and Akt phosphorylation.[18] The previous reports showing that there is a cross-talk between EGFR and IGF-1R, and that the simultaneous targeting of these RTK induces a synergistic inhibition of growth in HCC cells, might give this hypothesis credibility.[25,26]

The reduction in the dominant negative effect of RXR-α phosphorylation by combining ACR plus BCAA might activate the transcriptional activity of retinoic acid responsive element (RARE).[5,10] This is associated with the increased expression of RAR-β and $p21^{CIP1}$ mRNA because the promoter region of these genes contains RARE.[27,28] RAR-β, which is also a receptor for ACR, can exert tumor-suppressive effects in cancer cells.[29] Therefore, the induction of RAR-β by the treatment with ACR plus BCAA might have played a critical role in inhibiting the growth of HCC xenografts in the present study. In addition, this induction might be, at least in part, associated with $p21^{CIP1}$ upregulation by ACR plus BCAA because introduction of the $p21^{CIP1}$ gene into cells induces RAR-β expression and sensitizes cancer cells to retinoid treatment.[30] This hypothesis may be supported by recent reports that a substantial induction of RAR-β and $p21^{CIP1}$ produces positive feedback effects on the expression of RAR-β.[5,12]

Acyclic retinoid has an agonistic activity for both RAR and RXR.[2] Therefore, the reduction of the dominant-negative effect of RXR-α phosphorylation and the induction of the RAR-β expression by the combination of ACR plus BCAA might exert a significant inhibition of growth in the HCC xenografts. Because this study shows the possibility that the combination treatment consisting of ACR plus BCAA is an effective regimen for the treatment of HCC, we presume that this combination might also be useful for the prevention of HCC. In order to confirm

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this prediction, future studies are required to determine whether this combination treatment prevents the development of HCC using chemically-induced liver carcinogenesis in a rodent model with, for example, diethylnitrosamine.

In conclusion, this study, as well as prior ones,[5,11–14] indicates that the combination chemoprevention using ACR as a key agent might be an effective strategy for the prevention and treatment of HCC. Among such regimens, particularly combining ACR with BCAA might hold promise as a clinical modality for the chemoprevention of HCC because clinical trials have shown that both of these agents can significantly prevent the development of HCC without causing any adverse effects.[3,15]

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Preventive effects of branched-chain amino acid supplementation on the spontaneous development of hepatic preneoplastic lesions in C57BL/KsJ-*db/db* obese mice

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Obesity and its associated disorders, such as non-alcoholic steatohepatitis, increase the risk of hepatocellular carcinoma. Branched-chain amino acids (BCAA), which improve protein malnutrition in patients with liver cirrhosis, reduce the risk of hepatocellular carcinoma in these patients with obesity. In the present study, the effects of BCAA supplementation on the spontaneous development of hepatic premalignant lesions, foci of cellular alteration, in *db/db* obese mice were examined. Male *db/db* mice were given a basal diet containing 3.0% of either BCAA or casein, a nitrogen-content-matched control of BCAA, for 36 weeks. On killing the mice, supplementation with BCAA significantly inhibited the development of foci of cellular alteration when compared with casein supplementation by inhibiting cell proliferation, but inducing apoptosis. BCAA supplementation increased the expression levels of peroxisome proliferator-activated receptor-γ, $p21^{CIP1}$ and $p27^{KIP1}$ messenger RNA and decreased the levels of c-*fos* and cyclin D1 mRNA in the liver. BCAA supplementation also reduced both the amount of hepatic triglyceride accumulation and the expression of interleukin (IL)-6, IL-1β, IL-18 and tumor necrosis factor-α mRNA in the liver. Increased macrophage infiltration was inhibited and the expression of IL-6, TNF-α, and monocyte chemoattractant protein-1 mRNA in the white adipose tissue were each decreased by BCAA supplementation. BCAA supplementation also reduced adipocyte size while increasing the expression of peroxisome proliferator-activated receptor-α, peroxisome proliferator-activated receptor-γ and adiponectin mRNA in the white adipose tissue compared with casein supplementation. These findings indicate that BCAA supplementation inhibits the early phase of obesity-related liver tumorigenesis by attenuating chronic inflammation in both the liver and white adipose tissue. BCAA supplementation may be useful in the chemoprevention of liver tumorigenesis in obese individuals.

Introduction

Obesity is a serious health problem worldwide since it often causes a number of medical disorders, including metabolic syndrome and type 2 diabetes mellitus. Recent evidence also indicates that obesity and its related metabolic abnormalities are associated with an increased risk of developing hepatocellular carcinoma (HCC (1–5)). Non-alcoholic fatty liver disease (NAFLD) is a hepatic manifestation of metabolic syndrome and a subset of patients with this disease can progress to non-alcoholic steatohepatitis (NASH), which involves the risk of developing chronic hepatitis, cirrhosis and HCC (6–8). Obesity and diabetes mellitus have been shown to increase the risk of developing HCC also in patients with viral hepatitis (3,5). A state of chronic inflammation caused by insulin resistance and hepatic steatosis is considered to play a critical role in the development of HCC in several obesity-related pathophysiological conditions (2,6–10). Therefore, obese patients, especially those with complications of NASH or chronic viral hepatitis, are at high risk for developing HCC, and targeting chronic inflammation might be an effective strategy for preventing obesity-related liver carcinogenesis (11).

Branched-chain amino acids (BCAA), which are a group of three essential amino acids comprising valine, leucine and isoleucine, are used clinically to improve protein malnutrition in patients with liver cirrhosis (12,13). Oral supplementation with BCAA prevents progressive hepatic failure and improves event-free survival in patients with chronic liver diseases (14,15). Moreover, a multicenter, randomized controlled trial has reported that long-term oral BCAA supplementation reduced the risk of developing HCC in patients with chronic viral hepatitis; however, the effect was evident only in the patients who are obese (3). The results seen in that clinical trial are considered to be associated with the improvement of insulin resistance achieved by BCAA supplementation (13,16). In fact, BCAA supplementation inhibited the development of carcinogen-induced liver and colorectal carcinogenesis in obese mice by improving insulin resistance (17,18). Treatment with BCAA also suppressed insulin-induced proliferation of HCC cells by antagonizing the anti-apoptotic function of insulin (19).

In addition to improving protein malnutrition and glucose metabolism, BCAA supplementation has been reported to reduce lipid deposition in the liver in recent rodent studies (17,20). Supplementation with BCAA also retarded excess weight gain and reduced epididymal white adipose tissue (WAT) weight in mice that fed a high-fat diet (20). Because chronic low-grade systemic inflammation produced by excess lipid storage in WAT and liver is involved in both the development of NASH and the obesity-related liver tumorigenesis (2,6–10), BCAA supplementation may prevent the development of liver neoplasms in obese mice by reducing excess fat accumulation in WAT and by improving liver steatosis, thereby attenuating inflammation in these organs.

The spontaneous development of hepatic preneoplastic lesions, foci of cellular alteration (FCA), have been previously reported to be enhanced in obese and diabetic C57BL/KsJ-*db/db* (*db/db*) mice, when compared with C57B6 or C57BL/KsJ-+/+ mice, genetic controls for *db/db* mice (17). In the present study, we examined the effects of BCAA supplementation on the spontaneous development of FCA in *db/db* mice while focusing on the attenuation of inflammation in both the liver and the WAT. In addition, we investigated whether BCAA supplementation alters adipocyte size and the expression of peroxisome proliferator-activated receptor (PPAR)-α, PPAR-γ and adiponectin, which are key regulators of inflammatory signaling in obese adipose tissue (21–25), in the WAT of *db/db* mice.

