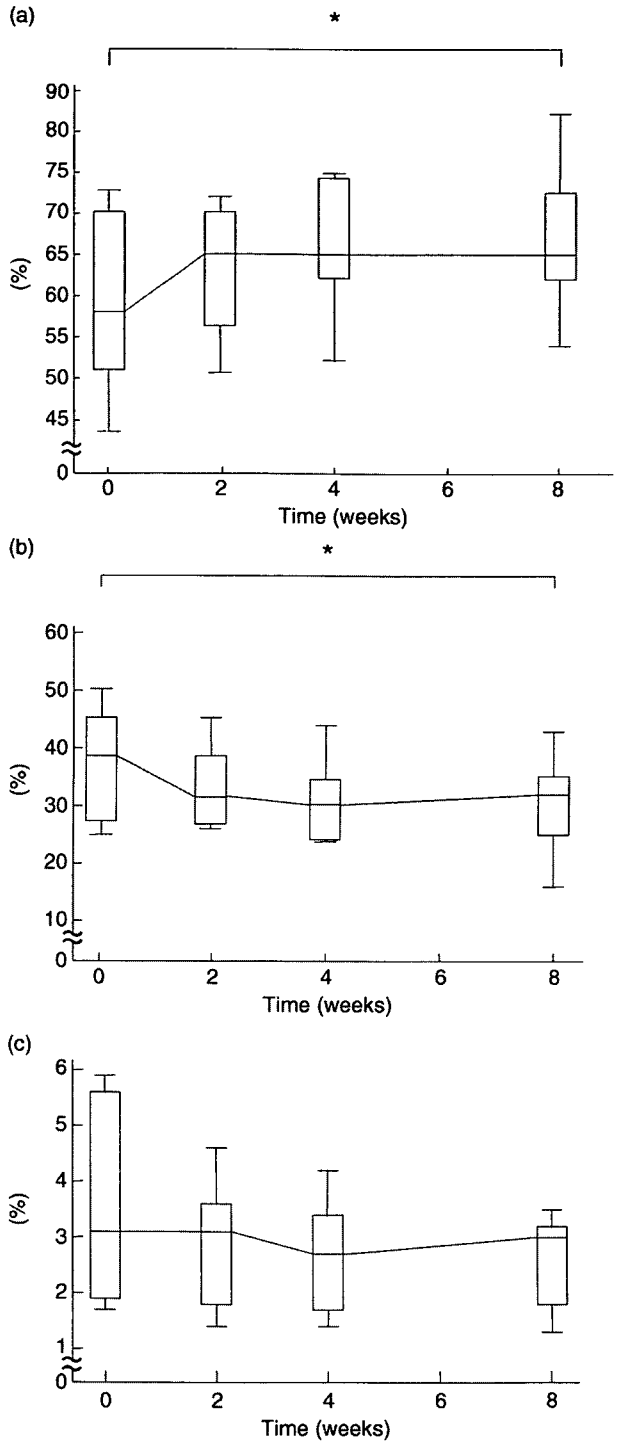

Figure 2 Changes in (a) HMA, (b) HNA-1 and (c) HNA-2 after branched-chain amino acid (BCAA) supplementation in cirrhotics. Vertical bars indicate the range and the boxes represent 25, 50 and 75 percentile values. HMA, human mercaptalbumin; HNA, human non-mercaptalbumin. $^*P < 0.05$ by the Wilcoxon's test.

induces oxidative stress and promotes disease progression.[9,10] In assessing hepatic fibrosis in previous cohort studies,[17] obesity and presence of superimposed NASH were independently associated with the stage of fibrosis. The oxidative stress associated with insulin resistance syndrome could result in the production of ROS, and peroxidation of membrane lipids produces toxic by-products.[18] Such a process could exaggerate the ongoing injury from chronic hepatitis C, further promoting hepatic fibrosis and carcinogenesis.[19] In our study, LPO tended to decrease after BCAA supplementation. Such a decrease might be derived from an increased proportion of reduced albumin. Although again being speculative, this action might partly explain the prevention of liver carcinogenesis by BCAA in a recent report.[19]

The mechanism of the rise in reduced albumin with BCAA has never been understood well, but our hypothesis is as follows. In liver cirrhosis, the whole-body turnover kinetics of albumin is impaired (i.e. synthesis and degradation rates are reduced), and biological half-life is prolonged.[20] In addition, there is high oxidative stress in intracellular and extracellular fluids in chronic liver disease as represented by increased ratios of 8-OhdG/Cr and LPO/Cr (Miwa *et al.*, 2005, unpubl. obs). Therefore, longer exposure to higher oxidative stress alters serum albumin from reduced form to oxidized form. BCAA supplementation recovers the impaired turnover of albumin, leading to an increased production rate and near normal half-life.[19] As albumin is synthesized and secreted by hepatic parenchymal cells as reduced form, serum albumin might recover a higher proportion of reduced form after BCAA supplementation. One important finding is Zn-reduction during BCAA treatment as shown in Table 2. Zinc acts at multiple steps in amino acid- and insulin-regulated intracellular signaling pathways, including mTOR.[21] Thus, enhanced protein synthesis might have promoted the consumption of Zn.

