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]$P < 0.05$.

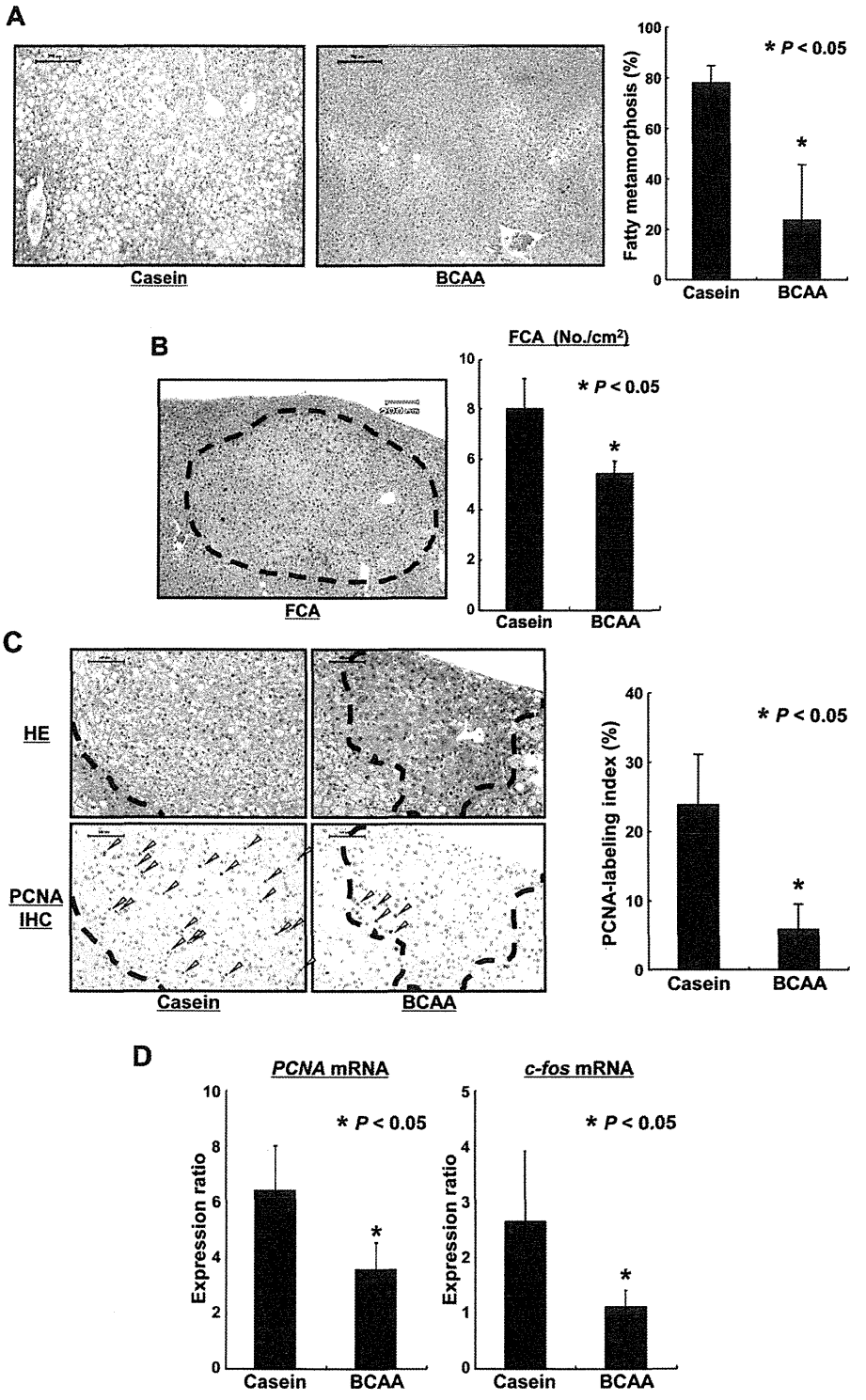

Fig. 1. Effects of BCAA supplementation on the hepatic steatosis, development of FCA, and on the expression of PCNA and c-*fos* mRNA in the livers of the *db/db* mice. (A) Histopatology (H&E staining) and a morphometric analysis of fatty metamorphosis in the liver of the casein-supplemented and the BCAA-supplemented *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.

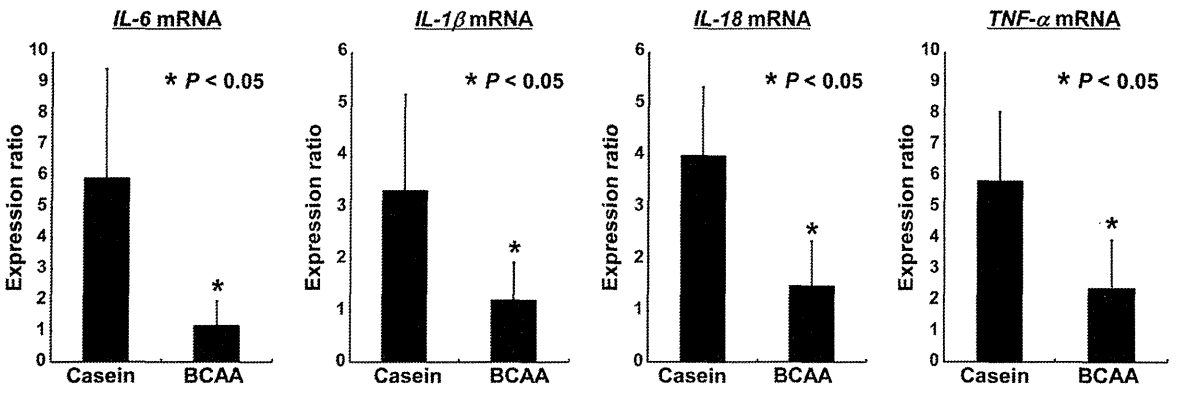

Fig. 2. Effect of BCAA supplementation on the expression of IL-6, IL-1β, IL-18 and TNF-α mRNA in the livers of the *db/db* mice. The expression levels of IL-6, IL-1β, IL-18 and TNF-α mRNA in the liver of the casein-supplemented and the BCAA-supplemented mice were examined by quantitative real-time RT–PCR using specific primers. The values are expressed as the mean ± the SEM. *$P < 0.05$ versus the casein-supplemented group.

findings suggesting any toxicity of BCAA to major organs, including the liver, kidney and spleen.

