auto KAINOS; KAINOS Laboratories, Inc., Ammonia-L; Serotec Co., Ltd., respectively). PT was measured by automated coagulation analyzer (ACL-TOP, Mitsubishi Kagaku Iatron, Inc.) using a commercial kit (RecombiPlasTin, Instrumentation Laboratory, Ltd.). Plasma amino acids were measured by high performance liquid chromatography on Amino acid analyzer (L-8500, Hitachi, Ltd.).

Measurement of D_2 binding sites and designation of ROIs

A SET-1400W10 PET scanner (Shimadzu, Japan, at the Cyclotron Research Center, Iwate Medical University) was used for the study. Static scans were obtained for 80 min after intravenous injection of 75 Bq of ^{11}C-*N*-MSP. Regional brain site levels were divided by those in the D_2 receptor-free cerebellum. The pixel values in twelve ROIs corrected for the pixel value of the cerebellum were measured to determine dopamine D_2 receptor binding.

ROIs were analyzed using three dimensional stereotaxic ROI template (3DSRT). 3DSRT is a fully automated ROI-based analysis program whereby ROIs grouped into 12 segments are designated on each hemisphere of the brain by computer processing, and the use of this template program has been shown to allow objective and reproducible assessment of SPECT data in the designated ROIs. The template is described in detail elsewhere (Takeuchi et al. 2002). A typical image using ^{11}C-*N*-MSP PET with 3DSRT in a healthy control subject is presented in Fig. 1.

Statistical analysis

Mann–Whitney's *U* test was employed for testing the significant of the differences between subgroups of cirrhotic patients. Correlation analyses were carried out for the biochemical data, prothrombin data, amino acid analyses, dopamine D_2 receptor binding as well as other laboratory parameters (Alb, T.Bil, NH_3, PT%, Plt, Tyr, Phe, BCAA), Child–Pugh scores and age. For analysis of continuous variables, Pearson's correlation coefficient was used, and $p<0.05$ was considered to indicate statistical significance. For the two continuous variables which cannot be considered to follow a normal distribution (T.Bil and NH_3) values were log-transformed prior to the analysis. For discrete variables (Child–Pugh score and age), Spearman's rank and sum correlation coefficient was determined, and $p<0.05$ was considered to indicate statistical significance.

For multivariate analysis, linear multiple regression analysis was carried out, with the binding of dopamine D_2 receptors in each ROI of the brain serving as the dependent variable. The following were adopted as independent variables based on the results of the simple correlation analysis: Alb, T.Bil, NH_3, PT%, Plt, Tyr, Phe and BCAA, age, sex, history of alcohol dependence, encephalopathy and ascites. For multivariate analysis, dummy variables were incorporated into the history of encephalopathy, history of ascites and gender of the patients.

Statistical analyses were carried out with Dr SSPS II for Windows 11.0.1.J standard version (SPSS Inc.).

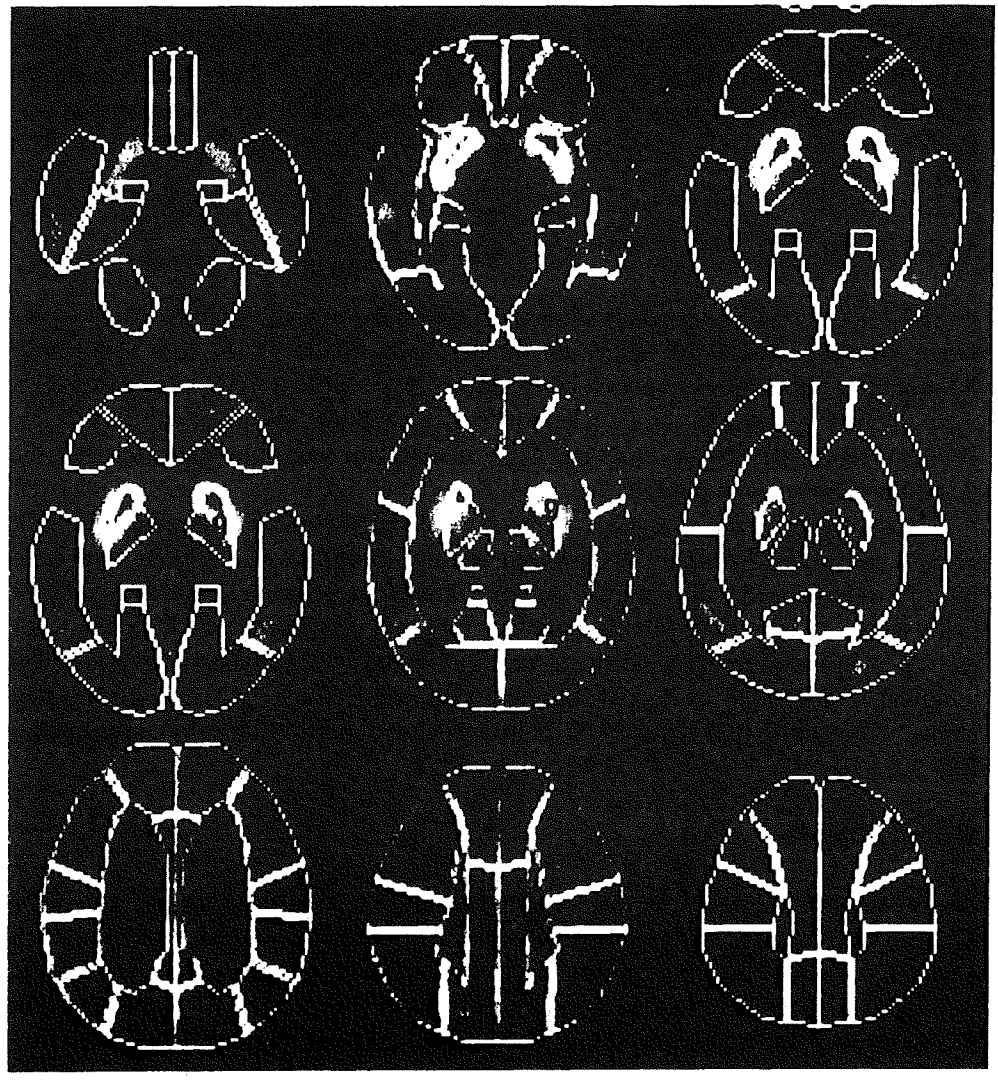

Fig. 1 Positron emission computed tomography. (PET) images using 11C-N-methylspiperone in a healthy volunteer subject with ROI delineation (*white*) by 3DSRT. Number (*red*) shows each region of the brain. 1: Callosomarginal, 2: Precentral, 3: Central, 4: Parietal, 5: Angular, 6: Temporal, 7: Occipital, 8: Pericalloral, 9: Lenticular Nucleus, 10: Thalamus, 11: Hippocampus

Results

Dopamine D_2 receptor binding in each ROI of the brain in healthy controls and liver cirrhosis patients

The dopamine D_2 receptor binding was lower in cirrhotic patients compared to that in the healthy controls in all ROI's investigated and in the thalamus and hippocampus these reductions were statistically significant (Table 2).

D_2 receptor binding was also lower in cirrhotic patients with a history of alcohol dependence where, again, the difference was statistically significant in thalamus

Table 2 Comparison of regional brain dopamine D_2 receptor binding in cirrhotic patients and healthy controls

Region of interest	D_2 receptor binding		p
	Cirrhosis (n=28)	Control (n=3)	
Callosomarginal	0.71±0.15	0.81±0.17	0.24
Precentral	0.90±0.15	1.00±0.18	0.26
Central	0.36±0.23	0.50±0.27	0.23
Parietal	0.36±0.26	0.45±0.20	0.26
Angular	0.63±0.48	0.80±0.59	0.23
Temporal	1.07±0.09	1.16±0.09	0.09
Occipital	0.93±0.23	1.03±0.18	0.42
Pericallosal	0.66±0.29	0.72±0.27	0.18
Lenticular nuclei	1.74±0.21	1.96±0.18	0.12
Thalamus	0.86±0.13*	1.11±0.11	0.01
Hippocampus	0.88±0.10*	1.04±0.05	0.01

Values are means±SD
*p<0.01 by Mann–Whitney U test, significantly different from control

(p<0.01) and hippocampus (p<0.02) (Table 3). Dopamine D_2 receptor binding was higher in all ROI in cirrhotic patients with history of overt HE and the difference was statistically significant for the pericallosal region and the hippocampus (Table 4). On the other hand, receptor binding between cirrhotic patients with a history of ascites was not significantly different from patients with no such history (data not shown).

Correlations between D_2 binding sites and clinical biochemical data in cirrhotic patients

Simple correlation analysis was performed in the 28 patients except for plasma amino acid analysis where data was unavailable in nine of these patients.

Table 3 Brain D_2 receptor binding in cirrhotic patients with or without alcohol dependence

Region of interest	History of alcohol dependence		p
	Yes (n=13)	No (n=15)	
Callosomarginal	0.68±0.11	0.73±0.17	0.39
Precentral	0.86±0.16	0.93±0.15	0.45
Central	0.30±0.20	0.41±0.26	0.32
Parietal	0.29±0.22	0.41±0.28	0.28
Angular	0.51±0.52	0.73±0.45	0.28
Temporal	1.04±0.08	1.10±0.09	0.15
Occipital	0.85±0.24	1.01±0.20	0.11
Pericallosal	0.56±0.30	0.74±0.26	0.20
Lenticular nuclei	1.69±0.16	1.78±0.24	0.27
Thalamus	0.80±0.10*	0.92±0.14	0.01
Hippocampus	0.82±0.07*	0.92±0.10	0.02

Values are means±SD
*Significantly different from patients with no history of alcohol dependence by Mann–Whitney U test (p values shown in right hand column)

Table 4 Brain D_2 receptor binding in cirrhotic patients with or without history of overt hepatic encephalopathy

Region of interest	History of overt hepatic encephalopathy		p
	Yes (n=6)	No (n=22)	
Callosomarginal	0.81±0.24	0.68±0.10	0.16
Precentral	1.00±0.18	0.87±0.14	0.07
Central	0.55±0.35	0.31±0.17	0.07
Parietal	0.57±0.37	0.30±0.19	0.06
Angular	0.98±0.31	0.53±0.49	0.12
Temporal	1.13±0.11	1.06±0.08	0.15
Occipital	1.12±0.20	0.88±0.22	0.12
Pericallosal	0.92±0.29*	0.59±0.25	0.02
Lenticular nuclei	1.89±0.24*	1.70±0.18	0.05
Thalamus	0.96±0.19	0.83±0.11	0.12
Hippocampus	0.97±0.12*	0.85±0.08	0.02