In conclusion, BCAA supplementation in cirrhotic patients reduces the oxidized albumin and raises the reduced albumin. In addition, reduced albumin is known to scavenge intravascular ROS. Taken together, BCAA supplementation is effective in leading to not only a quantitative rise but also a favorable qualitative alteration of serum albumin in patients with liver cirrhosis. Thus, long-term BCAA supplementation could prevent the disease progression, but this possible effect needs to be confirmed in prospective studies.

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Characterization of CD133$^+$ hepatocellular carcinoma cells as cancer stem/progenitor cells ☆

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Received 22 September 2006
Available online 2 November 2006

Abstract

The CD133 antigen, identified as a hematopoietic stem cell marker, appears in various human embryonic epithelia including the neural tube, gut, and kidney. We herein investigated whether CD133$^+$ cells isolated from human hepatocellular carcinoma cell lines possess cancer stem/progenitor cell-like properties. Among the three cell lines studied, the CD133 antigen was found to be expressed only on the surface of Huh-7 cells. CD133$^+$ cells from Huh-7 performed a higher *in vitro* proliferative potential and lower mRNA expressions of mature hepatocyte markers, glutamine synthetase and cytochrome P450 3A4, than CD133$^-$ population of Huh-7 cells. When either CD133$^+$ or CD133$^-$ cells were subcutaneously injected into SCID mice, CD133$^+$ cells formed tumors, whereas CD133$^-$ cells induced either a very small number of tumors or none at all. Taken together, the identification of CD133$^+$ cells could thus be a potentially powerful tool to investigate the tumorigenic process in the hepatoma system and to also develop effective therapies targeted against hepatocellular carcinoma.

Keywords: CD133; Stem/progenitor cells; Hepatocellular carcinoma

Cancer is believed to arise from a series of sequential mutations that occur as a result of genetic instability and/or environmental factors [1–3]. On the other hand, only a small proportion of tumor cells have been demonstrated to be able to form colonies *in vitro* [4–6]. To distinguish between these potentials, it is therefore necessary to identify the clonogenic cells in these tumors with markers that distinguish these cells from other nontumorigenic cells. In the hematopoietic system, stem/progenitor cells can be isolated using monoclonal antibodies against cell surface markers that identify clonal self-renewing and multipotent rare subpopulations [7,8]. Many tumors may also have their origins in normal stem cells. Stem cell biology could provide new insights into cancer biology, thus leading us to the notion that tumors might contain cancer stem cells (CSCs) with an indefinite proliferative potential that drive the formation and growth of tumors [9] In human leukemia, tumor clones are organized as a hierarchy that originates from rare leukemia stem cells. Such cells possess extensive proliferative and self-renewal potential and are responsible for maintaining the tumor clone [9,10]. Hepatocellular carcinoma (HCC) is typically comprised of morphologically diverse cells that express a variety of hepatic lineage markers. It has recently been reported that subpopulations with CSCs function exist in some HCC cell lines [11].

☆ *Abbreviations:* CSCs, cancer stem cells; HCC, hepatocellular carcinoma; SCID, severe combined immunodeficient; FITC, fluorescein isothiocyanate; PE, phycoerythrin; APC, allophycocyanin; Alb, albumin; AFP, alfa-fetoprotein; GS, glutamine synthetase; CYP3A4, cytochrome P450 3A4.

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doi:10.1016/j.bbrc.2006.10.128

The monoclonal antibody against CD133 recognizes the CD 133 antigen: a five-transmembrane glycoprotein, localized to either membrane protrusions or microvilli. Antibodies to CD133 have been used to isolate human hematopoietic stem cells [12], endothelial stem cells, neurons, and glial stem cells [13]. CD133 is also expressed by the intestine-derived epithelial cell line Caco-2 where it is down-regulated upon differentiation [14]. Some investigators reported that $CD133^+$ cells represent a more primitive cell population than their $CD34^+$ counterparts [12,15]. Recently, $CD133^+$ cells in medulloblastoma and glioblastoma were prospectively isolated as a subpopulation that exhibits stem cell properties *in vitro* [16,17]. However, little is known about the implications of CD133 in HCC.