Effects of BCAA supplementation on the spontaneous development of FCA, the proliferation activity in FCA and the expression levels of PCNA and c-fos messenger RNA in the livers of the db/db mice

At sacrifice, FCA developed in the liver of all experimental mice regardless of the treatment. It was found that supplementation with BCAA significantly decreased the number of FCA when compared with casein supplementation ($P < 0.05$, Figure 1B). An immunohistochemical analysis to detect PCNA showed the mean PCNA-labeling index for FCA in the BCAA-supplemented mice to be significantly lower than that in the casein-supplemented mice ($P < 0.05$, Figure 1C). In the whole liver, BCAA supplementation also inhibited the expression levels of PCNA and c-*fos* messenger RNA (mRNA) in comparison with casein supplementation

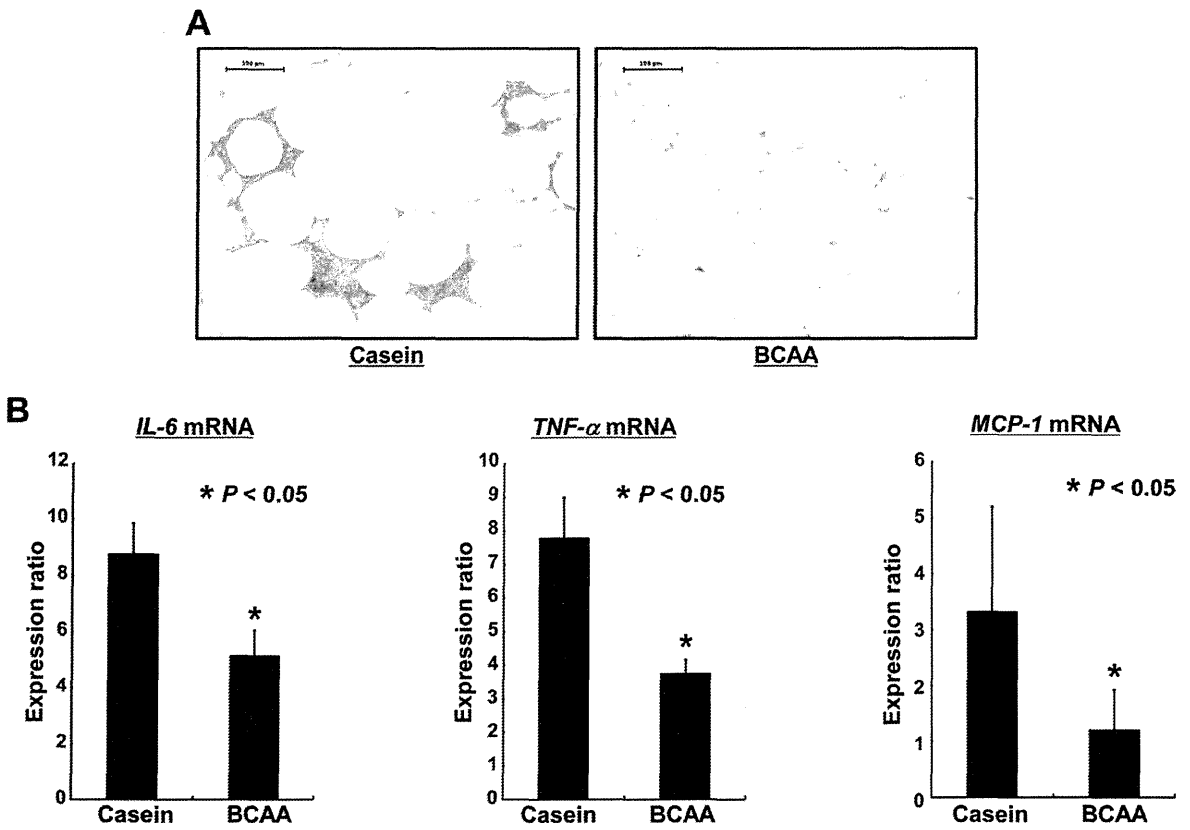

Fig. 3. Effect of BCAA supplementation on macrophage infiltration and the expression of IL-6, TNF-α and MCP-1 mRNA in the WAT of the *db/db* mice. (A) The F4/80 immunohistochemical analyses were performed in the periorchis WAT of the casein-supplemented and the BCAA-supplemented mice to show macrophage infiltration. (B) The expression levels of IL-6, TNF-α and MCP-1 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.

($P < 0.05$, 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).