Materials and methods

Mice and diets

Male *db/db* mice (4 weeks old) were obtained from Japan SLC (Shizuoka, Japan) and humanely maintained at Gifu University Life Science Research Center in accordance with Institutional Animal Care Guidelines. BCAA and casein were obtained from Ajinomoto (Tokyo, Japan). The BCAA composition (2:1:1.2 = leucine:isoleucine:valine) was set at the clinical dosage used for the treatment of decompensated liver cirrhosis in Japan (3,14).

Abbreviations: BCAA, branched-chain amino acids; FCA, foci of cellular alteration; HCC, hepatocellular carcinoma; H&E, hematoxylin and eosin; IL: interleukin; NAFLD, non-alcoholic fatty liver disease; NASH, non-alcoholic steatohepatitis; PCNA, proliferating cell nuclear antigen; PPAR, peroxisome proliferator-activated receptor; RT–PCR, reverse transcription–PCR; SEM, standard error mean; TNF-α, tumor necrosis factor-α; WAT, white adipose tissue.

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Experimental procedure

The experimental protocol was approved by the Institutional Committee of Animal Experiments of Gifu University. At 5 weeks of age, a total of 10 *db/db* mice were divided into two groups. The mice in Group 2 (n = 5) were given a basal diet (CRF-1, Oriental Yeast, Tokyo, Japan) supplemented with 3.0% BCAA (w/w) through the end of the experiment, whereas the mice in Group 1 (n = 5) were given a basal diet supplemented with 3.0% casein (w/w) that served as a nitrogen-content-matched control for the BCAA-treated group. At 41 weeks of age (after 36 weeks of supplementation with the experimental diet), all of the mice were killed using CO_2 asphyxiation and the development of FCA was analyzed.

Histopathology and measurement of adipocyte size

Maximum sagittal sections of each liver lobe (six sublobes) and WAT obtained from the periorchis were used for histological examination. The tissue specimens were fixed in 10% buffered formaldehyde and then embedded in paraffin. The sections (4 µm thick) were cut from the tissue blocks and stained with hematoxylin and eosin (H&E). The presence of FCA, which are phenotypically altered hepatocytes showing swollen and basophilic cytoplasm and hyperchromatic nuclei, was determined according to the criteria described previously (26). The multiplicity of the FCA was assessed on a per unit area basis (per cm^2). Fatty metamorphosis (% of fatty degeneration) was determined on the H&E-stained liver section using the BZ-Analyzer-II software (KEYENCE, Osaka, Japan (27)).

To evaluate adipocyte size, 10 adipocytes from each stained section (a total of 50 adipocytes) in each group were analyzed using a fluorescence microscope BZ-9000 (KEYENCE). Adipocyte size was measured and averaged using the BZ-Analyzer-II (KEYENCE). The unit of mean adipocyte size was square micrometers (μm^2).

Immunohistochemical analysis of proliferating cell nuclear antigen and F4/80

Immunohistochemical staining of proliferating cell nuclear antigen (PCNA), a G_1-to-S phase marker, was performed to estimate the cell proliferative activity of FCA using an anti-PCNA antibody (1:100; Santa Cruz Biotechnology, Santa Cruz, CA, USA). On the PCNA-immunostained sections, the cells with intensively reacted nuclei were considered to be positive for PCNA, and the indices (%) were calculated in 20 FCA randomly selected from each group (28).

Immunohistochemical staining to detect F4/80, a mature macrophage marker, was also performed to estimate the presence of macrophage infiltration in the WAT. After endogenous peroxidase activity was blocked with H_2O_2, the sections were incubated with a F4/80 primary antibody (1:50; AbD Serotec, Oxford, UK) for 30 min at 37°C. Subsequently, the sections were incubated with biotinylated secondary antibodies against the primary antibodies (Dako, Carpinteria, CA, USA) and then incubated with avidin-coupled peroxidase. The sections were then developed with 3,3′-diaminobenzidine using Dako Liquid DAB Substrate-Chromogen System (Dako) and counterstained with hematoxylin.

Hepatic lipid analysis

Approximately 200 mg of frozen liver was homogenized, and the lipids were extracted using a chloroform:methanol (2:1 v/v) solution, as described by Folch *et al.* (29). The levels of triglycerides in the livers of the mice were measured using the triglyceride E-test kit (Wako Pure Chemical, Osaka, Japan) according to the manufacturer's protocol (17).

RNA extraction and quantitative real-time reverse transcription–PCR analysis

Total RNA was isolated from the livers and adipose tissues of the mice using the RNeasy Mini Kit and RNeasy Lipid Tissue Mini Kit (Qiagen, Hilden, Germany), respectively. Total RNA (1 µg) was used for the synthesis of the first strand of complementary DNA using the SuperScript III First-Strand Synthesis System (Invitrogen, Carlsbad, CA, USA). Quantitative real-time reverse transcription (RT)-PCR was performed using specific primer sets that amplify PCNA, c-*fos*, interleukin (IL)-6, IL-1β, IL-18, tumor necrosis factor-α (TNF-α), monocyte chemoattractant protein-1 (MCP-1), adiponectin, PPAR-α, PPAR-γ, Bax, Bcl-2, $p21^{CIP1}$, $p27^{KIP1}$, cyclin D1 and β-actin genes. The sequences of these primers, which are obtained from the PrimerBank (http://pga.mgh.harvard.edu/primerbank/), are given in Table I. Each sample was analyzed on a LightCycler 1.0 (Roche Diagnostics, GmbH, Mannheim, Germany) with SYBR Premix Ex Taq (TaKaRa Bio, Shiga, Japan). The expression level of each gene was normalized to the β-actin expression level using the standard curve method (17).

Statistical analysis

All data were expressed as the mean ± the standard error mean (SEM). Differences between the two groups were analyzed using Student's t-test. All analyses were conducted using JMP 8.0 (SAS Institute Inc., Cary, NC, USA). Values with $P < 0.05$ were considered to be significant.

Table I. Primer sequences

Gene		Primer sequence
PCNA	F	5′-TTTGAGGCACGCCTGATCC-3′
	R	5′-GGAGACGTGAGACGAGTCCAT-3′
c-fos	F	5'-CGGGTTTCAACGCCGACTA-3'
	R	5′-TTGGCACTAGAGACGGACAGA-3′
IL-6	F	5′-CTGCAAGAGACTTCCATCCAG-3′
	R	5-AGTGGTATAGACAGGTCTGTTGG-3′
IL-1β	F	5′-GCAACTGTTCCTGAACTCAACT-3′
	R	5-ATCTTTTGGGGTCCGTCAACT-3′
IL-18	F	5′-GTGAACCCCAGACCAGACTG-3′
	R	5′-CCTGGAACACGTTTCTGAAAGA-3′
TNF-α	F	5′-CAGGCGGTGCCTATGTCTC-3′
	R	5′-CGATCACCCCGAAGTTCAGTAG-3′
adiponectin	F	5′-TGTTCCTCTTAATCCTGCCCA-3′
	R	5′-CCAACCTGCACAAGTTCCCTT-3′
PPAR-α	F	5′-AGAGCCCCATCTGTCCTCTC-3′
	R	5′-ACTGGTAGTCTGCAAAACCAAA-3′
PPAR-γ	F	5′-TCGCTGATGCACTGCCTATG-3′
	R	5′-GAGAGGTCCACAGAGCTGATT-3′
MCP-1	F	5′-TTAAAAACCTGGATCGGAACCAA-3′
	R	5′-GCATTAGCTTCAGATTTACGGGT-3′
Bax	F	5′-AGACAGGGGCCTTTTTGCTAC-3′
	R	5′-AATTCGCCGGAGACACTCG-3′
Bcl-2	F	5′-ATGCCTTTGTGGAACTATATGGC-3′
	R	5′-GGTATGCACCCAGAGTGATGC-3′
$p21^{CIP1}$	F	5′-CCTGGTGATGTCCGACCTG-3′
	R	5′-CCATGAGCGCATCGCAATC-3′
$p27^{KIP1}$	F	5′-TCAAACGTGAGAGTGTCTAACG-3′
	R	5′-CCGGGCCGAAGAGATTTCTG-3′
Cyclin D1	F	5′-GCGTACCCTGACACCAATCTC-3′
	R	5′-ACTTGAAGTAAGATACGGAGGGC-3′
β-actin	F	5′-GGCTGTATTCCCCTCCATCG-3′
	R	5′-CCAGTTGGTAACAATGCCATGT-3′