Values are means±SD
*Significantly different from patients with no history of hepatic encephalopathy by Mann–Whitney U test (p values shown in right hand column)

Dopamine D_2 receptor binding in thalamus and hippocampus showed significant positive correlations with serum total bilirubin and negative correlations with prothrombin times. D_2 receptor binding in thalamus was positively correlated with serum phenylalanine levels and Child–Pugh scores whereas D_2 receptor binding in callosomarginal, precentral, central, parietal, occipital, pericallosal cortices and hippocampus showed significant positive correlations with the age of the patient (Table 5). Using multiple linear regression analysis, D_2 receptor binding in hippocampus was significantly associated with two variables (plasma tyrosine concentrations and a history of HE). D_2 receptor binding in thalamus revealed a

Table 5 Correlations between clinical and laboratory parameters and D_2 receptor binding in cirrhotic patients

Variables	Region of Interest	Correlation coefficient	P
Child–Pugh scores[a]	Thalamus	0.452	0.016
Serum bilirubin	Thalamus	0.431	0.022
	Hippocampus	0.419	0.027
Prothrombin time activity	Thalamus	−0.480	0.010
Phenylalanine (plasma)	Thalamus	0.457	0.049
Age[a]	Callosomarginal	0.408	0.031
	Precentral	0.439	0.019
	Central	0.419	0.027
	Parietal	0.392	0.039
	Occipital	0.402	0.034
	Pericallosal	0.429	0.023
	Hippocampus	0.451	0.016

No significant correlations were observed between D_2 receptor binding and serum albumin, ammonia, tyrosine, branched chain or aromatic amino acids or platelet count.
Order Correlation coefficient. Association of other continuous variables to cerebral D_2 receptor binding activities was examined by Pearson's correlation coefficient
[a] Association of Age and Child–Pugh scores to D_2 receptor binding was examined by Spearman Rank

significant positive correlation with plasma phenylalanine and a negative correlation with the plasma levels of BCAA. D_2 receptor binding in the lenticular nuclei was correlated with prothrombin time.

Discussion

The present study is the first to describe using PET significant alterations of binding sites for ^{11}C-*N*-MSP in the brains of cirrhotic patients indicative of alterations of dopamine D_2 receptors in these patients. Binding site densities were found to be heterogeneous in distribution with up to five-fold differences observed between lenticular nuclei (known to be rich in D_2 receptors) and other cortical and subcortical structures. ^{11}C-*N*-MSP binding sites in thalamus and hippocampus of cirrhotic patients were significantly reduced compared to healthy controls. A previous study using SPECT and ^{123}I-iodobenzamide as ligand revealed decreased D_2 receptor sites in striatum of a cirrhotic patient (Weissenborn et al. 2000). However, in contrast to the patient population in the present study in which no patients had overt neurological symptoms, the patient studied by SPECT showed clear extrapyramidal symptoms. Earlier neurochemical studies in autopsied brain tissue from cirrhotic patients who died in hepatic coma also showed a significant loss of D_2 sites in globus pallidus/putamen (Mousseau et al. 1993) and it was suggested that loss of these sites could be the consequence of manganese deposition in the brains of these patients (Spahr et al. 1996). Manganese accumulation is the most likely cause of T_1-weighted signal hyper-intensities observed in pallidum by magnetic resonance imaging (Pomier-Layrargues et al. 1995) and such signal hyperintensities were observed in all patients enrolled in the present study. However, findings from the present study of a lack of decrease in D_2 sites in lenticular nuclei do not support the notion of a toxic effect of manganese on D_2 sites suggesting that the toxic effects of manganese occur at later stages of liver decompensation associated with overt encephalopathy. Findings from the present study of a selective loss of thalamic D_2 sites in cirrhotic patients could be expected to lead to altered levels of brain excitability and relate to previous PET findings of increased cerebral blood flow and glucose utilization in thalamus of similar patients (Lockwood et al. 1991).

Decreased D_2 binding site decreases were found to be more severe in alcoholic cirrhotic patients compared to non alcoholic cirrhotics suggesting that alcohol (or one of its metabolites) could play a contributory role. A substantial body of evidence suggests that the brain dopamine system is implicated in the central nervous system effects of alcohol (Heinz et al. 2004) and results of a previous SPECT study suggest a role for decreased D_2 binding sites in alcohol dependence (Ebert et al. 2002). In the autopsy study of Mousseau et al. (1993), showing loss of D_2 sites, the etiology of cirrhosis was alcoholic in all cases. Taken together, these findings strongly suggest that exposure to alcohol (or its metabolites) in addition to liver-derived toxins contributes to the loss of D_2 sites in the brains of alcoholic cirrhotics.

In contrast to the apparent effects of alcohol, a previous history of overt HE did not result in greater decreases of D_2 sites. On the contrary, D_2 binding sites were significantly increased in lenticular nuclei, pericallosal area and hippocampus of patients who manifested previous episodes of HE compared to those patients who

did not. This apparently counter-intuitive finding might suggest the presence of an alternative or additional mechanism to explain the pathogenesis of HE in these patients. On the other hand, these finding could relate to decreased synaptic concentrations of dopamine resulting in upregulation of postsynaptic D_2 receptors in the brains of these patients. Studies in autopsied brain tissue from cirrhotic patients who died in hepatic coma provide evidence for a dopamine deficit; such evidence includes increased activities of monoamine oxidase (the enzyme responsible for dopamine degradation) (Mousseau et al. 1997) and increased brain concentrations of dopamine metabolites (Bergeron et al. 1989). The notion of dopaminergic deficit has clinical correlates in HE. Both the dopamine precursor amino acid and L-DOPA (Lunzer et al. 1974) and the dopamine receptor agonist bromocriptine (Morgan et al. 1980) improve the motor coordination and performance in speed-based psychometric tests in patients with chronic HE.

The magnitude of D_2 sites in thalamus of cirrhotic patients in the present study were significantly correlated with indices of severity of liver failure such as Child–Pugh scores, serum bilirubin and prothrombin times. No significant correlations were observed between D_2 site densities and serum ammonia, albumin or amino acids with the exception of phenylalanine (correlation $p<0.05$ with D_2 sites). Phenylalanine is a precursor amino acid for the synthesis of catecholamines. However, in the case of dopamine, the rate-limiting enzyme is tyrosine hydroxylase and results of the present study showed no correlation between D_2 sites and circulating levels of tyrosine. It is unlikely therefore that alterations of D_2 sites in the brain of cirrhotic patients is a consequence of altered availability of dopamine precursor amino acids.

D_2 binding sites in cirrhotic patients showed a clear correlation with patient age in both cortical and hippocampal structures, a finding that could relate to loss of cholinergic neurons in these brain structures that are known to express D_2 receptors (Finch and Roth 1999). A greater loss of D_2 sites from these neurons could explain the more severe cognitive dysfunction that is observed in older cirrhotic patients following portal decompression by TIPS or in aged portacaval-shunted animals (Audet and Butterworth 1998). If confirmed, these findings might indicate a potentially beneficial effect of D_2 receptor agonists in this population of patients.

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Review Article

Strategy and mechanism for the prevention of hepatocellular carcinoma: Phosphorylated retinoid X receptor α is a critical target for hepatocellular carcinoma chemoprevention

Masahito Shimizu,[1] Koji Takai and Hisataka Moriwaki

Department of Medicine, Gifu University Graduate School of Medicine, Gifu, 501-1194 Japan

(Received October 16, 2008/Revised October 22, 2008/Accepted October 23, 2008/Online publication December 5, 2008)

Hepatocellular carcinoma (HCC) is a major health care problem worldwide. The prognosis of patients with HCC is poor because even in the early stages when surgical treatment might be expected to be curative, the incidence of recurrence in patients with underlying cirrhosis is very high due to multicentric carcinogenesis. Therefore, strategies to prevent recurrence and second primary HCC are required to improve the prognosis. One of the most practical approaches to prevent the multicentric development of HCC is 'clonal deletion' therapy, which is defined as the removal of latent (i.e. invisible) (pre)malignant clones from the liver in a hypercarcinogenic state. Retinoids, a group of structural and functional analogs of vitamin A, exert their biological function primarily through two distinct nuclear receptors, retinoic acid receptors and retinoid X receptors (RXR), and abnormalities in the expression and function of these receptors are highly associated with the development of various cancers, including HCC. In particular, a malfunction of RXRα due to phosphorylation by the Ras–mitogen-activated protein kinase signaling pathway is profoundly associated with the development of HCC and thus may be a critical target for HCC chemoprevention. Acyclic retinoid, which has been clinically shown to reduce the incidence of a post-therapeutic recurrence of HCC, can inhibit Ras activity and phosphorylation of the extracellular signal-regulated kinase and RXRα proteins. In conclusion, the inhibition of RXRα phosphorylation and the restoration of its physiological function as a master regulator for nuclear receptors may be a potentially effective strategy for HCC chemoprevention and clonal deletion. Acyclic retinoid, which targets phosphorylated RXRα, may thus play a critical role in preventing the development of multicentric HCC. (*Cancer Sci* 2009; 100: 369–374)

Hepatocellular carcinoma is the fifth most common cancer worldwide and the third most common cause of cancer mortality. HCC is unique in that it usually occurs within an established background, chronic liver disease and cirrhosis. The development of HCC is frequently associated with chronic inflammation of the liver induced by a persistent infection with hepatitis B virus or hepatitis C virus. Therefore, this cancer is a major health care problem in Eastern as well as Western countries where hepatitis virus infection is endemic.[1,2] Patients with viral liver cirrhosis are a high-risk group for HCC because the annual rate for this cancer in those patients is approximately 7%. Even in the early stages when surgical treatment might be expected to be curative, the incidence of recurrence in patients with underlying cirrhosis is approximately 20–25% a year. Therefore, the recurrence rate at 5 years after curative treatment may exceed 70%.[3–6] In addition, at least one-third of secondary tumors are primary *de novo* cancers.[7] Based on these clinical characteristics, the prognosis of patients with HCC is poor. Thus, it is a task of pressing urgency to develop more effective strategies for the chemoprevention of HCC and, for this purpose, there is a critical need to elucidate the molecular mechanisms underlying liver carcinogenesis.

Cancer chemoprevention is defined as an approach where a natural or synthetic chemical compound works to arrest or reverse premalignant cells by using physiological pathways.[8] We previously reported that, in a clinical trial, the administration of ACR, a novel synthetic retinoid (Fig. 1), reduced the incidence of post-therapeutic HCC recurrence and improved the survival rate of patients.[9–11] We have also revealed that a malfunction of RXRα, a nuclear retinoid receptor, due to aberrant phosphorylation is associated with carcinogenesis in the liver.[12–14] The aim of the present paper is to review the evidence that ACR exerts its chemopreventive effects on the development of HCC by targeting p-RXRα. In addition, the concept of 'clonal deletion', which is one of the most practical approaches to preventing multicentric HCC development, is reviewed and the possibility of 'combination chemoprevention', which uses ACR as a key drug and might be an effective strategy to prevent this malignancy by pharmacological synergism, is discussed.