We herein investigated whether $CD133^+$ cells isolated from human HCC cell lines possess cancer stem/progenitor cell-like properties in culture and in severe combined immunodeficient (SCID) mice. Our findings provide a novel model of hepatoma biology in which a defined subset of cells drives tumorigenesis, as well as generating tumor cell heterogeneity. Identification of this tumorigenic population of cancer cells should allow the search for molecules expressed in these cells that could serve as targets to eliminate this critical population of cancer cells.

Materials and methods

Cell lines and culture conditions. Huh-7 (a human hepatoma cell line) and HepG2 cells (a human hepatoblastoma cell line) obtained from the Japanese Collection of Research Bioresources (Tokyo, Japan) were cultured in RPMI 1640 medium (Sigma–Aldrich, Irvine, UK) containing 1% penicillin and streptomycin (Gibco-BRL, Rockville, MD, USA), 12.5 mM Hepes buffer (Sigma–Aldrich), and supplemented with 0.2% lactoalbumin (Sigma–Aldrich) or 10% heat-inactivated fetal bovine serum (Sigma–Aldrich), respectively. Hc cells (a human fetal hepatocyte cell line) obtained from Applied Cell Biology Research Institute (Kirkland, WA, USA) were cultured in CS–C complete medium (Cell System, Kirkland, WA, USA) supplemented with 1% penicillin and streptomycin (Gibco-BRL).

Flow cytometry. The expression of surface markers was analyzed with two or three-color FACS using a FACS-vantage flow cytometer (Becton–Dickinson, San Jose, CA, USA). The cells were incubated at 4 C for 30 min with phycoerythrin (PE)-conjugated anti-CD133/2 (Miltenyi Biotec, Bergisch Gladbach, Germany) in combination with fluorescein isothiocyanate (FITC)-conjugated anti-CD34 (Miltenyi Biotec) following treatment with FcR blocking reagent (Miltenyi Biotec) to inhibit unspecific or Fc receptor mediated binding of antibodies to nontarget cells. For the three-color FACS analysis allophycocyanin (APC)-conjugated anti-CD117 (Miltenyi Biotec), PE-conjugated anti-CD29 (BD Pharmingen), and FITC-conjugated anti-CD44 (BD Pharmingen) were used. Dead cells were eliminated using 0.5 μg/ml propidium iodide (PI) (Calbiochem, San Diego, CA, USA). The labeled cells were analyzed and separated with FACS-vantage, and then the data were analyzed using CELLQuest software (Becton–Dickinson Immunocytometry Systems, San Jose, CA, USA). Gating was implemented based on negative-control staining profiles.

Cell proliferation assays. Cell proliferation was examined on days 0, 3, 5, and 7 after inoculation by means of a cell proliferation assay using Cell Counting Kit (Wako, Tokyo, Japan) according to the manufacturer's instructions. The optical density was measured using the Original Multiskan JX (Thermo Electron Corporation, San Jose, CA, USA) at a wavelength of 570 nm.

Reverse-transcription polymerase chain reaction analysis (RT-PCR). Total RNA was isolated from cells using ISOGEN (Wako). cDNA was synthesized from 1 μg of total RNA, with an oligo(dT) primer and Moloney Murine Leukemia Virus (M-MLV) reverse transcriptase (Gibco-BRL) according to the manufacturer's instructions. The cDNA samples were subjected to PCR amplification with specific primers. PCR was performed under linear conditions in order to reflect the original