Results

General observations

Body, liver, kidney and fat (WAT of the periorchis and retroperitoneum) weights and hepatic triglyceride levels of the two groups measured at the end of the study are listed in Table II. The mean liver weight and mean level of triglycerides in the livers of the mice in the BCAA supplementation group were found to be significantly less than those in the mice in the casein-treated group ($P < 0.05$). BCAA supplementation also improved macrovesicular steatosis, which was observed in the casein-fed mice ($P < 0.05$, Figure 1A), suggesting that BCAA supplementation inhibits hepatomegaly by improving the accumulation of lipids in the liver. Other measurements did not differ significantly between the two groups. All of the mice remained healthy, and no clinical signs indicating toxicity of BCAA were observed during the experiment. Histopathologically, there were no

Table II. Body, liver, kidney and fat weights and hepatic triglyceride levels of the experimental mice

Treatment	No. of mice	Body wt (g)	Relative wt (g/100 g body wt) of			Hepatic triglyceride (mg/100 mg liver tissue)
			Liver	Kidney	Fat[a]	
Casein	5	67.9±7.9[b]	7.1±1.5	0.9±0.1	9.1±2.1	13.9±2.8
BCAA	5	68.4±2.7	5.1±0.6[c]	0.9±0.1	11.0±2.5	6.8±4.4c

[a]White adipose tissue of the periorchis and retroperitoneum.
[b]Mean ± SEM.
[c]$P < 0.05$.

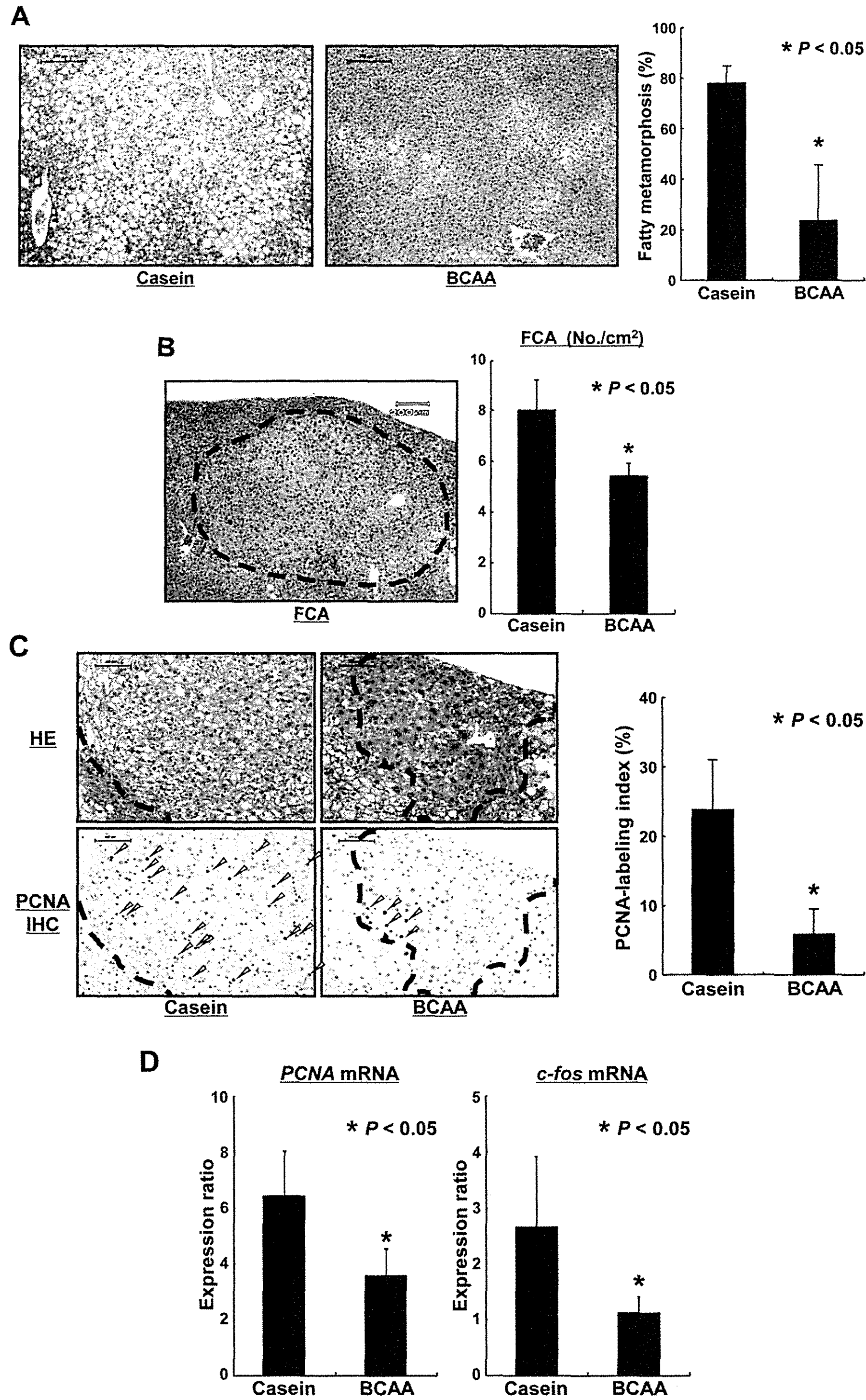

Fig. 1. Effects of BCAA supplementation on the hepatic steatosis, development of FCA, and on the expression of PCNA and c-*fos* mRNA in the livers of the *db/db* mice. (A) Histopatology (H&E staining) and a morphometric analysis of fatty metamorphosis in the liver of the casein-supplemented and the BCAA-supplemented *db/db* mice. (B) A representative photograph of FCA that spontaneously developed in the *db/db* mice (H&E staining) and the average number of FCA in the casein-supplemented and the BCAA-supplemented groups. (C) Representative photographs of H&E staining and the PCNA-immunohistochemical analysis of the FCA developed in the livers of the casein-supplemented and the BCAA-supplemented mice (left panels). The PCNA-labeling indices of the FCA developed in the livers of each group were determined by counting the PCNA-positive nuclei (arrowheads) in the FCA (right panel). * $P < 0.05$ versus the casein-supplemented group. (D) The expression levels of PCNA and c-*fos* mRNA in the liver of the casein-supplemented and the BCAA-supplemented mice were examined by quantitative real-time RT–PCR using specific primers. The values are expressed as the mean ± the SEM. *$P < 0.05$ versus the casein-supplemented group.

($P < 0.05$, Figure 1D). These findings suggest that BCAA supplementation prevents the development of FCA, at least in part, by reducing cell proliferation.