Retinoids and their receptors

Retinoids, a group of structural and functional analogs of vitamin A, exert fundamental effects on the regulation of epithelial cell growth, differentiation, and development.[15,16] A small portion of dietary retinoids is converted to RA, which is an active metabolite of retinoids. Retinoids exert their biological functions primarily by regulating gene expression through two distinct nuclear receptors, the RXR and the RAR, which are both composed of three subtypes (α, β, and γ) that are characterized by a modular domain structure. RXR is specific for 9-*cis* RA, whereas RAR binds both 9-*cis* RA and all-*trans*

[1]To whom correspondence should be addressed. E-mail: shimim-gif@umin.ac.jp

Abbreviations: ACR, acyclic retinoid; AFP-L3, lectin-reactive α-fetoprotein isoform 3; Erk, extracellular signal-regulated kinase; HCC, hepatocellular carcinoma; 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; p-RXR, retinoid X receptor; RA, retinoic acid; RAR, retinoic acid receptor; RTK, receptor tyrosine kinase; RXR, retinoid X receptor; VK_2, vitamin K_2.

doi: 10.1111/j.1349-7006.2008.01045.x

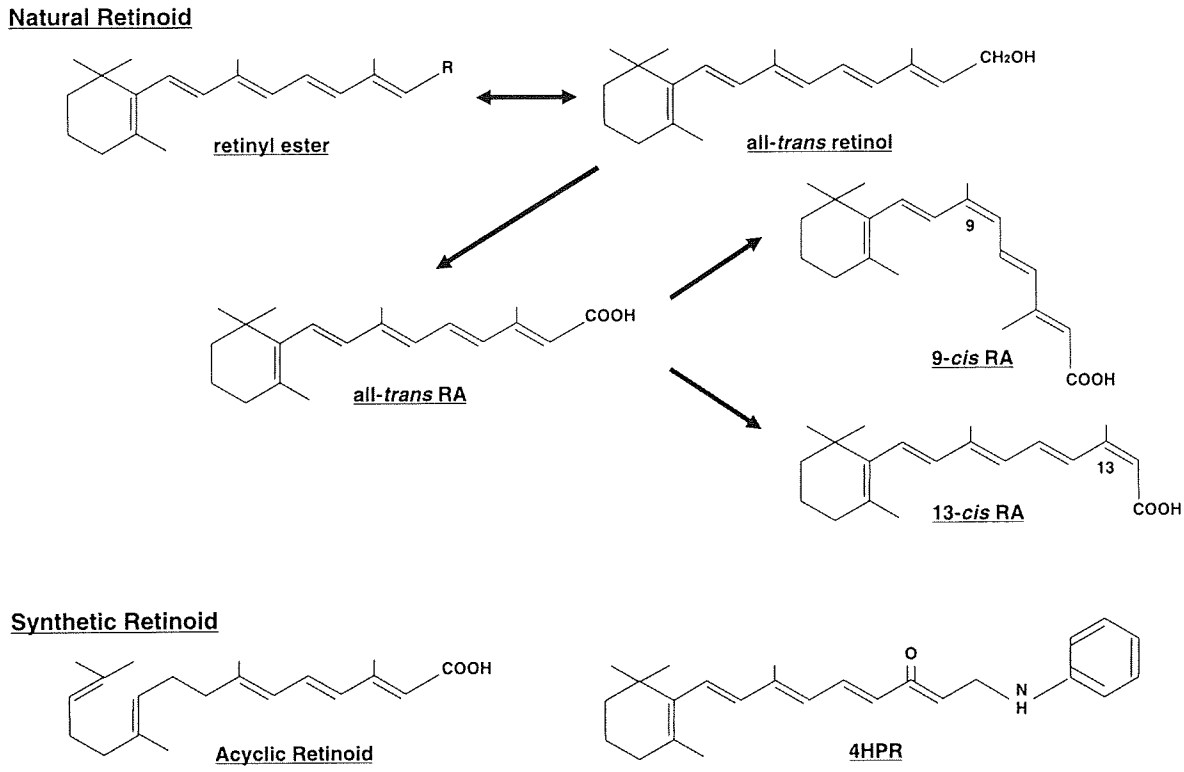

Fig. 1. Chemical structures of natural and representative synthetic retinoids. Retinyl esters (mainly retinyl palmitate; R, fatty acid), stored in the liver stellate cells, are hydrolyzed to retinol, which is then transported to target cells through the circulation after binding to retinol-binding protein. Retinoic acid (RA) is biosynthesized from retinol via the intermediate metabolite retinal by oxidization in the cells of peripheral tissues. Three well-known isomers of RA, all-*trans* RA, 9-*cis* RA, and 13-*cis* RA activate the retinoid receptor retinoic acid receptor (RAR), whereas only 9-*cis* RA activates the other receptor, retinoid X receptor (RXR). A number of synthetic retinoids have been developed to carry out their pharmacological applications including cancer chemoprevention. Acyclic retinoid and *N*-(4-hydroxyphenyl) retinamide (4HPR) successfully prevented the development of hepatocellular carcinoma and breast cancer, respectively, in clinical trials (see review reference[27]).

retinoic acid (Fig. 1). Nuclear retinoid receptors are ligand-dependent transcription factors. After ligand binding, RXR form a homodimer as well as heterodimer with RAR, which interacts with the retinoid X response element or the RAR responsive element located in the promoter region of the target genes, thereby modulating gene expression. RXR also form a heterodimer with other nuclear receptors, such as peroxisome proliferator-activated receptor.[17] Among the retinoid receptors, RXRα is thought to be one of the most important receptors with respect to regulation of fundamental cell activities, including normal cell proliferation and metabolism, and act as the master regulator of nuclear receptors.[15,16]

In addition to the binding of specific ligands, recent studies have also revealed that phosphorylation processes are crucial for regulating RAR- and RXR-mediated transcriptional activity.[18,19] For instance, the phosphorylation of RXRα at its N-terminal domain plays a role in the activation of a subset of RA-responsive genes and in the antiproliferative effect of RA, indicating that RXRα 'positively' regulates the transactivation of target genes through phosphorylation.[20] In contrast, there are some reports that show the phosphorylation of RXRα to 'negatively' modulate the function of its heterodimeric binding partners. Indeed, MAPK-mediated phosphorylation of the omega loop of the RXRα ligand binding domain impairs the transcriptional activity of RXR–RAR[12,21] and RXR–vitamin D_3 receptor[22,23] heterodimers. These 'negative' effects of RXRα via its phosphorylation might be associated with certain types of human diseases, including malignant disorders.[12,24–26]

Hepatocellular carcinoma and RXRα phosphorylation

Because retinoids and their receptors play an essential role in normal cell proliferation and differentiation, abnormalities in the expression and function of these molecules are highly associated with the development of various human malignancies and therefore might be critical targets for cancer chemoprevention and chemotherapy.[27] HCC is no exception in this concern. In the rodent model, we found that retinol was locally deficient in the HCC but not in the adjacent normal liver tissues and this was associated with aberrant metabolism of retinol.[28] The expression of RXRα was also decreased not only in HCC and adenoma, but also in glutathione S-transferase placental form-positive foci, a precancerous lesion of HCC, suggesting that the repression of RXRα occurs even in an early stage of liver carcinogenesis.[29]

In addition, we have previously shown that hepatocarcinogenesis is accompanied by the accumulation of the phosphorylated (i.e. inactivated) form of RXRα.[30] Specifically, RXRα protein is anomalously phosphorylated at serine and threonine residues, and accumulated both in human HCC tissue as well as in HCC cell lines.[12] Phosphorylation at serine 260 of RXRα, a consensus site of MAPK, is closely linked to its retarded degradation, low transcriptional activity, and the promotion of cancer cell growth. In addition, the abrogation of phosphorylation by MAPK-specific inhibitors restored the degradation of RXRα in a ligand-dependent manner.[12,31] Furthermore, in a normal liver and in non-proliferating hepatocyte cultures, RXRα is

doi: 10.1111/j.1349-7006.2008.01045.x

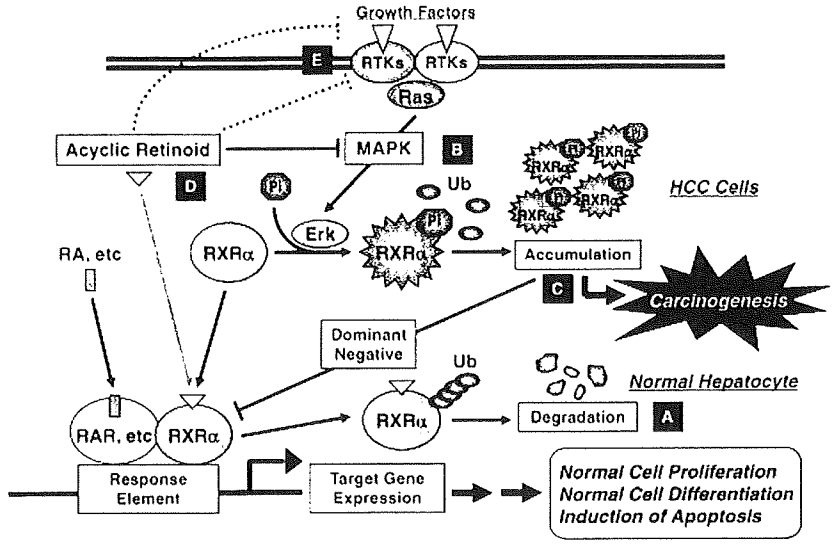

Fig. 2. Retinoid refractoriness due to phosphorylation of retinoid X receptor (RXR) α and its restoration by acyclic retinoid (ACR) in hepatocellular carcinoma (HCC) cells. In normal hepatocytes, when the ligand binds to and activates RXRα, the receptor becomes able to heterodimerize with other nuclear receptors, such as retinoic acid receptor (RAR), and then activates the expression of target genes, which may regulate normal cell proliferation and differentiation, by binding to the specific response elements. Thereafter, RXRα dissociates from the dimer, is ubiquitinated (Ub), and is degraded by the proteasome. The whole process from ligand binding to proteasomal breakdown of RXRα is estimated to take approximately 6 h (A).[14] In HCC cells, the Ras–mitogen-activated protein kinase (MAPK) pathway is highly activated and phosphorylates RXRα at serine residues, thus impairing dimer formation and the subsequent transactivation functions of the receptor. Furthermore, phosphorylated RXRα (pi-RXRα) escapes from ubiquitination and proteasomal degradation (B). Therefore, pi-RXRα accumulates and interferes with the physiological function of the remaining unphosphorylated RXRα, presumably, in a dominant-negative manner, thereby playing a critical role in the development of HCC (C). ACR is not only a ligand for RXRα but also suppresses the Ras–MAPK signaling pathway, inhibiting phosphorylation of RXRα, restoring the function of the receptor, and thus subsequently activating the transcriptional activity of the responsive element (D). ACR also directly or indirectly inhibits the ligand (i.e. specific growth factor)-dependent receptor tyrosine kinase (RTK) activities in cancer cells (E). These effects may contribute to the inhibition of extracellular signal-regulated kinase (Erk) and RXRα phosphorylation, thus causing inhibition of the growth of HCC cells.