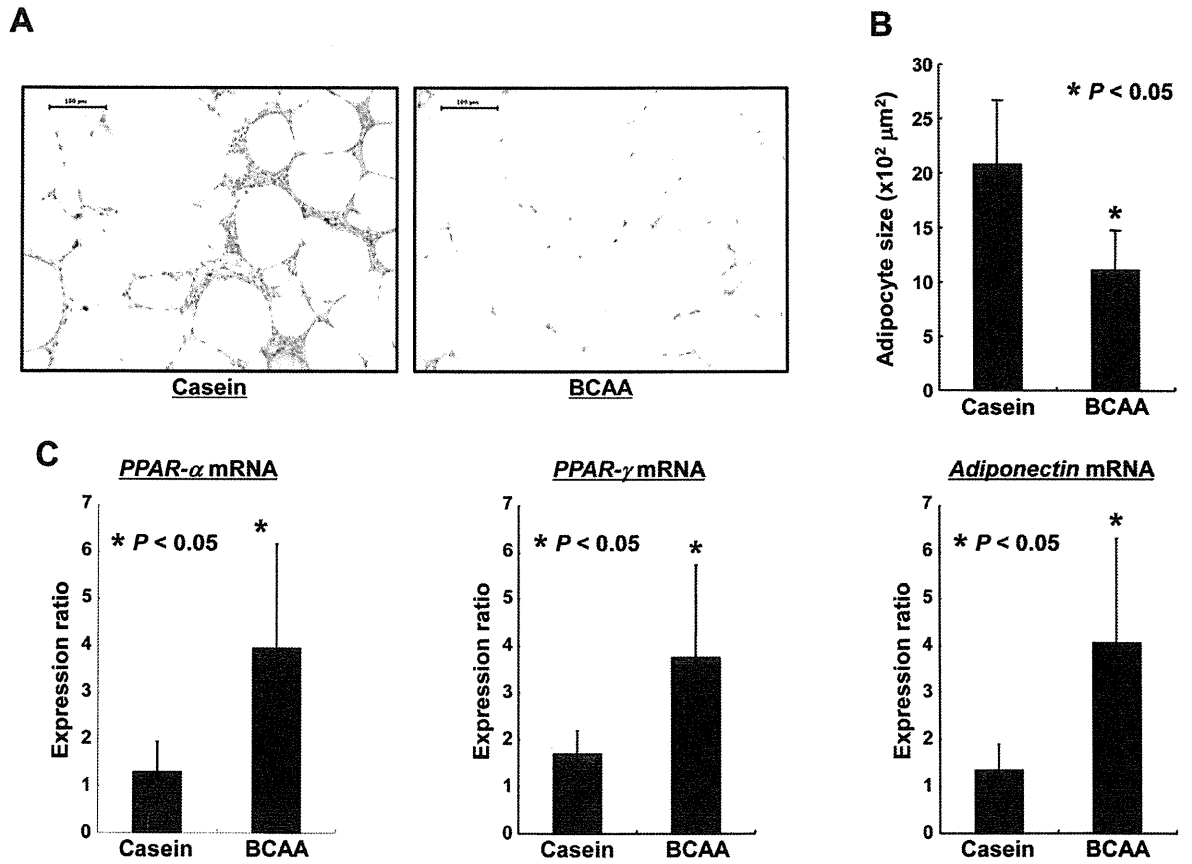

Fig. 4. Effect of BCAA supplementation on adipocyte size and the expression of PPAR-α, PPAR-γ and adiponectin mRNA in the WAT of the *db/db* mice. (A) The histopathology of the periorchis WAT of the casein-supplemented and the BCAA-supplemented mice (H&E staining). (B) The H&E staining images of the 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.

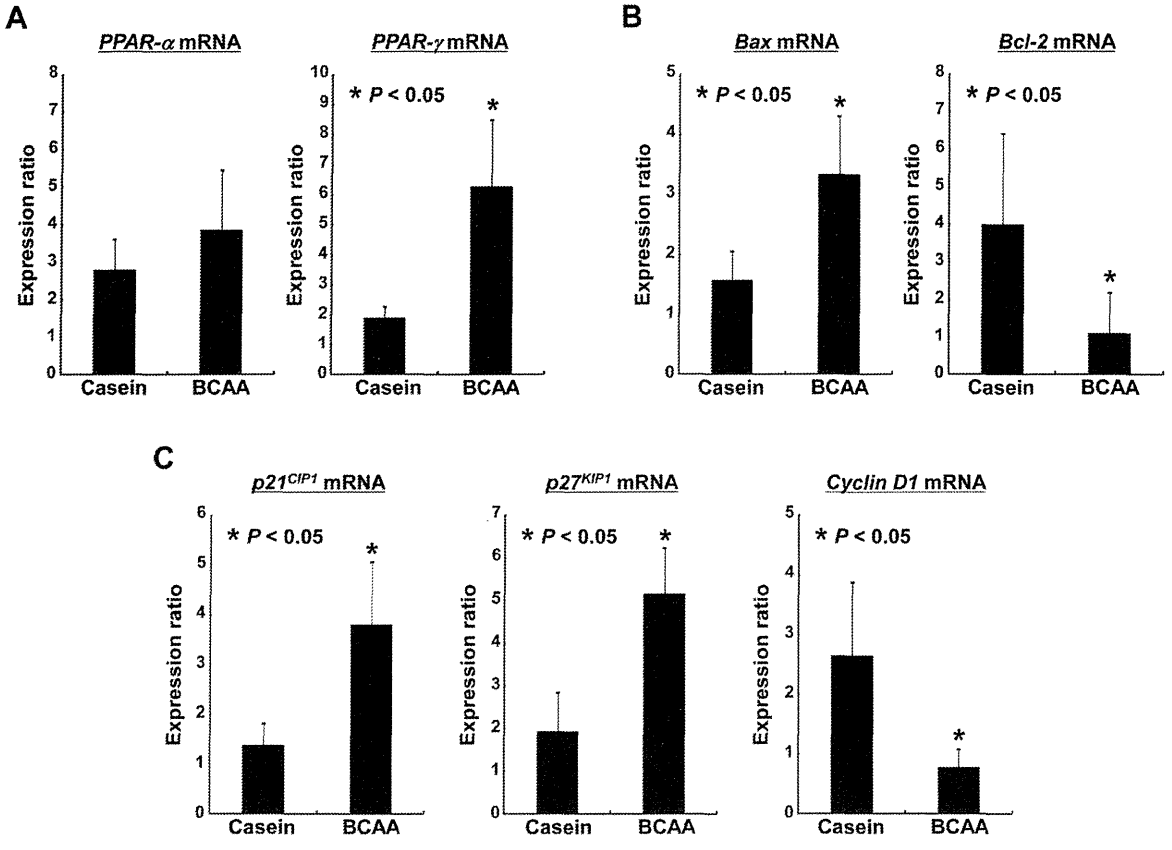

Fig. 5. Effect of BCAA supplementation on the expression of PPAR-α, PPAR-γ, Bax, Bcl-2, $p21^{CIP1}$, $p27^{KIP1}$ and cyclin D1 mRNA in the livers of the *db/db* mice. The expression levels of (A) PPAR-α and PPAR-γ, (B) Bax and Bcl-2 and (C) $p21^{CIP1}$, $p27^{KIP1}$ and cyclin D1 mRNA in the liver of the casein-supplemented and the BCAA-supplemented mice were examined using quantitative real-time RT–PCR with specific primers. The values are expressed as the mean ± SEM. *$P < 0.05$ versus the casein-supplemented group.

Moreover, BCAA supplementation increased the expression of PPAR-α mRNA, which can be a key regulator of inflammatory signaling (23,24), in the WAT of the *db/db* mice. Furthermore, the expression levels of PPAR-γ, a master regulator of adipocyte differentiation, and its downstream adiponectin mRNA, which also possesses the ability to suppress proinflammatory signaling (24,25), in the WAT were both significantly upregulated by BCAA supplementation ($P < 0.05$, Figure 4C).