Effects of BCAA supplementation on the expression levels of IL-6, IL-1β, IL-18 and TNF-α mRNA in the livers of the db/db mice

Chronic inflammation induced by the excessive production of storage lipids plays a role in obesity-related liver carcinogenesis (2,6–10). Therefore, the effects of BCAA supplementation on the expression levels of proinflammatory cytokines IL-6, IL-1β, IL-18 and TNF-α mRNA, which are central mediators of chronic inflammatory diseases (2,6–10), in the livers of the *db/db* mice were determined. Quantitative real-time RT–PCR revealed that in comparison with the casein-supplemented mice, the experimental mice showed significantly decreased expression levels of mRNA in the liver following BCAA supplementation ($P < 0.05$, Figure 2). These findings suggest that BCAA supplementation attenuates chronic inflammation in the livers of obese and diabetic *db/db* mice.

Effects of BCAA supplementation on macrophage infiltration and the expression level of IL-6, TNF-α and MCP-1 mRNA in the WAT of the db/db mice

Macrophages play important roles in inflammation in obese adipose tissue (21,22). Therefore, whether BCAA supplementation attenuates chronic inflammation or inhibits increased infiltration of macrophages in WAT was examined. Immunohistochemical analysis performed with an antibody to F4/80 revealed the presence of apparent macrophage infiltration in the periorchis WAT of the casein-supplemented *db/db* mice; however, the infiltration was markedly inhibited by BCAA supplementation (Figure 3A). The expression levels of IL-6 and TNF-α mRNA in the WAT were also reduced by BCAA supplementation. Additionally, supplementation with BCAA significantly inhibited the expression of MCP-1 mRNA ($P < 0.05$, Figure 3B), which plays a role in the recruitment of macrophages into obese adipose tissue (30,31). These findings suggest that inhibition of macrophage infiltration and subsequent attenuation of chronic inflammation in WAT by BCAA supplementation are, at least in part, associated with the suppression of MCP-1 expression.

Effects of BCAA supplementation on adipocyte size and expression levels of PPAR-α, PPAR-γ, and adiponectin mRNA in the WAT of the db/db mice

The induction of inflammation in obese adipose tissue is associated with increased adipocyte size (21,22). Therefore, whether BCAA supplementation alters the histology of WAT was next examined. Histological analysis showed that in addition to the inhibition of macrophage infiltration, BCAA supplementation reduced the size of adipocyte (Figure 4A). The average adipocyte size observed in the BCAA-supplemented mice was significantly smaller than that observed in the casein-supplemented mice ($P < 0.05$, Figure 4B).

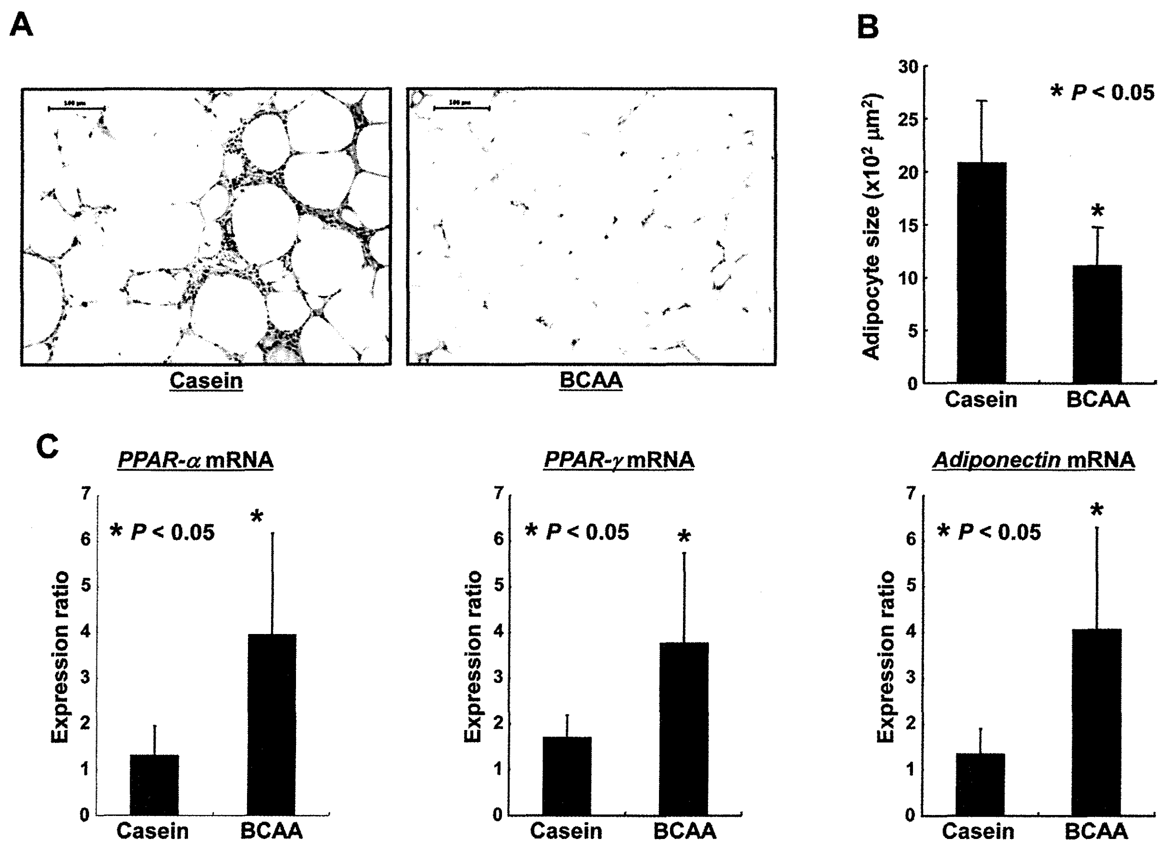

Fig. 4. Effect of BCAA supplementation on adipocyte size and the expression of PPAR-α, PPAR-γ and adiponectin mRNA in the WAT of the *db/db* mice. (A) The histopathology of the periorchis WAT of the casein-supplemented and the BCAA-supplemented mice (H&E staining). (B) The H&E staining images of the adipose tissues were analyzed using a fluorescence microscope BZ-9000, and adipocyte size was measured using a BZ-Analyzer-II. (C) The expression levels of PPAR-α, PPAR-γ and adiponectin mRNA in the periorchis WAT of the casein-supplemented and the BCAA-supplemented mice were examined by quantitative real-time RT–PCR using specific primers. The values are expressed as the mean ± the SEM. *$P < 0.05$ versus the casein-supplemented group.

spontaneous development of hepatic neoplastic lesions because increases in these proinflammatory cytokines, which are accompanied by lipid accumulation in the liver, are critically involved in obesity-related liver carcinogenesis (2,6–10). The preventive effects of obesity-related liver tumorigenesis by targeting IL-6 and TNF-α expression and liver steatosis are also demonstrated in other rodent studies (28,35,36). In addition, the alleviation of hepatic steatosis with BCAA supplementation, which might be associated with the effects of improving insulin resistance (13), is consistent with previous reports (17,20).

In addition to the benefits observed in the liver, the present study also showed that BCAA supplementation significantly attenuates chronic inflammation in the WAT of *db/db* mice. Macrophage infiltration into WAT, which is accompanied by IL-6 and TNF-α production, is an early contributing event for the development of chronic low-grade systemic inflammation (21,22). MCP-1 plays a crucial role in the recruitment of macrophages into obese adipose tissue (30,31). MCP-1 is also capable of inducing steatosis in hepatocytes, indicating that secretion of this chemokine by adipose tissue may induce steatosis not only by recruiting macrophages but also by acting directly on hepatocytes (37). In addition, upregulation of IL-6, TNF-α and MCP-1 in WAT is critically involved in the induction of systemic insulin resistance (21,22), which is a key factor for accelerating obesity-related liver carcinogenesis (2,6–10). Therefore, the inhibition of enhanced adipose tissue inflammation, that is increased macrophage infiltration and IL-6, TNF-α and MCP-1 expression, by BCAA supplementation is important in preventing the development of steatosis and subsequent liver tumorigenesis in obese mice.