unphosphorylated and highly ubiquitinated, thus rendering it sensitive to proteasome-mediated degradation. In contrast, p-RXRα is resistant to ubiquitination and proteasome-mediated degradation in both human HCC tissues and a human HCC cell line.[14] In addition, the phosphorylation of RXRα abolishes its ability to form heterodimers with RARβ and this might be associated with uncontrolled cell growth and resistance to retinoids.[13] These findings suggest that the accumulation of p-RXRα (i.e. non-functional RXRα) may interfere with the function of normal RXRα in a dominant-negative manner, thereby playing a critical role in the development of HCC (Fig. 2). Therefore, the inhibition of RXRα phosphorylation and the restoration of its physiological function as a master regulator of nuclear receptors, such as heterodimeric activity with other nuclear receptors, may be an effective and important strategy for inhibiting the growth of HCC cells.

Chemoprevention of HCC by ACR: Experimental study

Acyclic retinoid (NIK-333; Kowa Pharmaceutical Co., Tokyo, Japan) is a synthetic retinoid and has an agonistic activity for both RXR and RAR.[32,33] In experimental studies, this agent has demonstrated several beneficial effects on inhibition of HCC development. For instance, ACR inhibits chemically induced hepatocarcinogenesis in rats as well as spontaneously occurring hepatoma in mice.[28] ACR also inhibits growth and induces apoptosis in human HCC-derived cells and this might be associated with induction of apoptosis and cell differentiation in these cancer cells.[33–37] In a human HCC cell line, ACR causes an arrest of the cell cycle in G_0–G_1, increases cellular levels of the $p21^{CIP1}$ protein, and decreases levels of the cyclin D1 protein.[38] Moreover, recent studies indicated that ACR is not only the ligand for RXRα. Indeed, in human HCC-derived cells, ACR restores the function of RXRα by inactivating the Ras–Erk signaling system and thereby dephosphorylating RXRα, although 9-*cis* RA fails to suppress phosphorylation of the Erk protein and subsequent RXRα phosphorylation.[31] Both *in vivo*[39,40] and *in vitro*[41,42] studies have demonstrated that ACR reduces the development of HCC and prevents growth of cancer cells by inhibiting the activation of RTK, which play a critical role in stimulation of the Ras–MAPK signaling pathway.[43] Therefore, in addition to direct inhibition of the Ras–Erk signaling system[31] ACR may also cause dephosphorylation of the Erk and RXRα proteins by inactivating RTK, the upstream molecules of Ras, and thus restoring the function of RXRα. These findings suggest that ACR is a promising agent for the chemoprevention of HCC and that p-RXRα is a useful molecular target of ACR (Fig. 2).

Chemoprevention of HCC by ACR: Clinical study

The chemopreventive effects of ACR on recurrent and secondary HCC were confirmed in patients who received anticancer treatment for an initial HCC in a double-blind and placebo-controlled clinical study.[9–11] Oral administration of ACR (600 mg per day) for 12 months significantly reduced the incidence of post-therapeutic HCC recurrence in patients who underwent potentially curative treatments.[9] The survival rate was also significantly improved by the administration of this compound after a median follow up of 62 months.[10] Moreover, the preventive effects of ACR lasted up to 199 weeks after randomization (or 151 weeks after completion of ACR administration).[11] Therefore, administration of ACR for only 12 months confers a long-term effect over several years, without

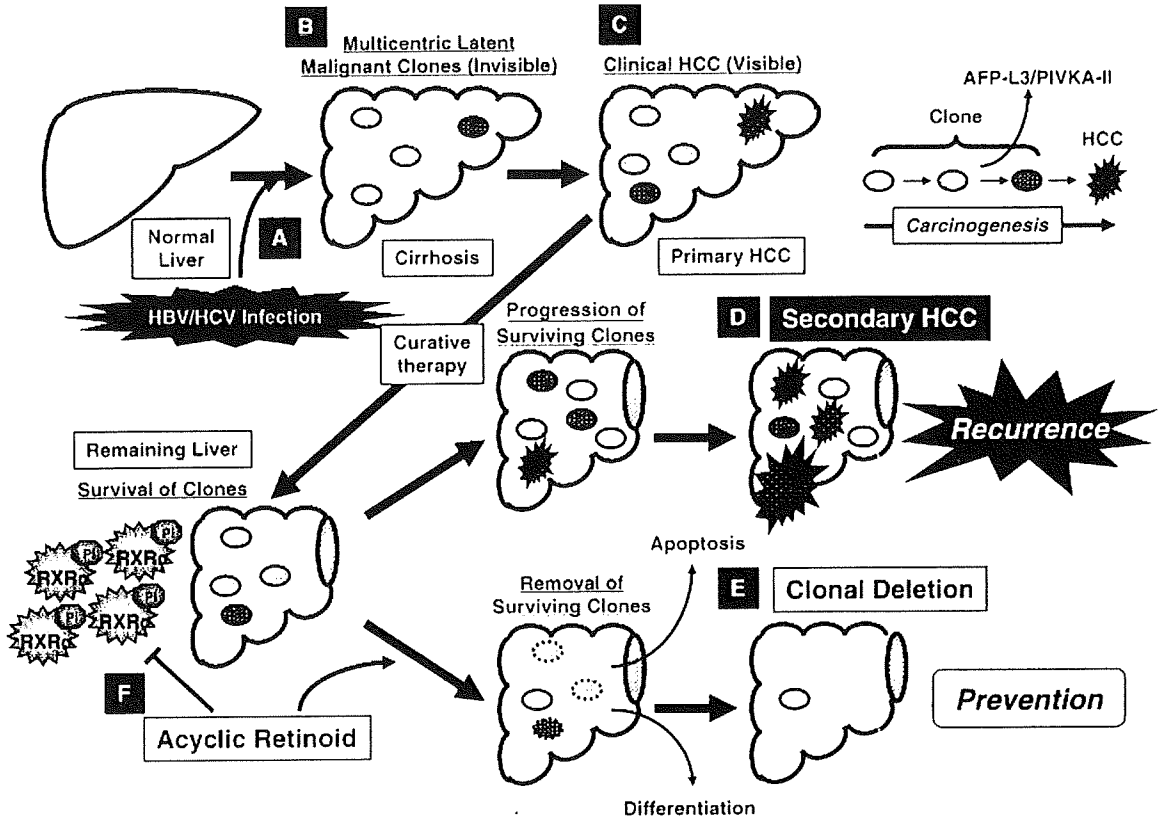

Fig. 3. The concept of 'clonal deletion'. Persistent inflammation caused by hepatitis B virus (HBV) or hepatitis C virus (HCV) infection transforms the liver into a 'precancerous field' (A). Therefore, the high incidence of hepatocellular carcinoma (HCC) as well as its recurrence in cirrhotic patients strongly suggests the presence of latent malignant clones that arise through multicentric carcinogenesis and are undetectable clinically by image analysis (invisible) (B). These multiple clones demonstrate different grades of malignancy (atypia) in the cirrhotic liver and, at some point, turn into clinical HCC (visible) (C). Even when primary HCC could be found in an early stage and surgical treatment might be expected to be curative, other clones still survive in the remaining liver and thus grow into secondary HCC again (D). Therefore, the eradication of such transformed clones, referred to as 'clonal deletion', may be one of the most effective strategies to prevent secondary HCC (E). Clinical experience suggests that acyclic retinoid (ACR), which inhibits phosphorylation (Pi) of retinoid X receptor (RXR) α (F), reduces the recurrence of HCC on the basis of this concept because this agent causes a decrease in the serum levels of lectin-reactive α-fetoprotein isoform 3 (AFP-L3) and protein induced by vitamin K absence or antagonist-II (PIVKA-II), which are produced by latent malignant clones, by eradicating or inhibiting these clones. Once such clones are deleted, the preventive effect on HCC lasts several years without any continuous administration of ACR. In fact, 1-year administration of ACR inhibited secondary HCC for the next 3 years.[11] Therefore, this agent can significantly improve the survival rate of such patients.

causing any severe adverse effects of retinoids, such as dry skin, cheilitis, or conjunctivitis. However, headache or hyperlipidemia was reported in one case ACR.[9] A phase II/III trial of this compound to test its effect in preventing second primary HCC is currently proceeding as a large-scale randomized controlled study. This trial is scheduled to be completed around 2009–10 and it is expected to yield positive results.

Concept of 'clonal deletion'

Pathologically, the high incidence of the development of second primary HCC may be explained by its characteristic mode of carcinogenesis, multicentric carcinogenesis, which is also expressed by the term 'field cancerization'.[44] Once a liver is exposed to continuous carcinogenic insults, such as hepatitis virus infection, the whole liver is regarded as a precancerous field that possesses multiple as well as independent premalignant or latent malignant clones. Therefore, the most effective strategy for HCC chemoprevention is the deletion of latent malignant clones (clonal deletion) as well as inhibition of the evolution of such clones (clonal inhibition) before they expand into a clinically detectable tumor. We therefore propose the benefits of 'clonal deletion' therapy for the prevention of HCC recurrence, which is defined as the removal of latent malignant (or premalignant) clones that are invisible by diagnostic imaging from the liver in a hypercarcinogenic state[11,45–47] (Fig. 3).

This concept has been clinically demonstrated and implemented in a clinical trial using ACR. Indeed, in that trial, ACR significantly reduced the serum levels of AFP-L3, which indicates the presence of latent (i.e. invisible) HCC cells in the remnant liver, after 12 months of administration.[45] The administration of ACR also caused a decrease in the serum levels of protein induced by PIVKA-II, which may also be produced by latent HCC cells.[11] These results strongly suggest that ACR deleted such malignant clones producing AFP-L3 or PIVKA-II before they expanded to clinically detectable tumors, thereby inhibiting second primary HCC. Therefore, once such latent clones are eradicated or inhibited, it may take several years for the next cancer clone to arise clinically.[11] Moreover, ACR also prevented the appearance of AFP-L3 in patients who had been negative at entry, although there was a significant increase in the incidence of AFP-L3-positive patients in the placebo group and these patients had a significantly higher risk of second primary HCC.[45] This is also one of the reasons for the long-term benefit of ACR after only a

doi: 10.1111/j.1349-7006.2008.01045.x

12-month treatment with this agent.[11] Therefore, we suggest that the concept of 'clonal deletion' seems more of a therapy rather than prevention and that ACR is a more affirmative agent to inhibit the development of HCC (Fig. 3).