Effects of BCAA supplementation on the expression levels of PPAR-α, PPAR-γ, Bax, Bcl-2, $p21^{CIP1}$, $p27^{KIP1}$ and cyclin D1 mRNA in the livers of the db/db mice

Recent studies have revealed the activation of PPAR-γ to exert a beneficial effect against HCC by inducing apoptosis and cell-cycle arrest (32,33). Therefore, in addition to the WAT (Figure 4C), whether BCAA supplementation also increases the expression levels of PPAR-γ in the liver was next examined. The expression levels of PPAR-γ mRNA in the liver were found to be significantly increased by BCAA supplementation ($P < 0.05$), whereas this agent did not alter the levels of PPAR-α mRNA (Figure 5A). Supplementation with BCAA increased the levels of Bax mRNA, which accelerates apoptosis, and decreased the levels of Bcl-2 mRNA, an anti-apoptotic member of the Bcl-2 family, in the livers of the experimental mice (Figure 5B, $P < 0.05$). There were also significant increases in the expression levels of $p21^{CIP1}$ and $p27^{KIP1}$ mRNA and decreases in the levels of cyclin D1 mRNA in the livers of the mice supplemented with BCAA (Figure 5C, $P < 0.05$).

Discussion

Obesity, which is implicated in the development of NAFLD and NASH, has been shown to increase the risk of developing HCC (1–5). The present study, a NAFLD mice model in which obesity and severe steatosis were developed, clearly indicates that dietary supplementation with BCAA effectively prevents the spontaneous development of liver preneoplastic lesions in *db/db* mice through the inhibition of cell proliferation. These findings are consistent with our recent report that BCAA supplementation suppresses the chemically induced liver tumorigenesis in obese mice by improving insulin resistance (17). We considered that the results of the present study showed an equivalent significance to those of the previous experiment (17) because NAFLD that has not yet progressed to NASH can induce hepatocyte proliferation and hepatic hyperplasia, both of which initiate the hepatic neoplastic process in obesity (34). Therefore, targeting NAFLD, a hyperproliferative field, through intervention using specific agents such as BCAA supplementation might be an effective strategy for preventing obesity-related liver carcinogenesis.

In various obesity-related metabolic disorders, substantial evidence has shown that chronic inflammation caused by obesity contributes to the progression of NAFLD to NASH and finally to HCC (2,6–10). Hepatic steatosis, which is a source of inflammation, also promotes the development of HCC (8,9). Therefore, reduction of lipid accumulation and attenuation of chronic inflammation in the liver achieved by BCAA supplementation play a critical role in the suppression of the spontaneous development of hepatic neoplastic lesions in obese mice. The inhibition of the expression of IL-6 and TNF-α by BCAA supplementation is particularly important in the suppression of the

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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Conflict of Interest Statement: The authors declare that they have no competing interests.

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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.

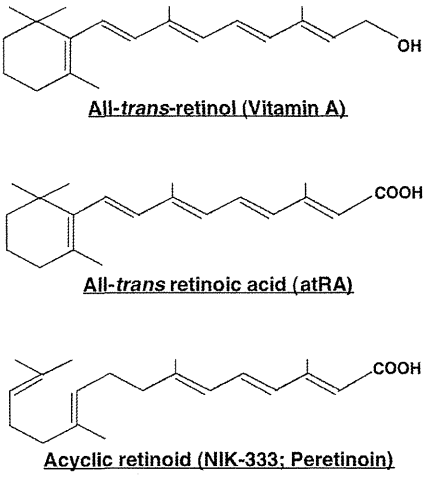

Fig. (1). The chemical structures of all-*trans* retinol (vitamin A), all-*trans* retinoic acid (atRA), and acyclic retinoid (ACR). ACR is the same substance as NIK-333 and peretinoin.

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

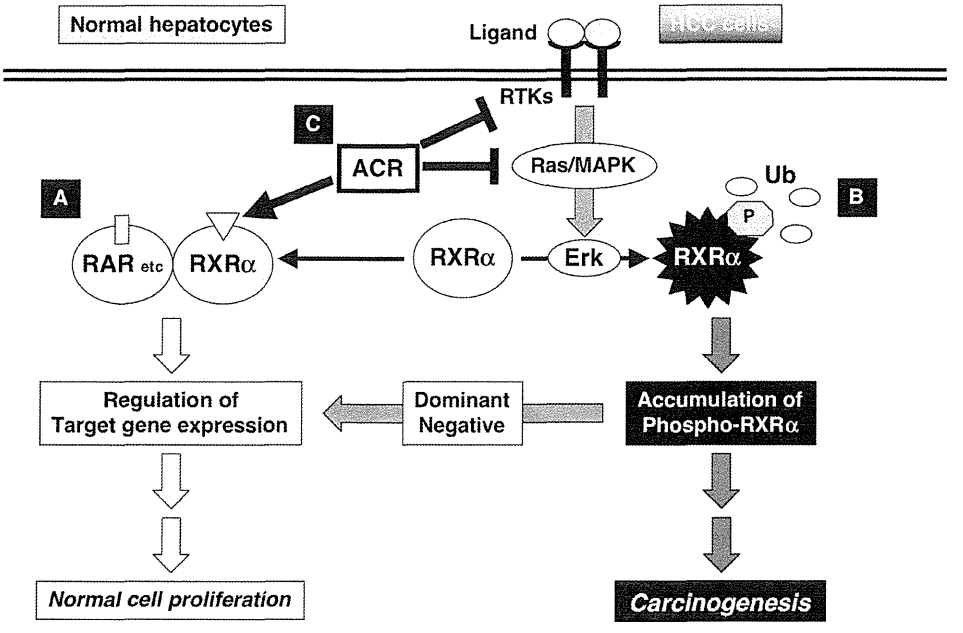

Fig. (2). Retinoid refractoriness due to phosphorylation of RXRα, and its restoration by ACR in liver carcinogenesis. When ACR binds to and activates RXRα in normal hepatocytes, it forms homo- and/or heterodimers with other nuclear receptors, including RARs, resulting in expression of the target genes 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