The present study demonstrated that adipocyte size in BCAA-supplemented mice is much smaller than that in control mice. This finding might be associated with the effects of BCAA on the induction of PPAR-α and PPAR-γ in WAT because activation of these nuclear receptors significantly prevents adipocyte hypertrophy (24,38). An increase in the number of small adipocytes induces adiponectin and its receptors, which downregulates the production of IL-6 and TNF-α, thereby reducing obesity-related inflammation in adipose tissue (24,25). A lack of adiponectin enhances the progression of hepatic steatosis and tumor formation in a mice model of NASH (39), whereas this adipokine alleviates hepatic steatosis by decreasing TNF-α production (40). Moreover, the induction of adiponectin plays a role in the suppression of chemically induced liver tumorigenesis in obese mice (28). Therefore, in the present study, the effects of BCAA on the upregulation of PPAR-α, PPAR-γ and adiponectin achieved by inhibiting adipocyte hypertrophy may contribute to preventing obesity-related liver tumorigenesis.

In addition to the WAT, the present study also showed the first evidence that BCAA supplementation increases the mRNA level of PPAR-γ, but not that of PPAR-α, in the livers of obese mice. The precise mechanisms underlying the upregulation of the expression of PPAR-γ in the liver by BCAA have not yet been clarified. However, these findings are significant when considering the prevention of liver carcinogenesis because PPAR-γ is regarded to be an antitumorigenic factor in HCC, whereas the role of PPAR-α in HCC development is contradictory (32,33,41). The overexpression of PPAR-γ suppresses the growth of HCC cells by reducing cell proliferation and inducing apoptosis (32). The activation of PPAR-γ by its ligand also inhibits the proliferation of HCC cells by upregulating the $p21^{CIP1}$ and $p27^{KIP1}$ expression, which thus leads to the G_1 arrest of the cell cycle (33). These reports (32,33), together with the results of the present study showing that BCAA supplementation increases the expression of PPAR-γ, Bax, $p21^{CIP1}$ and $p27^{KIP1}$ mRNA and decreases the expression of Bcl-2 and cyclin D1 mRNA, suggest that the induction of apoptosis and regulation of cell-cycle progression induced by BCAA via the upregulation of PPAR-γ in the liver may also help to inhibit the development of FCA.

Finally, it should be noted again that improved insulin resistance achieved from BCAA supplementation, which has been demonstrated in several basic and clinical studies (13,16), is critical to suppress the development of neoplasms in both the liver and the colon of obese mice (17,18). Because chronic inflammation occurring in WAT plays a role in systemic insulin resistance (30,31), BCAA supplementation might prevent the spontaneous development of hepatic preneoplastic lesions via the attenuation of adipocyte inflammation and the subsequent improvement of insulin resistance. These findings suggest that in addition to the liver, as shown in the present and previous studies (17,42), WAT might be a critical target for BCAA to exert chemopreventive properties in obesity-related liver carcinogenesis.

In conclusion, supplementation with BCAA may be an effective strategy for the chemoprevention of HCC, especially in obese patients who are at an increased risk of developing HCC. The results of the present study further strengthen our hypothesis that targeting obesity-induced pathologic conditions, such as chronic inflammation, might be effective for preventing liver carcinogenesis in obese individuals (11).

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Role of Acyclic Retinoid in the Chemoprevention of Hepatocellular Carcinoma: Basic Aspects, Clinical Applications, and Future Prospects

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Abstract: The poor prognosis for hepatocellular carcinoma (HCC) is associated with its high rate of recurrence in the cirrhotic liver. Therefore, development of effective strategies for preventing recurrence and secondary tumors will improve the clinical outcome of HCC patients. A malfunction of the retinoid X receptor-α (RXRα) due to phosphorylation by the Ras-MAPK signaling pathway is profoundly associated with liver carcinogenesis, and thus, may be a promising target for HCC chemoprevention. Acyclic retinoid (ACR), which inhibits Ras-MAPK activation and RXRα phosphorylation, successfully prevents HCC recurrence, thus improving patient survival. The fundamental concept of HCC chemoprevention by ACR is "clonal deletion," which is defined as the removal of latent malignant clones from the liver before they expand into clinically detectable HCC. "Combination chemoprevention" using ACR as a key drug holds great promise of a new effective strategy for the prevention of HCC because of its synergism. ACR is also expected to prevent the development of HCC in obese people, who are at an increased risk to HCC, because this agent significantly inhibits obesity-related liver tumorigenesis in the rodent model. Here, we review the detailed effects of ACR on preventing HCC development, especially based on the results of our basic and clinical research.

Keywords: Acyclic retinoid, chemoprevention, clonal deletion, combination therapy, HCC, obesity, phosphorylated RXRα.

INTRODUCTION

Retinoids, a group of structural and functional derivatives of vitamin A, have fundamental effects on cellular activities, including growth, differentiation, and apoptosis, as well as on morphology [1-3]. Because of this, loss of retinoid activity or responsiveness is linked to carcinogenesis, including the development of hepatocellular carcinoma (HCC). Targeting retinoid abnormalities may therefore be an effective strategy for the prevention and/or treatment of certain types of malignancies [4, 5]. We have reported in clinical trials that the administration of acyclic retinoid (ACR), which is the same substance as NIK-333 and Peretinoin (Kowa Pharmaceutical Co., Tokyo, Japan; Fig. 1), reduces the incidence of post-therapeutic recurrence of HCC, and thus, improves patient survival from this malignancy (for the most recent results, see Ref. 9) [6-9]. Many experimental studies have revealed the pleiotropic effects of ACR in the prevention of HCC and suppression of cancer cell growth [10-12]. Among these effects, targeting the phosphorylation of retinoid X receptor-α (RXRα), which is closely involved in liver carcinogenesis [13], is one of the critical mechanisms of inhibition of HCC development by ACR [14-17].

The aim of this article is to review the evidence that a malfunction of RXRα due to phosphorylation plays a critical role in liver carcinogenesis, and that ACR prevents the development of HCC by targeting this retinoid receptor. We also review the concept of "clonal deletion" therapy, a practical approach to preventing HCC development, and the possibility of "combination chemoprevention", which uses ACR as a key drug. In addition, the possibility that ACR can inhibit the development of liver tumorigenesis associated with HCC risk factors such as obesity and insulin resistance is also discussed.

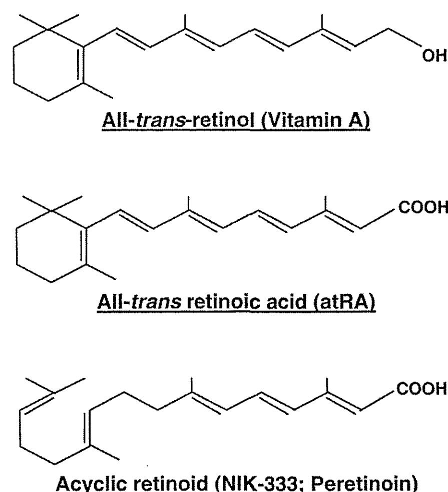

Fig. (1). The chemical structures of all-*trans* retinol (vitamin A), all-*trans* retinoic acid (atRA), and acyclic retinoid (ACR). ACR is the same substance as NIK-333 and peretinoin.