Possibility of 'combination chemoprevention' with ACR

The combined use of two or more agents is often advantageous as it may permit lower clinical dosages, consequently decreasing the overall toxicity and thus providing the potential for synergistic effects between specific agents, including retinoids.[24,48] Therefore, the beneficial effects, such as synergism, between ACR and other agents to inhibit the growth of HCC cells have been examined. For instance, ACR acts synergistically with IFN in suppressing growth and inducing apoptosis in human HCC cell lines and this synergism was associated with the upregulation of type 1 IFN receptor expression by ACR.[49] The combination of ACR plus OSI-461, a potent derivative of sulindac sulfone, exerts synergistic inhibition of cell growth and induction of apoptosis in HepG2 human HCC cells.[50] In addition, the combination of ACR plus VK_2 also synergistically induced apoptosis and inhibited the growth of HCC cells without affecting the growth of normal human hepatocytes.[51] The findings that both IFN and VK_2 enhance the effects of ACR seem to be of interest because these agents are expected to reduce the development and recurrence rates of HCC.[52,53]

In the above study,[51] VK_2 inhibited phosphorylation of the RXRα protein through the inhibition of Ras activation and Erk phosphorylation, and the inhibition of RXRα phosphorylation by VK_2 was enhanced when the cells were cotreated with ACR. In addition, ACR and trastuzumab, the humanized anti-HER2 monoclonal antibody, cooperatively inhibit the activation of HER2 and its downstream signaling pathways, subsequently inhibiting the phosphorylation of RXRα and the growth of HCC cells.[54] Therefore, ACR may support the effect of the agents that target RTK, thus cooperatively or synergistically inhibiting HCC by targeting RXRα phosphorylation. These findings, together with those of previous reports,[39–41] suggest that the combination of ACR plus a specific agent that targets RTK and the Ras–MAPK signaling pathway may be able to inhibit the phosphorylation of RXRα and it may therefore be a promising strategy to prevent the development of HCC.

Conclusion

The very high incidence of secondary HCC is mainly responsible for the poor prognosis of patients with this malignancy. This fact suggests that, in turn, the establishment of a new effective strategy to prevent the recurrence of HCC will significantly improve the outcome of these patients and thus be an urgent task worldwide. One of the most practical approaches to prevent the development of HCC is 'clonal deletion' and clinical trials using ACR theoretically proved the significance of this therapy in HCC chemoprevention.[11,45] Experimental studies strongly suggest that phosphorylated RXRα is associated with HCC carcinogenesis and thus may be a critical target for HCC chemoprevention. ACR, which targets phosphorylated RXRα, may therefore play a critical role in preventing the development of HCC when it is used alone or combined with other agents.

Acknowledgments

This work was supported in part by Grants-in-Aid from the Ministry of Education, Science, Sports, and Culture of Japan (no. 18790457 to M.S. and no. 17015016 to H.M.).

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doi: 10.1111/j.1349-7006.2008.01045.x

Supplementation with Branched-chain Amino Acids Inhibits Azoxymethane-induced Colonic Preneoplastic Lesions in Male C57BL/KsJ-*db/db* Mice

Masahito Shimizu,[1] Yohei Shirakami,[1] Junpei Iwasa,[1] Makoto Shiraki,[1] Yoichi Yasuda,[1] Kazuya Hata,[3] Yoshinobu Hirose,[2] Hisashi Tsurumi,[1] Takuji Tanaka,[4] and Hisataka Moriwaki[1]

Abstract **Purpose:** Obesity and related metabolic abnormalities, including insulin resistance and activation of the insulin-like growth factor (IGF)/IGF-I receptor (IGF-IR) axis, are risk factors for colon cancer. Supplementation with branched-chain amino acids (BCAA) reduces the risk of liver cancer in cirrhotic patients who are obese, and this has been associated with an improvement of insulin resistance. The present study examined the effects of BCAA on the development of azoxymethane (AOM)-initiated colonic premalignant lesions in C57BL/KsJ-*db/db* (*db/db*) mice that were obese and had hyperinsulinemia.

Experimental Design: Male *db/db* mice were given 4 weekly s.c. injections of AOM (15 mg/kg of body weight) and then they were fed a diet containing 3.0% BCAA or casein, a nitrogenc content–matched control diet, for 7 weeks.

Results: Feeding with BCAA caused a significant reduction in the number of total aberrant crypt foci and β-catenin accumulated crypts, both of which are premalignant lesions of the colon, compared with the control diet–fed groups. BCAA supplementation caused a marked decrease in the expression of IGF-IR, the phosphorylated form of IGF-IR, phosphorylated glycogen synthase kinase 3β, phosphorylated Akt, and cyclooxygenase-2 proteins on the colonic mucosa of AOM-treated mice. The serum levels of insulin, IGF-I, IGF-II, triglyceride, total cholesterol, and leptin were also decreased by supplementation with BCAA.

Conclusion: BCAA supplementation in diet improves insulin resistance and inhibits the activation of the IGF/IGF-IR axis, thereby preventing the development of colonic premalignancies in an obesity-related colon cancer model that was also associated with hyperlipidemia and hyperinsulinemia. BCAA, therefore, may be a useful chemoprevention modality for colon cancer in obese people.

Colorectal cancer (CRC) is a major health problem worldwide. Recent evidence indicates that the risk of CRC is elevated in patients with metabolic syndrome, also called insulin resistance syndrome, which is commonly associated with obesity and related metabolic abnormalities (1, 2). Obesity is the main determinant of insulin resistance and hyperinsulinemia, which is also a possible risk factor for CRC (3). CRC occurs more frequently in patients with diabetes mellitus, a condition associated with hyperinsulinemia (4, 5). Insulin has growth-promoting properties in CRC cells, and exogenous insulin injection stimulates the growth of CRC precursors in rodent models (6–8). In addition, elevated circulating levels of insulin causes alterations in the insulin-like growth factor (IGF)/IGF-I receptor (IGF-IR) axis, which is involved in the development and progression of CRC (9, 10). Therefore, increased insulin resistance and abnormalities in the IGF/IGF-IR axis might be a critical target to prevent the development of obesity-related malignancies, including CRC. For instance, (-)-epigallocatechin gallate, the major biologically active component of green tea, inhibited the development of colonic premalignant lesions in an obesity-related colon cancer that was associated with improvement in insulin resistance and inhibition of the IGF/IGF-IR axis (11).

Diet supplementation with branched-chain amino acids (BCAA; leucine, isoleucine, and valine) has been suggested to improve protein malnutrition in patients with liver cirrhosis (12). Recent studies have revealed that BCAA is useful for both preventing progressive hepatic failure and improving event-free survival in patients with chronic liver diseases, such as liver cirrhosis, and these beneficial effects are associated with the improvement of insulin resistance by BCAA (13–15). In addition, oral supplemental treatment with BCAA can reduce the risk of hepatocellular carcinoma in cirrhotic patients who

Authors' Affiliations: [1]Departments of Medicine and [2]Tumor Pathology, Gifu University Graduate School of Medicine; [3]BMR Laboratories, Sunplanet Co., Ltd, Gifu, Japan; and [4]Department of Oncologic Pathology, Kanazawa Medical University, Ishikawa, Japan

Received 8/10/08; revised 1/8/09; accepted 1/23/09; published OnlineFirst 4/14/09.
Grant support: Ministry of Education, Science, Sports and Culture of Japan Grants-in-Aid Nos. 18790457 (M. Shimizu) and 17015016 (H. Moriwaki).
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Requests for reprints: Masahito Shimizu, Department of Medicine, Gifu University Graduate School of Medicine, 1-1 Yanagido, Gifu 501-1194, Japan. Phone: 81-58-230-6313; Fax: 81-58-230-6310; E-mail: shimim-gif@umin.ac.jp.

doi:10.1158/1078-0432.CCR-08-2093

Translational Relevance

Obesity and related metabolic abnormalities, including insulin resistance and the activation of the insulin-like growth factor (IGF)/IGF-I receptor axis, are associated with colorectal cancer (CRC) development. Therefore, the prevention of CRC by targeting the dysregulation of energy homeostasis might be a promising strategy for obese people who are at increased risks of CRC. We believe that this study is novel and clinically relevant because this article is the first report indicating that supplementation with branched-chain amino acids (BCAA) effectively suppressed the development of azoxymethane-induced putative precursor lesions of colonic adenocarcinoma in C57BL/KsJ-*db/db* mice that are obese and developed diabetes mellitus. Our studies indicate that this suppressing effect of BCAA was associated with improvement of hyperlipidemia and hyperleptinemia. BCAA supplementation could also improve insulin resistance and exert a depressant effect on the IGF/IGF-IR axis. The current findings suggest the possibility of using BCAA as a chemopreventive agent for obesity-related malignancies.

are obese (with a body mass index ≥25; ref. 16). Obesity, hyperinsulinemia, and diabetes mellitus are possible risk factors for hepatocellular carcinoma, which commonly develops in cirrhotic livers (16–18). Based on these findings, BCAA supplementation in diet may also reduce the risk of other obesity-related human malignancies, including CRC, by improving insulin resistance. However, no detailed studies on whether BCAA can prevent the development of obesity-related CRC have yet been conducted.

In previous studies, we have established a useful preclinical animal model to determine the possible underlying mechanisms of how specific agents prevent the development of obesity-related CRC with the use of C57BL/KsJ-*db/db* (*db/db*) mice with obesity, hyperinsulinemia, and hyperleptinemia (19–21). The mice are susceptible to the colonic carcinogen azoxymethane (AOM) because the development of AOM-induced aberrant crypt foci (ACF) and β-catenin–accumulated crypts (BCAC), both of which are putative precursor lesions for colonic adenocarcinoma (22, 23), is enhanced in *db/db* mice compared with *db/+* or +/+ mice (19, 20). In the present study, we investigated in detail the effects of BCAA on the development of colonic premalignant lesions, ACF and BCAC, in *db/db* mice initiated with AOM, focusing on the improvement of hyperinsulinemia, hyperlipidemia, and hyperleptinemia. In addition, we also determined whether BCAA supplementation in the diet inhibits the activation of the IGF/IGF-IR axis in this animal model.