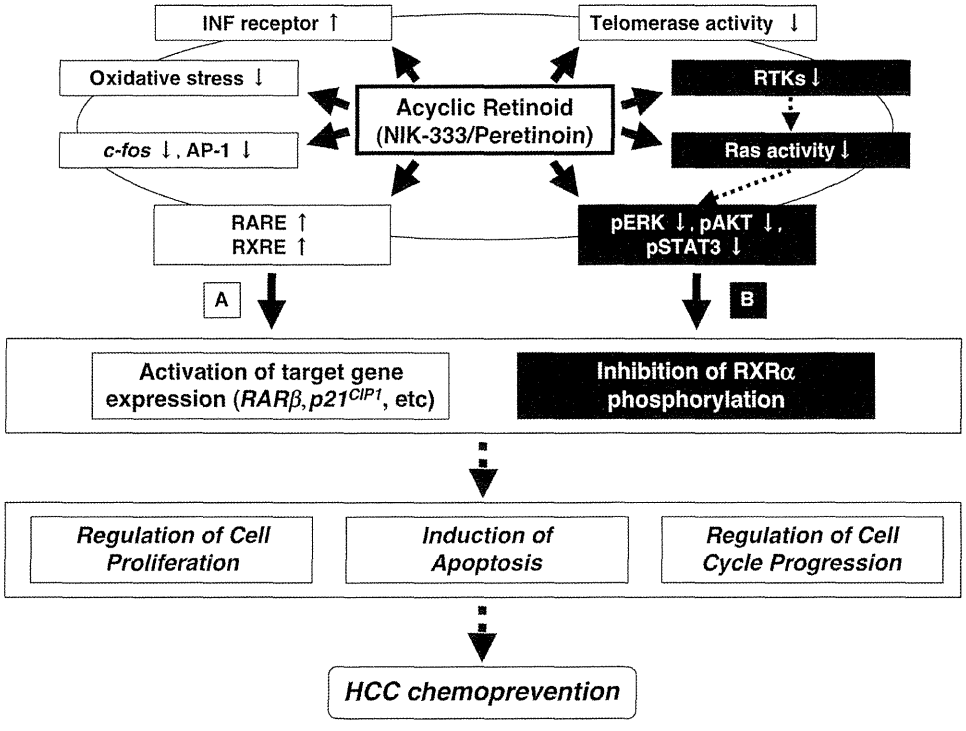

Fig. (3). Pleiotropic effects of ACR to prevent HCC development. One of the main effects of ACR is to activate the expression of its target genes such as *RARβ* and $p21^{CIP1}$ by upregulating the promoter activity of RARE and RXRE (**A**). In addition, ACR suppresses cancer cell growth by inhibiting 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

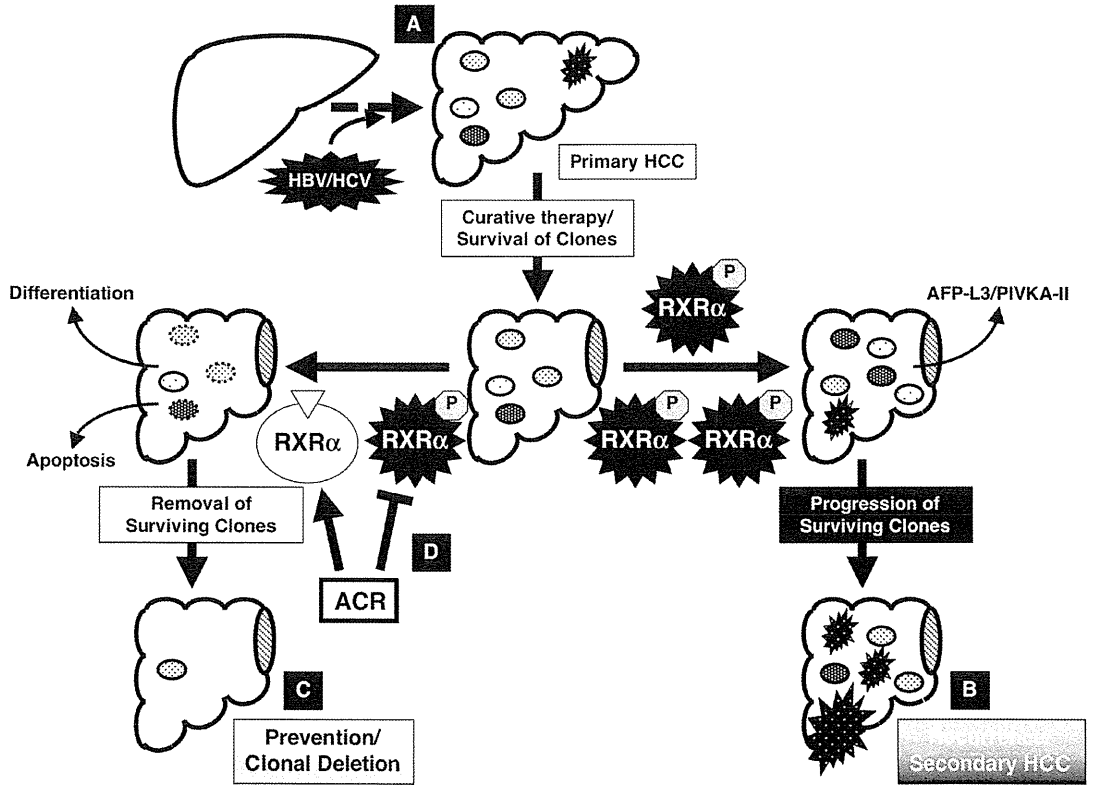

Fig. (4). Concept of "clonal deletion" therapy for HCC chemoprevention. Persistent inflammation caused by hepatitis viral infection transforms the liver into a precancerous setting, which consists of multiple latent malignant clones that can, at some point, develop into HCC (**A**). Even after early 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**).

upregulation of the expression of type-1 IFN receptor and Stat1 [48]. ACR also acts synergistically with vitamin K_2 (VK_2) in suppressing growth and inducing apoptosis in the human HCC cells [15]. These findings are significant when considering the clinical use of ACR because both IFN and VK_2 are expected to exert preventive effects on the development and recurrence of HCC [64-67]. Therefore, in the near future, "combination chemoprevention" using ACR as the key agent, such as ACR plus IFN or VK_2, may become one of the effective strategies to prevent the development of HCC. Synergistic growth inhibition caused by ACR plus VK_2 treatment is also observed in HL-60 human leukemia cells [68].

The expected mechanisms of how the combination of ACR and other specific agents synergistically exerts chemopreventive effects in HCC cells are schematically summarized in Fig. (**5**). Among these mechanisms, dephosphorylation of RXRα by targeting the Ras-MAPK signaling pathway or its upstream RTKs seems to be one of the most crucial approaches of ACR for inducing synergistic growth inhibition in HCC cells. Indeed, ACR and VK_2 cooperatively inhibit the activation of the Ras-MAPK signaling pathway, and thus, suppress the phosphorylation of RXRα and the growth of HCC cells [15]. Trastuzumab is a humanized monoclonal antibody against human epidermal growth factor receptor-2 (HER2), a member of the RTK family, and the combination of 9cRA [69] or ACR (unpublished data) and this antibody synergistically inhibits the activation of HER2, ERK, and Akt, subsequently dephosphorylating RXRα, and thus inhibiting growth and inducing apoptosis in the HCC cells. Combined treatment with ACR plus valproic acid, a histone deacetylase (HDAC) inhibitor, also acts synergistically to induce apoptosis and G_0–G_1 cell cycle arrest in the HCC cells by inhibiting phosphorylation of RXRα, ERK, Akt, and GSK-3β proteins [16]. In addition to HCC, the combination of ACR plus gemcitabine synergistically inhibits cell growth and induces apoptosis by inhibiting Ras activation in pancreatic cancer cells [62].