RETINOIDS AND THEIR RECEPTORS

Retinoids consist of natural and synthetic molecules. Natural retinoids include retinyl esters, retinol, and retinoic acid (RA). Large numbers of synthetic retinoids, including ACR, have also been developed (Fig. 1). Retinoids exert

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their biological functions primarily by regulating gene expression through 2 distinct nuclear receptors, the retinoic acid receptors (RARs) and RXRs. RARs bind to all-*trans*-RA (atRA), a drug for acute promyelocytic leukemia [18], and 9-*cis*-RA (9cRA) with similar affinities, whereas RXRs bind only to 9cRA. Both RAR and RXR are composed of 3 subtypes (α, β, and γ), which are characterized by a modular domain structure [1-3].

Like other members of the nuclear receptor superfamily, the retinoid receptors are ligand-dependent transcription factors. After ligand binding, RXRs form homodimers as well as heterodimers with RARs, which interact with the retinoid X response element (RXRE) or the retinoic acid receptor responsive element (RARE), located in the promoter region of target genes, thereby modulating gene expression [1-3]. In addition to forming a heterodimer with RARs, RXRs can also form heterodimers with several other nuclear receptors, thus indicating that RXRs act as common heterodimerization partners for various types of nuclear receptors [3]. Therefore, RXRs play an essential role in controlling normal cell proliferation, differentiation, and death (regulation of apoptosis), and act as master regulators of nuclear receptors [3]. On the other hand, these facts also suggest that altered expression and function of retinoids and their receptors, especially RXRs, are strongly associated with deviation from normal cell proliferation and death, which are key factors for cancer development [4, 5].

CLINICAL CHARACTERISTICS OF HCC

HCC is a major healthcare problem worldwide, accounting for 750,000 annual cases; approximately the same number of individuals (700,000) die from this malignancy each year [19]. HCC development is frequently associated with chronic inflammation and subsequent cirrhosis of the liver induced by a persistent infection with hepatitis B virus (HBV) or hepatitis C virus (HCV) [20, 21]. Patients with viral liver cirrhosis have a high risk for HCC with an annual rate of carcinogenesis of approximately 7%. The frequency of HCC recurrence after curative treatment is also very high in cirrhotic patients; the recurrence rate 5 years after definitive therapy may exceed 70% [22-25], suggesting that curative treatment for HCC is difficult once this malignancy has developed. In addition, no effective and established chemotherapeutic agents are currently available for this malignancy. These facts indicate the limited improvement in HCC prognosis, and therefore, there is an urgent need to develop more effective strategies for chemoprevention and chemotherapy of this malignancy. In particular, cancer chemoprevention, an approach wherein a natural or synthetic chemical compound arrests or reverses premalignant cells *via* physiological pathways [26], is one of the most promising and practical strategies for the treatment of HCC because the high-risk groups of this cancer, such as hepatitis virus-positive patients, are easily identified; thus, the strategy targets good candidates for intervention.

RXRα PHOSPHORYLATION AND LIVER CARCINOGENESIS

Abnormalities in retinoid signaling are prominently involved in the development of HCC. In a rodent model of liver carcinogenesis, retinol is locally deficient in the HCC but not in the adjacent normal liver tissues [27]. The *RARβ* gene, which is regarded as a tumor suppressor gene because of its ability to regulate cell growth and apoptosis [28], is an HBV integration site, and its expression is markedly decreased in human HCC [29, 30]. In a rat model of chemically-induced liver carcinogenesis, the expression levels of RARβ protein and mRNA are also decreased in HCC [31]. On the other hand, RARγ is overexpressed in the HCC tissues and cells; this is associated with growth of HCC cells, which suggests the oncogenic potential of this retinoid receptor in liver carcinogenesis [32].

Among retinoid receptors, RXRα alterations are particularly implicated in the development and progression of HCC. The expression of RXRα is decreased not only in HCC and liver cell adenoma, but also in glutathione *S*-transferase placental form-positive foci, a precancerous HCC lesion, in a rat model of chemically-induced liver carcinogenesis. These findings indicate that the repression of RXRα occurs even in the early stage of liver carcinogenesis [31]. In addition, we have previously shown that RXRα protein is anomalously phosphorylated at the serine and threonine residues, and that it accumulates in both surgically resected human HCC tissues and HCC cell lines [13, 33]. The activated extracellular signal-regulated kinase (ERK) is highly expressed in HCC cells, and constitutive phosphorylation at the serine at position 260 of RXRα, a mitogen-activated protein kinase (MAPK)/ERK consensus site, is closely associated with its retarded degradation, low transcriptional activity, and promotion of cancer cell growth; in turn, the abrogation of phosphorylation by a MAPK inhibitor restores the degradation of RXRα in a ligand-dependent manner [13, 14]. The phosphorylated form of RXRα (p-RXRα) is resistant to ubiquitination and proteasome-mediated degradation in human HCC cells, resulting in an accumulation of this phosphorylated protein within the HCC tissues [34]. In addition, p-RXRα abolishes its ability to form heterodimers with RARβ, and this is associated with uncontrolled cell growth and resistance to retinoids [35]. The analogous effects of phosphorylated RXRα in the negative modulation of its heterodimeric binding partners have also been reported in several studies [36-38]. These findings, therefore, suggest that the accumulation of p-RXRα, which is regarded as the non-functional form of RXRα, may interfere with the function of normal (unphosphorylated) RXRα in a dominant-negative manner, and thus, play a critical role in HCC development (Fig. **2**). The impact of RXRα phosphorylation on retinoid resistance, anti-apoptosis, and activation of cell growth is also revealed in other types of human malignant cells, including colon cancer and leukemia cells [39-41].

MECHANISMS OF ACR IN HCC CHEMOPREVENTION: RESULTS FROM EXPERIMENTAL STUDIES

As mentioned above, liver carcinogenesis is accompanied by the accumulation of p-RXRα because aberrant phosphorylation of RXRα abolishes its function as the master regulator of the nuclear receptor superfamily [13, 33-35]. Therefore, inhibition of RXRα phosphorylation and the associated restoration of its heterodimeric activity with other