Materials and Methods

Animals, chemicals, and diets. Four-week-old male homozygous *db/db* mice were obtained from Japan SLC, Inc.. All mice were maintained at the Gifu University Life Science Research Center according to the Institutional Animal Care Guidelines. AOM was purchased from Sigma Chemical Co.. BCAA and casein were obtained from Ajinomoto Co., Ltd.. The BCAA composition (2:1:1.2, leucine/isoleucine/valine) was set at the clinical dosage that is used for the treatment of hypoalbuminemia in patients with decompensated liver cirrhosis in Japan.

Experimental procedure. The animal experiment was approved by the Institutional Committee of Animal Experiments of Gifu University. A total of 54 male *db/db* mice were divided into 6 groups. At 5 wk of age, the mice in groups 1 to 3 were s.c. injected with AOM (15 mg/kg of body weight) weekly for 4 wk. As controls, the mice in groups 4 to 6 were given s.c. injections of saline. Groups 1 (12 mice) and 4 (6 mice) were fed a basal diet, corticotropin-releasing factor (CRF)-1 (Oriental Yeast Co., Ltd.), throughout the experiment. Groups 3 (12 mice) and 6 (6 mice) were given a basal diet containing 3.0% BCAA (weight for weight) for 7 wk, starting 1 wk after the last injection of AOM. The BCAA concentration (3.0%) was determined by the previous study, which indicated the same intake to improve insulin resistance in C57BL/6J mice (24). The mice in groups 2 (12 mice) and 5 (6 mice) were given a basal diet containing 3.0% casein (weight for weight). The casein-fed groups were served as nitrogen content–matched controls for the BCAA-treated groups to eliminate the possibility that the nitrogen content itself affects the promotion or the prevention of colonic premalignant lesions. At the termination of the study (16 wk of age), the mice were sacrificed by CO_2 asphyxiation to analyze the number of colonic ACF and BCAC.

Counting the number of ACF and BCAC. The ACF and BCAC were determined according to the standard procedures described previously (20, 21, 25). ACF are defined as single or multiple crypts that have altered luminal openings, exhibit thickened epithelia, and are larger than adjacent normal crypts (22). BCAC, which have high frequency mutations in the *β-catenin* gene, show histologic dysplasia with a disruption of the cellular morphology and an accumulation of this protein (Fig. 1A; ref. 23). BCAC do not have a typical ACF-like appearance because the lesion is not recognized on the mucosal surface like ACF and is only identified in the histologic sections of en face preparations. Both of these lesions are utilized as biomarkers to evaluate a number of agents for their potential chemopreventive properties (26). After the colons were fixed flat in 10% buffered formalin for 24 h, the mucosal surface of the colons were stained with methylene blue (0.5% in distilled water), and then the number of ACF were counted under a light microscope. Thereafter, the distal parts (5 cm from the anus) of the colon were cut to count the number of BCAC. To identify BCAC intramucosal lesions, the distal part of the colon (mean area, 0.7 cm^2 per colon) was embedded in paraffin, and then a total of 20 serial sections (4-μm thick each) per colon were made by an en face preparation (20, 21, 25). For each case, 2 serial sections were used to analyze BCAC.

Histopathology and immunohistochemical analyses for β-catenin and PCNA. Three serial sections were made from paraffin-embedded tissue blocks. Two sections were subjected to H&E staining for histopathology and β-catenin immunohistochemistry to count the number of BCAC. The other section was used for the proliferating cell nuclear antigen (PCNA), a G_1-to-S phase marker, immunohistochemistry to estimate the cell proliferative activity in the colonic mucosa. Immunohistochemical analyses for β-catenin and PCNA were done with the labeled streptavidin-biotin method (LSAB kit; DAKO) as previously described (20, 21). Anti–β-catenin antibody (1:1,000 final dilution) was obtained from Transduction Laboratories (catalogue no. 610154). Anti-PCNA antibody (1:100 final dilution) was from Santa Cruz Biotechnology, Inc. (sc-7907). Negative control sections were immunostained without the primary antibody. PCNA-positive cells in the colonic mucosa, which seemed normal by H&E staining, were counted and expressed as a percentage of the total number of normal crypt cells. The PCNA labeling index (%) was determined by counting at least 200 crypt cells in each mouse (a total of 1,000 crypt cells per group). Two experienced pathologists (Y. Hirose and T. Tanaka) immunohistologically determined the BCAC and PCNA-positive cells.

Protein extraction and western blot analysis. Total proteins were extracted from the scraped mucosa from the remaining colon of the AOM-treated mice (groups 1 to 3), and equivalent amounts of proteins

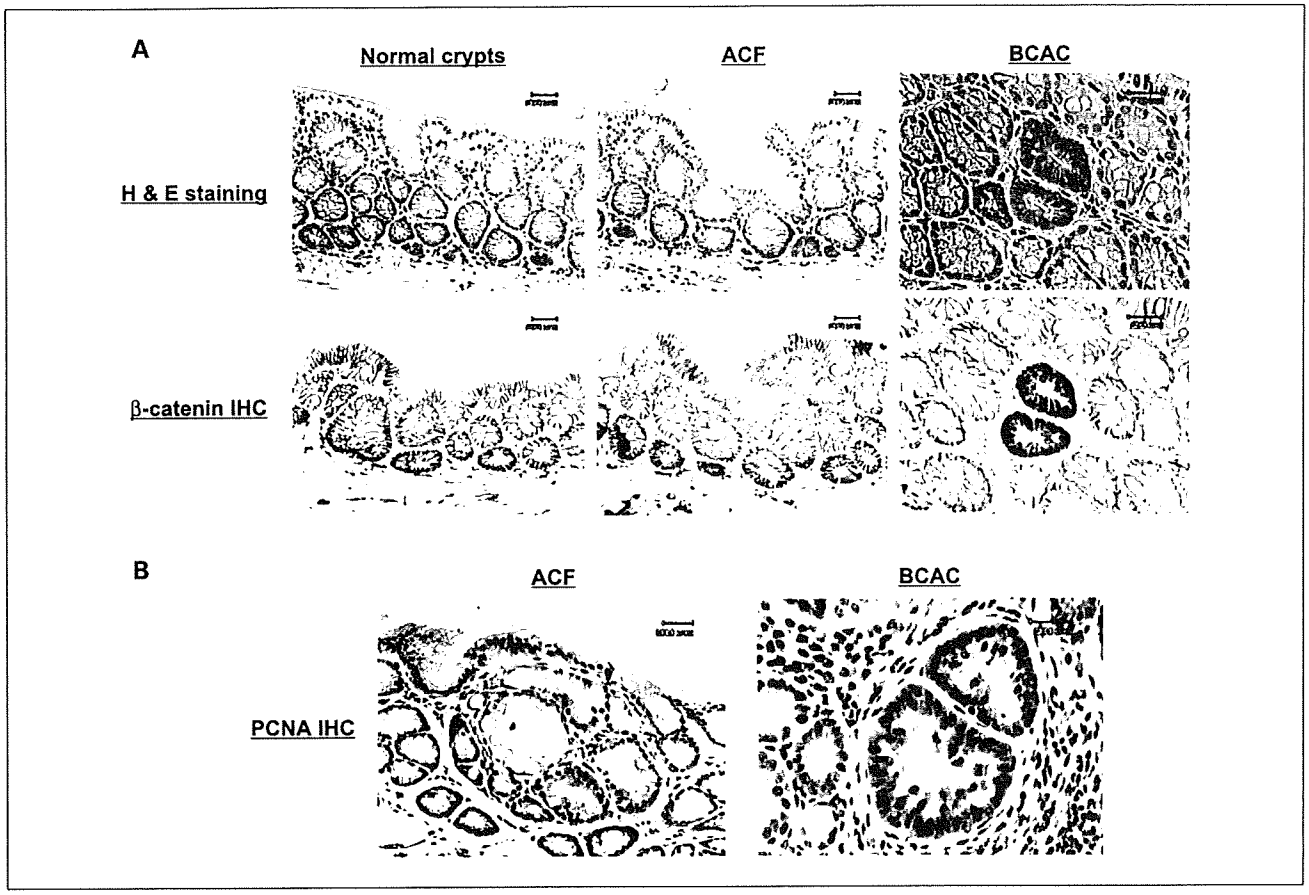

Fig. 1. Histopathology and immunohistochemical expression of β-catenin and PCNA proteins in ACF and BCAC. *A*, representative photographs of ACF and BCAC induced by AOM in *db/db* mice. Top, H&E staining; bottom, β-catenin immunohistochemistry. Left, normal crypts; middle, ACF; right, BCAC. The localization of the accumulated β-catenin protein is apparent in the cytoplasm and nucleus of atypical cryptal cells in BCAC. *B*, immunohistochemical pattern of PCNA protein in ACF and BCAC. The nuclear expression of the PCNA protein significantly increased in BCAC compared with ACF and surrounding normal crypts. Bar, 20 or 30 μm, respectively.

(40 μg per lane) were examined by a western blot analysis with the use of the primary antibodies for IGF-IR, phosphorylated IGF-IR (p-IGF-IR), phosphorylated glycogen synthase kinase 3β (p-GSK-3β), Akt, phosphorylated Akt (p-Akt), cyclooxygenase-2 (COX-2), and glyceraldehyde-3-phosphate dehydrogenase as described previously (11, 27, 28). An antibody to glyceraldehyde-3-phosphate dehydrogenase served as a loading control. The intensities of the blots were quantified with the NIH Image software version 1.62. The intensities of the blots found at the CRF-fed mice in each antibody was set at 1, and the changes in expression were shown as the fold difference.

Table 1. Body, liver, kidney, and white adipose tissue weights of the experimental mice

Group no.	Treatment	Diet	No. of mice	Final body weight (g)	Body length (cm)	BMI	Absolute organ weight		
							Liver	Kidney	White adipose tissue
1	AOM 15 mg/kg	CRF-1	12	49.7 ± 8.3*,†	9.25 ± 0.77	0.58 ± 0.05	2.64 ± 0.76	0.38 ± 0.04†	2.67 ± 0.64
2	AOM 15 mg/kg	Casein	12	51.7 ± 4.8	9.43 ± 0.37	0.58 ± 0.02	2.75 ± 0.48‡	0.41 ± 0.04	2.74 ± 0.37
3	AOM 15 mg/kg	BCAA	12	50.3 ± 5.0§	9.47 ± 0.25	0.56 ± 0.04	2.58 ± 0.64§	0.40 ± 0.05	2.51 ± 0.42
4	Saline	CRF-1	6	58.1 ± 2.5	9.63 ± 0.22	0.63 ± 0.02	3.35 ± 0.72	0.45 ± 0.06	3.02 ± 0.32
5	Saline	Casein	6	58.0 ± 2.1	9.70 ± 0.18	0.62 ± 0.01	3.87 ± 1.04	0.44 ± 0.04	2.70 ± 0.38
6	Saline	BCAA	6	58.5 ± 2.5	9.63 ± 0.17	0.63 ± 0.01	3.83 ± 0.86	0.44 ± 0.01	2.60 ± 0.35

*Mean ± SD.
†Significantly different from group 4 ($P < 0.05$).
‡Significantly different from group 5 ($P < 0.05$).
§Significantly different from group 6 ($P < 0.05$).