In addition to targeting RXRα phosphorylation, induction of nuclear receptors that dimerize with RXR, such as RAR and PPAR [39, 70], and recruitment of their ligands may also exert synergistic growth inhibition in cancer cells when combined with ACR. For instance, both valproic acid [16] and OSI-461 [50], a potent derivative of sulindac sulfone, enhance the ability of ACR to raise the cellular levels of RARβ, thereby markedly increasing the RARE and RXRE promoter activity and inducing apoptosis in the HCC cells. These findings suggest that, among the nuclear receptors, RARβ is considered the most preferable heterodimeric partner for RXRα in ACR-based combination chemoprevention.

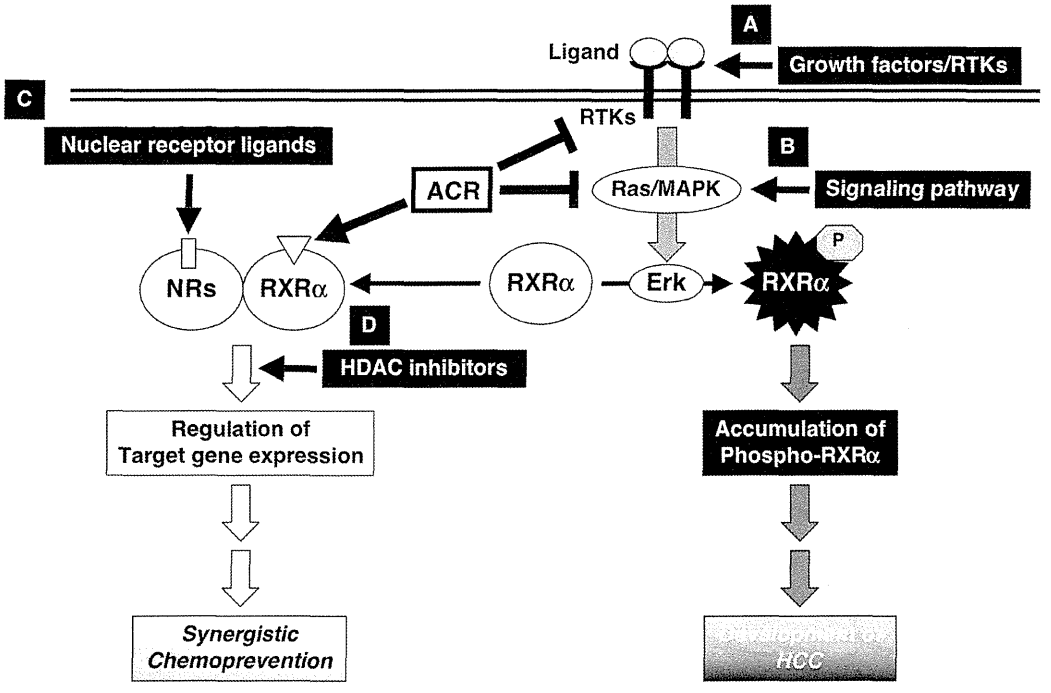

Fig. (5). "Combination chemoprevention" for HCC using ACR as the key agent. Dephosphorylation of RXRα and subsequent restoration of the function of this nuclear receptor are critical to prevent HCC development. Therefore, agents that target growth factors and their corresponding RTKs (**A**), as well as their related signaling pathways (**B**), including the Ras-MAPK that phosphorylates RXRα, may be good candidates to synergize with ACR for the chemoprevention of HCC. The ligands for the nuclear receptors (NRs) that heterodimerize with RXRs, such as RARs and PPARs (**C**), are also capable of enhancing the chemopreventive effect of ACR through the activation of target gene expression. HDAC inhibitors increase the expression of ACR-target genes by remodeling the chromatin template and increasing histone acetylation, suggesting that the combination of ACR and HDAC inhibitors may be a promising regimen for HCC chemoprevention (**D**).

FUTURE PROSPECTS OF ACR – 2: PREVENTION OF OBESITY-RELATED LIVER CARCINOGENESIS BY ACR

Recent studies have revealed that the risk of HCC is elevated in patients with metabolic syndrome, which is commonly associated with obesity and diabetes mellitus [20, 71, 72]. Several pathophysiological mechanisms linking obesity and liver carcinogenesis have been shown, including the emergence of insulin resistance and the subsequent inflammatory cascade [73]. Therefore, patients with obesity and insulin resistance comprise a high-risk group for HCC, and thus, are regarded as considerable targets for HCC chemoprevention. On the other hand, improving metabolic abnormalities such as insulin resistance and chronic inflammation by nutritional or pharmaceutical intervention might be an effective and attractive strategy to inhibit obesity-related liver carcinogenesis [74]. We have reported that the administration of branched chain amino acids (BCAA), which are used to improve protein malnutrition in patients with liver cirrhosis, and (-)-epigallocatechin-3-gallate, which is a major biologically active component of green tea, significantly prevents liver tumorigenesis in obese and diabetic *db/db* mice by targeting insulin resistance and improving chronic inflammation [75, 76]. A recent clinical trial also revealed that supplementation of food with BCAA reduced the risk of HCC in obese patients with chronic viral liver disease [72].

ACR effectively prevents the development of obesity-related liver carcinogenesis by inhibiting the activation of Ras and phosphorylation of ERK and RXRα in the liver of DEN-treated *db/db* mice. In this study, ACR administration also improved liver steatosis and insulin sensitivity, while attenuating the chronic inflammation induced by excessive fatty deposits [17]. Obesity and metabolic syndrome are the major healthcare problems in the present society, and the influences of metabolic abnormalities, in particular, such as promotion of cancers including HCC, are critical issues awaiting resolution. The results of this preclinical experiment [17] may encourage the clinical use of ACR for cirrhotic patients with obesity and diabetes, who are at a notably higher risk of developing HCC [20, 71, 72].