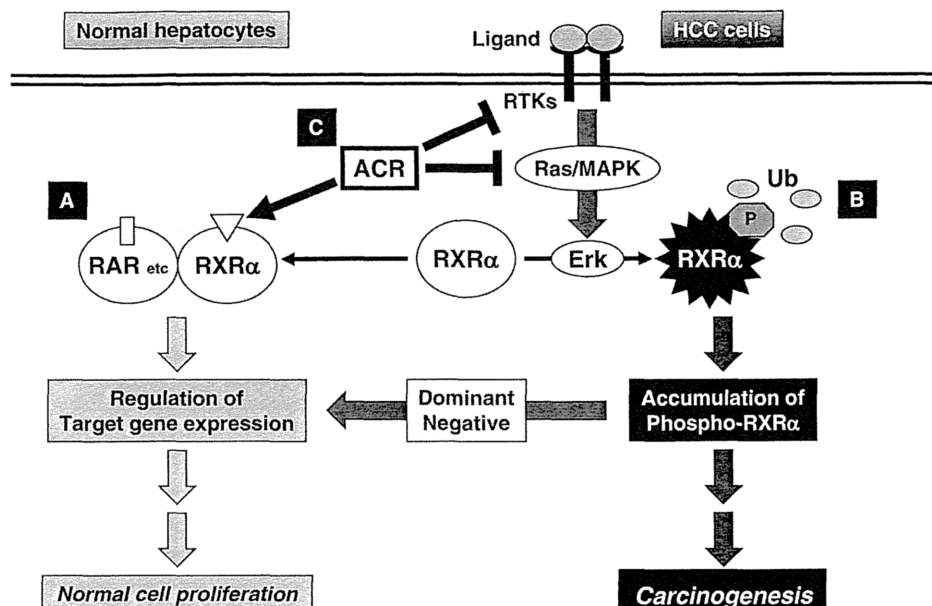

Fig. (2). Retinoid refractoriness due to phosphorylation of RXRα, and its restoration by ACR in liver carcinogenesis. When ACR binds to and activates RXRα in normal hepatocytes, it forms homo- and/or heterodimers with other nuclear receptors, including RARs, resulting in expression of the target genes that regulate normal cell proliferation and differentiation (**A**). In HCC cells, the Ras-MAPK pathway is highly activated and phosphorylates RXRα at serine residues, thus impairing dimer formation and the subsequent transactivation functions of the receptor. Furthermore, non-functional phosphorylated RXRα (p-RXRα) is sequestered from ubiquitin/proteasome-mediated degradation and accumulates in liver cells, interfering with the physiological function of the remaining unphosphorylated RXRα in a dominant negative manner, thereby playing a critical role in liver carcinogenesis (**B**). ACR is not only a ligand for RXRα but also a suppressor of the Ras-MAPK signaling pathway; it inhibits RXRα phosphorylation, thereby restoring the function of the receptor and activating the transcriptional activity of the responsive element. ACR also inhibits, directly or indirectly, the ligand (growth factor)-dependent RTK activities, which contribute to the inhibition of ERK and RXRα phosphorylation and suppression of growth in HCC cells (**C**).

nuclear receptors may be an effective and important strategy for the prevention and treatment of HCC. ACR, which was initially developed as an agonist for both RXR and RAR [42, 43], is a possible candidate for this goal [10-12]. ACR activates the promoter activity of RXRE and RARE; controls the expression of target genes including RARβ, $p21^{CIP1}$, and cyclin D1; induces apoptosis; and causes cell cycle arrest in G_0–G_1, thus, inhibiting the growth of human HCC-derived cells [14-16, 44-52]. This agent inhibits both chemically-induced liver carcinogenesis in rats and spontaneously occurring HCC in mice [27, 53-55]. The anti-tumor effects of ACR are also associated with inhibition of angiogenesis and repression of oxidative stress [56, 57]. A number of beneficial effects of ACR on the prevention of HCC development and inhibition of growth of HCC cells are summarized in Fig. (**3**).

Increasing evidence has indicated that, in addition to retinoid receptors, several types of growth factors and their corresponding receptor tyrosine kinases (RTKs), which play a role in the activation of the Ras-MAPK signaling pathway, are critical targets of ACR to inhibit cancer cell growth [46, 54-56, 58, 59]. These reports are important because aberrant activation of certain types of RTKs such as the epidermal growth factor receptor and the downstream Ras-MAPK pathway are closely associated with liver carcinogenesis and, therefore, are regarded as critical targets for HCC treatment [60, 61]. The activated Ras-MAPK pathway phosphorylates RXRα, and this may contribute to liver carcinogenesis [13, 33]. On the other hand, ACR restores RXRα function by inactivating the Ras-MAPK signaling system, leading to dephosphorylation of RXRα, although 9cRA is incapable of suppressing ERK and RXRα phosphorylation [14]. ACR inactivates Ras activation in human HCC and pancreatic cancer cells [15, 62]. In the liver of diethylnitrosamine (DEN)-treated *db/db* mice, ACR also inhibits the activation of Ras and the phosphorylation of ERK and RXRα proteins [17]. These findings suggest that ACR impedes the development of HCC and inhibits cancer cell growth, at least in part, by targeting the RTK-Ras-MAPK signaling pathway and subsequent RXRα phosphorylation. The role of RXRα phosphorylation in liver carcinogenesis and its inhibition by ACR are schematically represented in Fig. (**2**).

CHEMOPREVENTION OF HCC BY ACR: RESULTS FROM CLINICAL TRIALS

Findings from numerous preclinical experiments strongly indicate that ACR is a promising agent for the chemoprevention of HCC (Figs. **2** and **3**). Therefore, an early-phase randomized controlled clinical trial was conducted to test the chemopreventive effect of ACR on

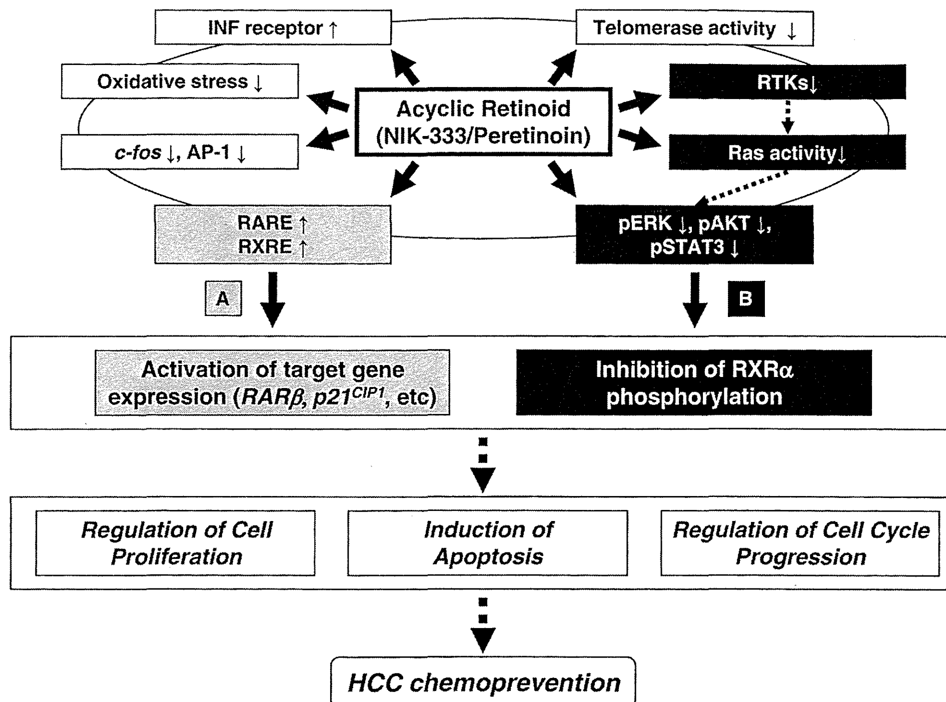

Fig. (3). Pleiotropic effects of ACR to prevent HCC development. One of the main effects of ACR is to activate the expression of its target genes such as *RARβ* and $p21^{CIP1}$ by upregulating the promoter activity of RARE and RXRE (**A**). In addition, ACR suppresses cancer cell growth by inhibiting the activation and expression of some types of RTKs, including EGFR, HER2, VEGFR-2, and FGFR, which contribute to the subsequent inhibition of Ras-MAPK activation and RXRα phosphorylation (**B**). ACR inhibits phosphorylation of Akt and Stat3 proteins. Induction of RARβ and restoration of RXRα function due to dephosphorylation by ACR leads to cooperative regulation of cell proliferation, cell cycle progression, and induction of apoptosis, thus preventing HCC development. ACR also induces the expression of the IFN receptor (INFR), inhibits the transcriptional activity of *c-fos* and AP-1 promoters, and downregulates telomerase activity in the HCC and squamous cell carcinoma cells. ACR also suppresses liver tumorigenesis by repressing oxidative stress.

secondary HCC in patients who underwent potentially curative treatment for initial HCC [6-8]. We randomly assigned 89 patients to undergo either ACR treatment (44 patients, 600 mg/day) or placebo (45 patients) for 12 months. In this trial, oral administration of ACR significantly reduced the incidence of recurrent or new HCCs after a median follow-up period of 38 months; 12 patients (27%) in the ACR group developed HCC as compared with 22 patients (49%) in the placebo group (P = 0.04) [6]. After a further follow-up to 62 months, ACR improved both the recurrence-free survival (P = 0.002) and overall survival (P = 0.04) [7]. The relative risk of the development of secondary HCC and death were 0.31 (95% confidence interval [CI], 0.12 – 0.78) and 0.33 (95% CI, 0.11 – 0.79), respectively [6, 7]. Moreover, the preventive effects of ACR lasted up to 50 months after randomization or 38 months after completion of ACR administration, indicating that administration for only 12 months conferred a long-term effect over several years [8]. No severe side effects were observed in this trial.