Table 2. Effects of BCAA on AOM-induced ACF and BCAC formation in the experimental mice

Group no.	Treatment	Diet	No. of mice	Length of colon (cm)	Total no. of ACFs per colon	Total no. of BCACs/cm^2
1	AOM 15 mg/kg	CRF-1	12	12.4 ± 1.4*	85.9 ± 8.1	11.7 ± 8.4
2	AOM 15 mg/kg	Casein	12	12.5 ± 0.5	83.4 ± 11.2	8.3 ± 3.9
3	AOM 15 mg/kg	BCAA	12	12.0 ± 0.7	54.5 ± 8.6†,‡	4.2 ± 6.7§
4	Saline	CRF-1	6	12.5 ± 1.0	0	0
5	Saline	Casein	6	11.5 ± 0.7	0	0
6	Saline	BCAA	6	11.3 ± 0.5	0	0

*Mean ± SD.
†Significantly different from group 1 ($P < 0.001$).
‡Significantly different from group 2 ($P < 0.001$).
§Significantly different from group 1 ($P < 0.05$).

Clinical chemistry. At sacrifice, blood samples were collected from the AOM-treated mice (groups 1-3) to measure the serum concentrations of insulin, leptin, triglyceride, total cholesterol, IGF-I, IGF-II, and BCAA. The serum triglyceride, total cholesterol, and BCAA levels were assayed as described previously (20, 29). The serum insulin, leptin, IGF-I, and IGF-II were determined by an enzyme immunoassay according to the manufacturer's protocol (R&D Systems).

Statistical analysis. The results were presented as the mean ± SD and were analyzed with the use of the GraphPad InStat software program version 3.05 (GraphPad Software) for Macintosh. Differences between groups were analyzed by one-way ANOVA or, as required, by two-way ANOVA. When ANOVA showed a statistically significant effect ($P < 0.05$), comparisons of each experimental group with the control group were then made with the use of the Tukey-Kramer multiple comparisons test. The differences were considered significant when the two-tailed P was <0.05.

Results

General observations. As shown in Table 1, the average body weights of groups 1 (CRF-1) and 3 (BCAA) in the AOM-injected mice at the termination of this experiment were smaller than those of the saline-injected groups 4 (CRF-1; $P < 0.05$) and 6 (BCAA; $P < 0.05$). The mean liver weights in the AOM-treated groups 2 (casein) and 3 (BCAA) were significantly lower than those in the saline-treated groups 5 (casein; $P < 0.05$) and 6 (BCAA; $P < 0.05$). Among CRF-1-fed mice, the mean kidney weight in the AOM-treated group 1 was also significantly lower than that of the saline-treated group 4 ($P < 0.05$). No significant difference was observed in the body length, body mass index, and mean white adipose tissue weight among the experimental mice. A histopathologic examination also revealed no alteration, thus suggesting the absence of toxicity of BCAA in the liver and kidney of the mice in groups 3 and 6 (data not shown).

Effects of BCAA supplementation on AOM-induced ACF and BCAC formations in db/db *mice.* Table 2 summarizes the total number of ACF and BCAC (Fig. 1) in the mice of all groups. ACF and BCAC developed in the colons of all the mice that received AOM (groups 1 to 3) but not in the colons of the mice that did not receive AOM (groups 4 to 6). Dietary supplementation with BCAA significantly decreased the number of total ACF compared with those of the CRF-1-fed (37% reduction; $P < 0.001$) and casein-supplemented groups (35% reduction; $P < 0.001$). Compared with the CRF-1-fed group, the administration of BCAA also significantly reduced the number of total BCAC (64% reduction; $P < 0.05$).

Effects of BCAA supplementation on the serum levels of BCAA in AOM-treated db/db *mice.* Because the colonic premalignant lesions developed only in the AOM-injected mice (Table 2), the following experiments were done among the mice thatz received AOM (groups 1 to 3). BCAA supplementation caused a significant increase in the serum concentrations of total BCAA (valine, isoleucine, and leucine; 1736 ± 179 nmol/mL) compared with the CRF-1-fed (882 ± 160 nmol/mL; $P < 0.001$) and casein-supplemented groups (853 ± 51 nmol/mL; $P < 0.001$). These findings suggest that supplementation with 3.0% BCAA is sufficient to raise the serum concentration of BCAA.

Effects of BCAA supplementation on the serum levels of total cholesterol, triglyceride, and leptin in AOM-treated db/db *mice.* As shown in Table 3, the serum levels of total cholesterol in the BCAA-supplemented mice were significantly lower than

Table 3. Serum levels of total cholesterol, triglyceride, and leptin in AOM-treated *db/db* mice

Group no.	Treatment	Diet	No. of mice	Total cholesterol (mg/dL)	Triglyceride (mg/dL)	Leptin (ng/dL)
1	AOM 15 mg/kg	CRF-1	12	185 ± 34*	244 ± 49	117 ± 18
2	AOM 15 mg/kg	Casein	12	186 ± 40	229 ± 40	133 ± 32
3	AOM 15 mg/kg	BCAA	12	141 ± 48†,‡	187 ± 48†	99 ± 23§

*Mean ± SD.
†Significantly different from group 1 ($P < 0.05$).
‡Significantly different from group 2 ($P < 0.05$).
§Significantly different from group 2 ($P < 0.01$).

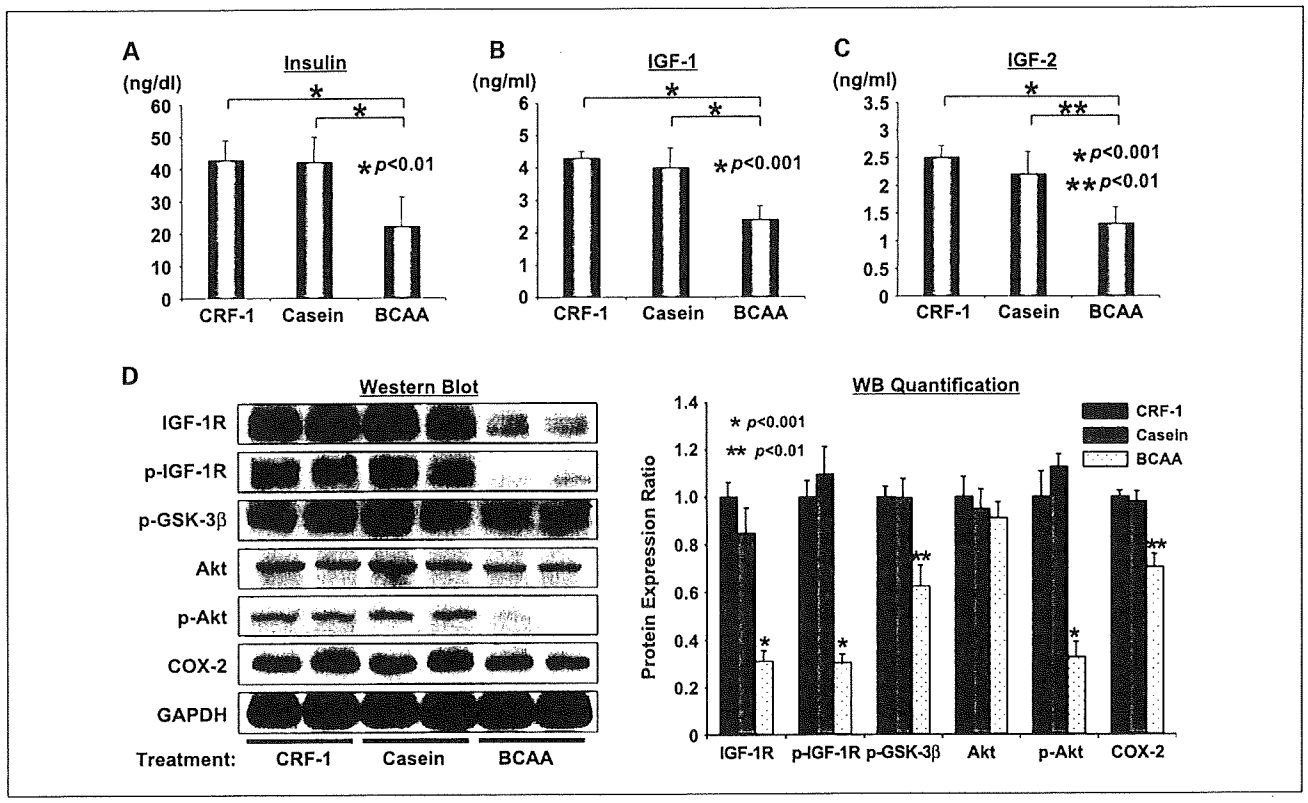

Fig. 2. The effect of BCAA supplementation on the serum levels of insulin, IGF-I, and IGF-II, and on the expression levels of the IGF-IR, p-IGF-IR, p-GSK-3β, Akt, p-Akt, and COX-2 proteins in AOM-treated *db/db* mice. *A* to *C*, the serum concentration of insulin (*A*), IGF-I (*B*), and IGF-II (*C*) were measured by an enzyme immunoassay. Bars, SD of triplicate assays. *D*, total proteins were extracted from the scraped colonic mucosa, and equivalent amounts of proteins were examined by a western blot analysis as described in Materials and Methods. Lanes, protein samples from two different mice in each group (*left*). The intensities of blots were quantitated by densitometry (*right*). Repeat western blots gave similar results. Values, mean ± SD. *, $P < 0.001$ and **, $P < 0.01$: significant differences obtained by comparison with CRF-1 – treated or casein-treated mice, respectively.

those in the CRF-1 – fed ($P < 0.05$) and casein-supplemented mice ($P < 0.05$). The mice supplemented with BCAA showed a significant decrease in the serum levels of triglyceride compared with the CRF-1 fed ($P < 0.05$). The serum leptin level of group 3 (BCAA) was also significantly lower than that of group 2 (casein; $P < 0.01$).