CONCLUSION

HCC prevention is an urgent issue demanding attention across the globe. One of the most practical approaches to prevent HCC is realization of the concept of "clonal deletion," and ACR is a promising candidate for this purpose because it may accomplish this concept. Experimental studies strongly suggest that RXRα phosphorylation is profoundly involved in liver carcinogenesis, and ACR mainly exerts chemopreventive effects on HCC *via* inhibition of RXRα phosphorylation. ACR-based combination chemoprevention, which is expected to exert synergism, holds a great possibility to perform a central role in HCC chemoprevention. Obese individuals, who are at an increased risk for HCC, might also be critical targets of ACR to prevent the development of this malignancy.

CONFLICT OF INTEREST

The authors declare that no conflicts of interest exist.

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ABBREVIATIONS

ACR	=	acyclic retinoid
AFP-L3	=	lectin-reactive α-fetoprotein factor 3
BCAA	=	branched chain amino acids
DEN	=	diethylnitrosamine
ERK	=	extracellular signal-regulated kinase
GSK-3β	=	glycogen synthase kinase-3β
HBV	=	hepatitis B virus
HCC	=	hepatocellular carcinoma
HCV	=	hepatitis C virus
HDAC	=	histone deacetylase
HER2	=	human epidermal growth factor receptor-2
IFN	=	interferon
MAPK	=	mitogen-activated protein kinase
PIVKA-II	=	protein induced by vitamin K absence or antagonist-II
PPAR	=	peroxisome proliferator-activated receptor
RA	=	retinoic acid
RAR	=	retinoic acid receptor
RARE	=	retinoic acid receptor responsive element
RTK	=	receptor tyrosine kinase
RXR	=	retinoid X receptor
RXRE	=	retinoid X response element
VK_2	=	vitamin K_2

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Review Article

Acyclic retinoid in chemoprevention of hepatocellular carcinoma: Targeting phosphorylated retinoid X receptor-α for prevention of liver carcinogenesis

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Abstract

One of the key features of hepatocellular carcinoma (HCC) is the high rate of intrahepatic recurrence that correlates with poor prognosis. Therefore, in order to improve the clinical outcome for patients with HCC, development of a chemopreventive agent that can decrease or delay the incidence of recurrence is a critical issue for urgent investigation. Acyclic retinoid (ACR), a synthetic retinoid, successfully improves HCC patient survival by preventing recurrence and the formation of secondary tumors. A malfunction of the retinoid X receptor-α (RXRα) due to phosphorylation by the Ras-MAPK signaling pathway plays a critical role in liver carcinogenesis, and ACR exerts chemopreventive effects on HCC development by inhibiting RXRα phosphorylation. Here, we review the relationship between retinoid signaling abnormalities and liver disease, the mechanisms of how RXRα phosphorylation contributes to liver carcinogenesis, and the detailed effects of ACR on preventing HCC development, especially based on the results of our basic and clinical research. We also outline the concept of "clonal deletion and inhibition" therapy, which is defined as the removal and inhibition of latent malignant clones from the liver before they expand into clinically detectable HCC, because ACR prevents the development of HCC by implementing this concept. Looking toward the future, we discuss "combination chemoprevention" using ACR as a key drug since it can generate a synergistic effect, and may thus be an effective new strategy for the prevention of HCC.

Keywords: Acyclic retinoid, chemoprevention, clonal deletion and inhibition, combination therapy, HCC, RXRα phosphorylation

INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most frequently occurring cancers in the world. The number of new cases per year is estimated to be 750,000; approximately the same number (700,000) of people die from this malignancy each year.[1,2] Chronic inflammation and subsequent cirrhosis of the liver, most cases of which are induced by persistent infection with hepatitis B virus (HBV) or hepatitis C virus (HCV), contribute to the development of HCC.[2,3] In order to improve the prognosis of HCC, several effective strategies to prevent the development of primary HCC and intrahepatic recurrence of this malignancy have been demonstrated in clinical trials. A meta-analysis reported that antiviral treatment reduces the risk of HBV-related HCC recurrence and decreases liver-related mortality as well as overall mortality.[4]

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For patients with HCV-related HCC, the effectiveness of interferon therapy for preventing the recurrence of HCC has been shown by meta-analyses.[5,6]

In addition to studies of antiviral treatment, other important trials using specific agents have been conducted to seek ways for preventing HCC development. For instance, supplementation with branched-chain amino acids reduces the risk of HCC in cirrhotic patients who are obese.[7] We reported the results of our prospective randomized study,[8-10] in which the oral administration of acyclic retinoid (ACR), a synthetic retinoid, inhibited the development of a second primary HCC and thus improved patient survival. The pleiotropic effects of ACR in the prevention of HCC and suppression of cancer cell growth have also been revealed in many experimental studies.[11-13] The aim of this article is to review the detailed effects of ACR in preventing the development of HCC, based on our clinical and basic research. In particular, we focus on the effects of ACR in targeting the phosphorylation of retinoid X receptor-α (RXRα), because malfunction of this nuclear receptor due to aberrant phosphorylation is closely involved in liver carcinogenesis.[14] We also review the concept of "clonal deletion and inhibition" therapy, a practical approach to preventing HCC development. Finally, we discuss the possibility of "combination chemoprevention," which uses ACR as a key drug and is expected to be an effective strategy that takes advantage of pharmacologic synergism to prevent the formation of HCC.

Retinoids and their receptors

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.[15-17] Retinoids exert their biologic functions primarily by regulating gene expression through two distinct nuclear receptors, the retinoic acid receptors (RARs) and RXRs, which are ligand-dependent transcription factors. Both RARs and RXRs are composed of three subtypes (α, β, and γ), which are characterized by a modular domain structure.[15-17] RARs can be activated by both all-*trans*-retinoic acid and 9-*cis*-retinoic acid with similar affinities, whereas RXRs are exclusively activated by 9-*cis*-retinoic acid.