A phase II/III clinical trial of ACR confirmed its effectiveness in preventing second primary HCCs in HCV-positive patients, who received curative treatment for primary or the first recurrence of HCC, in a multicenter large-scale (n = 401) randomized placebo-controlled trial with a median follow-up of 2.5 years. In this trial, oral administration of ACR (600 mg/day) was tolerated, and it had a strong effect on the prevention of second primary HCC with a hazard ratio of 0.27 (95% CI, 0.07 – 0.96) 2 years after treatment. Cumulative recurrence-free survival rates in the ACR-treated group were higher than those in the placebo group (during the first year: ACR, 71.9%; placebo, 66.0% and during 3 years: ACR, 43.7%; placebo, 29.3%), indicating that ACR reduced the recurrence of HCC, especially after 2 years of treatment [9]. Therefore, the results of these clinical trials [6-9, 63] suggest that ACR is a novel first-line therapy to reduce the development of second primary HCC. Application for governmental approval of this agent as an "HCC chemopreventive drug" in patients with liver cirrhosis is under progress in Japan.

"CLONAL DELETION" THERAPY: THE FUNDAMENTAL CONCEPT OF HCC CHEMOPREVENTION

It should be noted that, in an early-phase clinical trial [6-8], the serum levels of lectin-reactive α-fetoprotein factor 3 (AFP-L3), which indicates the presence of latent (*i.e.*, invisible) malignant clones in the remnant liver, were significantly reduced after ACR administration for 12 months. This agent also prevented the appearance of serum

AFP-L3 in patients whose AFP-L3 levels were negative at trial enrollment, whereas the number of patients whose serum AFP-L3 appeared *de novo* was significantly increased in the placebo group, and these patients had a significantly higher risk of developing secondary HCC [63]. These observations suggest the following 2 possibilities: (1) ACR deletes the AFP-L3-producing premalignant clones from the remnant liver before they expand into clinically detectable tumors ("clonal deletion") and (2) ACR also actively inhibits the development of AFP-L3-producing clones, which have the potential to become HCC ("clonal inhibition"). From these results, we have proposed new concepts of HCC chemoprevention, "clonal deletion" and "clonal inhibition," which are defined as the removal or inhibition, respectively, of latent malignant (or premalignant) clones that are invisible by diagnostic imaging from the chronically damaged liver when it is in a hyper-carcinogenic state. We believe that ACR prevents the development of HCC by implementing this concept, which is a key to HCC chemoprevention (Fig. **4**). Once the malignant clones are eliminated or inhibited from the remnant liver by ACR through the induction of apoptosis and cell differentiation [14-16, 42, 44-52], it takes several years for the clinical appearance of the next HCC clones. Therefore, only short-term administration (12 months) of ACR could exert long-term preventive effect on HCC development for several years after termination of treatment [8]. A recent phase II/III trial also demonstrated a similar result emphasizing the preventive effect of ACR on the development of second primary HCC 2 years after administration of this agent [9]. This finding suggests that ACR may mainly suppress *de novo* carcinogenesis in the cirrhotic liver, and we presume that this phenomenon is associated with the implementation of "clonal deletion" therapy by ACR.

FUTURE PROSPECTS OF ACR – 1: "COMBINATION CHEMOPREVENTION" OF HCC USING ACR AS THE KEY DRUG

There is growing interest in the combination therapy involving ACR with a variety of other agents because such a therapy often provides the potential for synergistic effects on growth inhibition in cancer cells. We have initially found that the combination of ACR and interferon (IFN)-β synergistically inhibits cell growth and induces apoptosis in HCC cells, and that this is associated with an ACR-induced

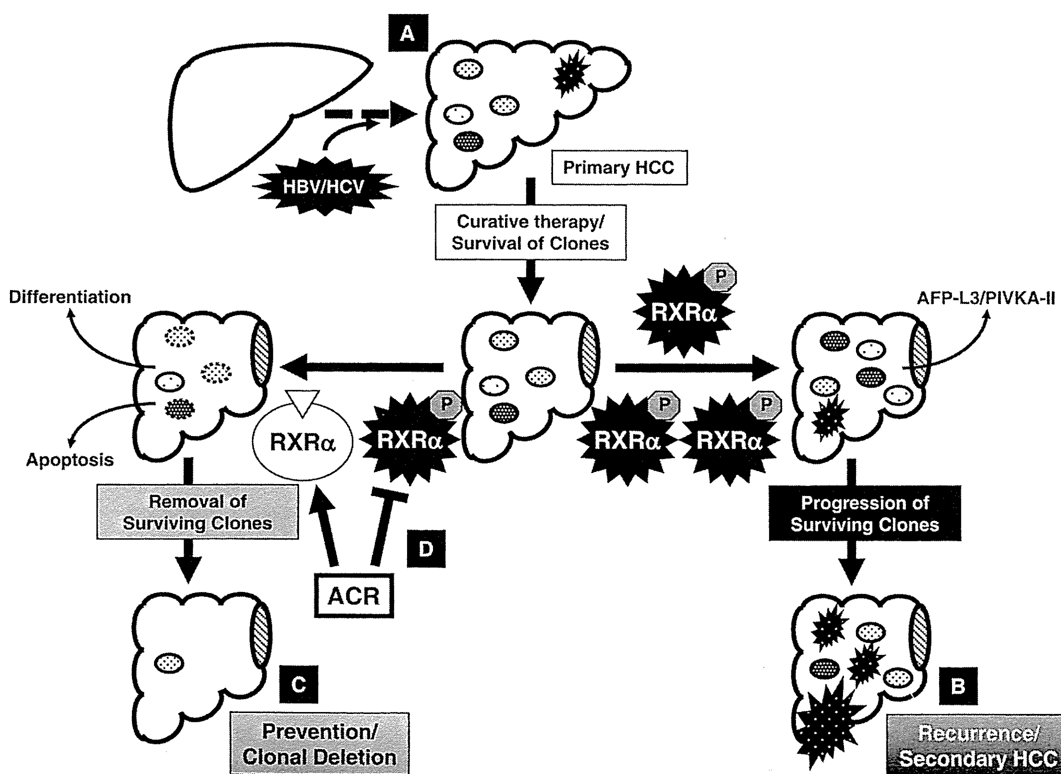

Fig. (4). Concept of "clonal deletion" therapy for HCC chemoprevention. Persistent inflammation caused by hepatitis viral infection transforms the liver into a precancerous setting, which consists of multiple latent malignant clones that can, at some point, develop into HCC (**A**). Even after early detection and removal of the primary HCC, the remaining clones survive in the liver and grow into secondary HCC lesions, which is a major cause of the poor prognosis for patients with this malignancy (**B**). Therefore, one of the most promising strategies to prevent secondary HCC is the deletion of such transformed clones by inducing cell differentiation or apoptosis before they expand into clinically detectable tumors (the concept of "clonal deletion") (**C**). ACR, which targets phosphorylated RXRα, prevents the recurrence and development of secondary HCC *via* the mechanism described by this concept (**D**).