Effects of BCAA supplementation on the serum levels of insulin, IGF-I, and IGF-II in AOM-treated db/db *mice.* Supplementation with BCAA caused a significant decrease in the serum levels of insulin (Fig. 2A) compared with the CRF-1 – fed ($P < 0.01$) and casein-supplemented mice ($P < 0.01$). Similarly, there was a significant decrease in the serum levels of both IGF-I (Fig. 2B) and IGF-II (Fig. 2C) in BCAA-supplemented mice compared with the CRF-1 – fed ($P < 0.001$ for each comparison) and casein-supplemented mice ($P < 0.001$ and $P < 0.01$, respectively).

Effects of BCAA supplementation on the expression levels of IGF-IR, p-IGF-IR, p-GSK-3β, p-Akt, and COX-2 proteins, and on cell proliferative activity in the colonic mucosa of AOM-treated db/db *mice.* Hyperinsulinemia and abnormal activation of the IGF/IGF-IR axis play a critical role in obesity-related CRC development (3, 6 – 10). Therefore, the effects of BCAA on the levels of IGF-IR and the phosphorylated (i.e., activated) form of IGF-IR proteins, and cell proliferation were examined in the colonic mucosa of AOM-treated mice. As shown in Fig. 2D, western blot analyses showed that BCAA supplementation caused a decrease in the levels of IGF-IR ($P < 0.001$ for each comparison) and p-IGF-1R ($P < 0.001$ for each comparison) proteins compared with the CRF-1 – fed and casein-supplemented mice. Supplementation with BCAA also decreased the expression levels of the phosphorylated (i.e., inactivated) form of GSK-3β ($P < 0.01$ for each comparison), the phosphorylated (i.e., activated) form of Akt ($P < 0.001$ for each comparison), and COX-2 ($P < 0.01$ for each comparison) proteins compared with the control groups. The finding that BCAA supplementation inhibited the phosphorylation of Akt is considered to be significant because the activation of this protein is one of the critical targets in the constitutive activation of the IGF/IGF-IR axis in colorectal carcinogenesis (30).

In addition, as shown in Fig. 3, the PCNA labeling index of nonlesional crypts in the BCAA-supplemented mice was significantly smaller than that of the CRF-1 – fed and casein-supplemented mice ($P < 0.001$ for each comparison), thus indicating that BCAA supplementation significantly inhibits cell proliferation in the colonic mucosa of the AOM-treated *db/db* mice.

Discussion

The present study clearly indicated that dietary supplementation with BCAA effectively suppressed the development of putative precursor lesions, ACF and BCAC (Fig. 1), for CRC

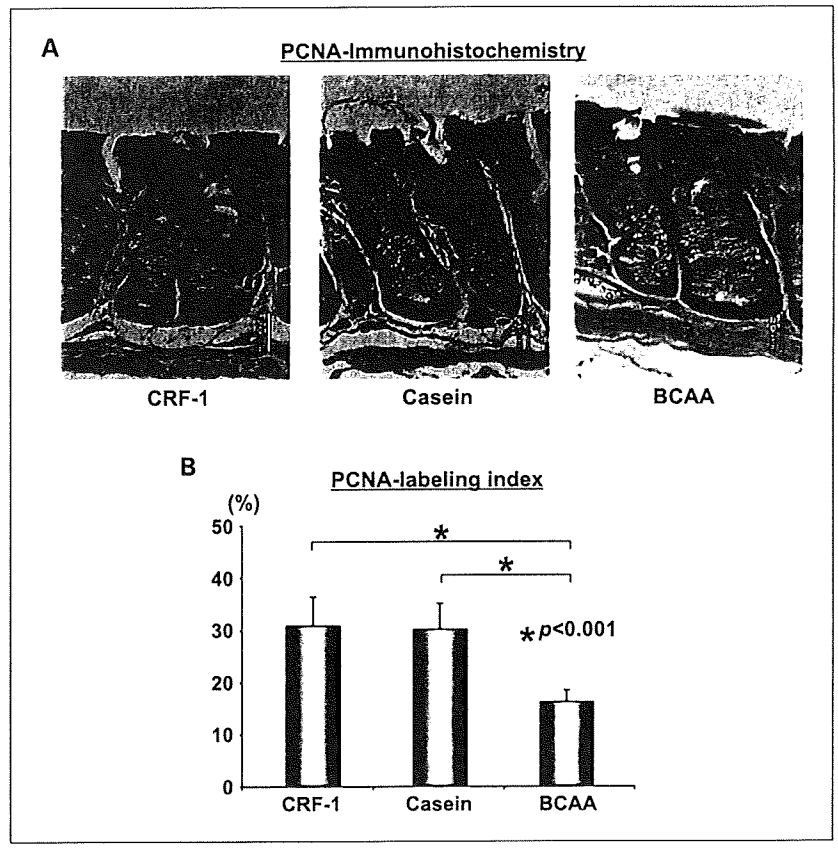

Fig. 3. The effect of BCAA supplementation on colonic epithelial cell proliferation in AOM-treated *db/db* mice. *A*, immunohistochemical staining of the normal crypts in the colon of AOM-treated *db/db* mice with anti-PCNA antibody. Sections of the colon were analyzed from CRF-1 – fed, casein-supplemented, and BCAA-supplemented mice, respectively. They were stained with anti-PCNA monoclonal antibody as described in Materials and Methods. Representative photographs from each group are shown. Bar, 20 μm. *B*, PCNA labeling index in the normal crypts in the colon of AOM-treated *db/db* mice. *Bars*, SD of triplicate assays.

(Table 2) by improving hyperlipidemia and hyperleptinemia in *db/db* mice (Table 3). The suppressive effect of BCAA in the early phase of obesity-related colorectal carcinogenesis was also associated, most likely, with the improvement of hyperinsulinemia (Fig. 2A) and the inhibition of cell proliferation on the colonic mucosa of experimental mice (Fig. 3). BCAA supplementation has also been reported to significantly decrease the incidence of hepatocellular carcinoma in patients with chronic liver disease if they had a body mass index score ≥25, and this effect might be associated with improvement of insulin resistance (15, 16, 31). Thus, BCAA might effectively prevent cancer development, at least in several organs, in obese subjects who are considered to have insulin resistance syndrome (3).

How can BCAA exert chemopreventive effects on obesity-related colorectal carcinogenesis? As described above, insulin resistance might be a critical target of BCAA in this beneficial effect because insulin has oncogenic properties on CRC cells. For instance, insulin stimulates the proliferation of CRC cells and promotes colorectal tumor growth in animal models (6–8). These reports, therefore, suggest that BCAA inhibits the development of colonic premalignant lesions (Table 2) and excessive cell proliferation in the colonic mucosa of AOM-injected *db/db* mice (Fig. 3) by improving insulin resistance (Fig. 2A). Recent studies by others have indicated that BCAA improves glucose tolerance by modulating insulin-independent glucose uptake into skeletal muscle in rodent models (32, 33). An improvement of insulin resistance and glucose tolerance by BCAA has also been shown by certain clinical trials (15, 31).

In addition, it is widely accepted that insulin resistance causes alterations in the IGF/IGF-IR axis, which may be closely associated with the development of CRC (9, 10, 30). For instance, the IGF-IR protein is overexpressed in BCAC compared with the surrounding normal cryptal cells (11). Therefore, the IGF/IGF-IR system is regarded as one of the effective targets with respect to the prevention of CRC (11). Our observations described herein comprise the first report showing that BCAA decreases the serum levels of IGF-I and IGF-II (Fig. 2B and C), thereby inhibiting the expression and activation of IGF-IR on the colonic mucosa of AOM-treated *db/db* mice (Fig. 2D). Our findings suggest that not only the improvement of insulin resistance but the inhibition of IGF/IGF-IR activation by BCAA plays a critical role in suppressing obesity-related and diabetes mellitus–related colorectal carcinogenesis.

The present study revealed that BCAA supplementation in the diet prevents the development of BCAC (Table 2), which is characterized by abundant β-catenin protein expression (23) and also accumulates the IGF-IR protein (11) while decreasing the expression levels of p-Akt and p-GSK-3β proteins on the colonic mucosa of AOM-treated *db/db* mice (Fig. 2D). Recent *in vitro* studies have indicated that insulin and the IGF/IGF-IR axis stabilize and activate the Wnt/β-catenin pathway, which is involved in the development of CRC (34, 35). GSK-3β, which can be phosphorylated by phosphatidylinositol 3-kinase/Akt via insulin or IGF treatment, is considered to be a key kinase for CRC development because the inactivation of GSK-3β leads to the dissociation of the adenomatous polyposis coli/axin/β-catenin complex and cytosolic β-catenin accumulation (36).

Free accumulated β-catenin translocates into the nucleus and forms a complex with the transcription factor T cell factor, thereby activating the transcription of target genes, including cyclin D1 and c-Myc, and thus contributing to abnormal proliferation and tumor progression (37, 38). Therefore, supplementation with BCAA, which targets insulin-associated and IGF-associated β-catenin accumulation by decreasing the levels of p-Akt and p-GSK-3β proteins (Fig. 2D), might be an effective strategy to prevent the development of CRC.

In addition to the beneficial effects mentioned above, BCAA has other physiologic activities that might be useful to prevent the development of CRC. For instance, supplementation with BCAA is capable of reducing the production of oxidative stress and microinflammation in patients with liver cirrhosis, which possibly leads to a decrease in the occurrence of hepatocellular carcinoma (39). In the current study, BCAA caused a decrease in the expression of the COX-2 protein in the colonic mucosa of AOM-treated *db/db* mice (Fig. 2D). COX-2 is one of the main mediators in the inflammatory signaling pathway and is certainly involved in CRC development; therefore, it might be a critical target for CRC chemoprevention (40). This effect might be explained by the inhibitory effect of BCAA on the IGF/IGF-IR axis because the activation of this axis mediates COX-2 expression (41, 42). Additional studies are required to clarify the direct effects of BCAA on inflammation and their relevance to the antitumor effects of this agent.

In summary, the prevention of CRC by targeting the dysregulation of energy homeostasis, especially insulin resistance and the activation of the IGF/IGF-IR axis, might be a promising strategy for obese people who are at an increased risk of CRC. BCAA seems to be a potentially effective and critical candidate for this purpose because this agent can improve insulin resistance while also exerting a depressant effect on the IGF/IGF-IR axis. The current findings, as well as those from a previous report (11), also suggest the possibility of using specific agents that target insulin resistance as chemopreventive agents for other obesity-related and diabetes mellitus–related malignancies. Therefore, insulin resistance–improving agents, including BCAA, are worthy of being further investigated as candidates for novel chemopreventive agents that may find a potential role in the society today, in which excessive body weight has been found to be associated with the risk of various types of human epithelial malignancies (43, 44).

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

We thank Yukari Nomura for her excellent technical assistance.

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