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.[15-17] In addition to forming a heterodimer with RARs, RXRs can also form heterodimers with several other nuclear receptors, indicating that RXRs act as common heterodimerization partners for various types of nuclear receptors.[16] For instance, RXRs interact with peroxisome proliferator-activated receptors (PPARs), which are receptors for fatty acids, thus regulating PPAR-mediated pathways and controlling energy homeostasis.[18] Therefore, RXRs function as auxiliary factors, determining the effects of other hormones, and acting as master regulators of nuclear receptors.[16]

Retinoid abnormalities and liver disease

The liver is one of the most important target organs for retinoid actions. Hepatocytes play central roles in the uptake and processing of dietary retinol in the liver and secrete retinol-binding protein into the plasma. Hepatic stellate cells (HSCs) are critically involved in the storage of retinoids in the liver, suggesting that the development of hepatic disease is highly correlated with impaired hepatic retinoid metabolism and storage.[19,20] In alcoholic patients, diminished hepatic retinoid storage is associated with progressively worsening stages of hepatic disease.[21] The loss of hepatic retinyl ester stores from the lipid droplets within HSCs leads to HSC activation and the development of liver fibrosis.[22,23] A progressive decrease in serum retinol levels has also been observed in patients diagnosed with liver cirrhosis compared with healthy subjects, and those patients with both cirrhosis and HCC had significantly lower levels than patients with cirrhosis alone.[24,25]

Several experimental studies using genetically engineered mice have revealed the pivotal effects of retinoids on fat metabolism in the liver. Yanagitani *et al.*[26] reported that RARα dominant-negative form transgenic mice developed steatohepatitis through the downregulation of hepatic mitochondrial β-oxidation activity of fatty acids. Studies in hepatocyte RXRα-deficient mice also demonstrated that RXRα plays vital roles in fatty acid and cholesterol metabolism in the liver.[27,28] These observations suggest that retinoids and their receptors are involved in the mediation of normal hepatic lipid metabolism.

Retinoid abnormalities and liver carcinogenesis

As previously noted, retinoids and their receptors, especially RXRs, play an essential role in controlling normal cell proliferation, differentiation, metabolism, and death (regulation of apoptosis).[15-17] On the other hand, these facts also suggest that the loss of retinoid activity or responsiveness is linked to deviation from normal cell proliferation and death, which are key factors for cancer development.[29,30] Indeed, it is well established that abnormalities in retinoid signaling are prominently involved in carcinogenesis in several organs, including the liver. In a rodent model of liver carcinogenesis,[31] retinol was observed to be locally deficient

in HCC lesions, but not in the adjacent normal liver tissues. Functional loss of retinoic acid leads to the occurrence of cellular dysplasia and cancer in the liver.[26] Overexpression of cellular retinoic acid-binding protein-II, which shows a high affinity for all-*trans*-retinoic acid, is associated with induction of retinoic acid resistance in HCC cells.[32] On the other hand, lecithin:retinol acyltransferase knockout mice, which possess increased retinoid signaling in the liver, are less susceptible to diethylnitrosamine (DEN)-induced hepatocarcinogenesis.[33] These findings suggest that supplementation with additional retinoids and improvement of retinoid signaling may be a promising strategy for the prevention and/or treatment of HCC.

In addition to retinoid depression, abnormalities in retinoid receptors are also associated with liver carcinogenesis and the growth of HCC cells. The *RARβ* gene, a tumor suppressor gene, is an HBV integration site, and the expression of this gene is markedly decreased in human HCC.[34,35] RARβ expression is also suppressed in liver cancer cell lines.[36,37] We have previously reported that the levels of RARβ protein and mRNA were decreased in HCC lesions in a rat model of chemically induced liver carcinogenesis.[38] On the other hand, RARγ is overexpressed in HCC tissues and cells, which is associated with the growth of HCC cells.[39] It has been reported that RARγ often resides in the cytoplasm of HCC cells and interacts with the p85α regulatory subunit of phosphatidylinositol 3-kinase, resulting in the activation of Akt and nuclear factor-κB, which are critical regulators of the growth and survival of cancer cells.[39,40] These reports support an oncogenic potential for RARγ in liver carcinogenesis.

RXRα phosphorylation and HCC

Among retinoid receptors, RXRα is most abundant in the liver; therefore, its alterations are particularly implicated in the development and progression of HCC. RXRα is reported to bind to the enhancer element of HBV and modulate viral replication.[41] 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, as seen in a rat model of chemically induced liver carcinogenesis.[38] These findings indicate that repression of RXRα occurs even in the early stage of liver carcinogenesis.

Moreover, we have shown that aberrant phosphorylation of RXRα is critically involved in liver carcinogenesis. Initially, we revealed that the RXRα protein is anomalously phosphorylated at the serine and threonine residues and that it was seen to accumulate in both surgically resected human HCC tissues and HCC cell lines, whereas in normal hepatocytes, RXRα is unphosphorylated and is broken into smaller peptides.[14,42] We previously reported that the phosphorylated form of RXRα protein was present in higher concentrations in HCC tissues than in noncancerous surrounding and normal liver tissues among all 10 cases examined.[14] 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 an MAPK inhibitor or transfection with unphosphomimic mutant RXRα restores the degradation of RXRα in a ligand-dependent manner.[14,43] Phosphorylated RXRα abolishes its ability to form heterodimers with RARβ, and this is associated with uncontrolled cell growth and resistance to retinoids.[44] Moreover, phosphorylated RXRα is resistant to proteolytic degradation via the ubiquitination/proteasome-mediated pathway in human HCC cells, resulting in an accumulation of this phosphorylated protein within the HCC tissues.[45] Therefore, in HCC tissues and cells, the accumulation of phosphorylated RXRα, which is regarded as the nonfunctional form of RXRα, may interfere with the function of normal (unphosphorylated) RXRα in a dominant-negative manner. Our observations suggest that not only retinoid depletion, but also malfunction of retinoid receptors, especially phosphorylation of RXRα, may play a critical role in HCC development [Figure 1].

Mechanisms of ACR in HCC chemoprevention

ACR, which is the same substance as NIK-333 and Peretinoin (Kowa Pharmaceutical Co., Tokyo, Japan), inhibits both chemically induced liver carcinogenesis in rats and mice and spontaneously occurring HCC in mice.[31,46-49] ACR was initially developed as an agonist for both RXR and RAR.[50,51] Therefore, this agent activates the promoter activity of RXRE and RARE and controls the expression of target genes, including RARβ, $p21^{CIP1}$, and cyclin D1, which results in induction of apoptosis, cell cycle arrest in G_0–G_1, and growth inhibition in human HCC-derived cells.[43,52-62] These findings suggest that ACR suppresses HCC, at least in part, by working as a ligand for retinoid receptors and controlling their target genes, especially RARβ and $p21^{CIP1}$.

On the other hand, many experimental studies have shown that ACR exerts chemopreventive effects in HCC cells by inhibiting RXRα phosphorylation. In human HCC cells, ACR restores RXRα function by inactivating the Ras–MAPK signaling system, leading to dephosphorylation of RXRα.[43] ACR also suppresses cancer cell growth by inhibiting the activation and expression of several