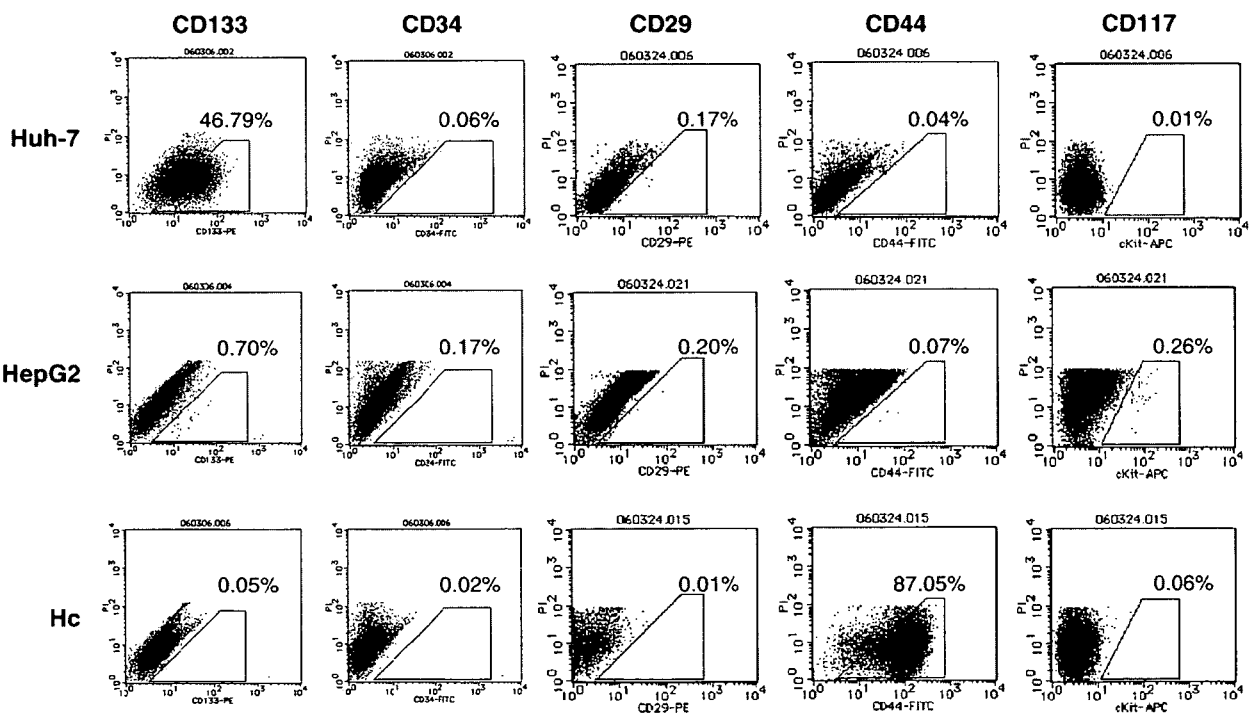

Fig. 1. The expression of cell surface markers was examined by FACS analysis. Huh-7, HepG2, and Hc cells were stained with fluorescence-conjugated anti-CD133, CD34, CD29, CD44, and CD117. The results shown are representative of at least five independent experiments.

amount of the specific transcripts. The PCR primers were as follows; albumin (Alb) (sense, 5′-CTCGATGAACTTCGGGATGA-3′; antisense 5′-AGCCTAAGGCAGCTTGACTT-3′), alfo-fetoprotein (AFP) (sense, 5′-CAGCGGCTGACATTATTATC-3′; antisense 5′-AAGCAGCAC GAGTTTTTGAA-3′), glutamine synthetase (GS) (sense, 5′-CCTGCTT GTATGCTGGAGTC-3′; antisense 5′-GAAAAGTCGTTGATGTTG GA-3′), cytochrome P450 3A4 (CYP3A4) (sense, 5′-CAAGACCCCT TTGTGGAAAA-3′; antisense 5′-TGCAGTTTCTGCTGGACATC-3′), β-actin (sense, 5′-GGGCATGGGGTCAGAAGGATT-3′; antisense 5′-GAGGCGTACAGGGATAGCAC-3′). Each PCR cycle consisted of a denaturation step for 1 min at 94 °C, an annealing step for 1 min at 58 °C (Alb), 57 °C (AFP), 59 °C (GS), 59 °C (CYP3A4), and 63 °C (β-actin), and an extension step for 2 min at 72 °C.

Cell transplantation into the SCID mice. To determine the ability of the cells separated by FACS-vantage for hepatic morphogenesis, growth, and functional cytodifferentiation *in vivo*, 1×10^7 $CD133^+$ or $CD133^-$ cells were resuspended in 200 μl PBS after sorting and injected subcutaneously into 6-week-old SCID female mice (CLEA, Tokyo, Japan). The grafts were removed at 6 weeks after transplantation, fixed with 10% buffered formalin, sectioned at a thickness of 4 μm, and stained with hematoxylin and eosin. To examine the expression of CD133 and CD34 in the transplanted cells, the grafts were treated with collagenase, and then the isolated cells were analyzed by FACS. The experiments were all conducted in accordance with the institutional guidelines established by the Gifu University Graduate School of Medicine.

Statistical analysis. All values in the figures and text are expressed as means ± SD. Any significance of differences among mean values was evaluated by Student's *t* test.

Results

FACS analysis for the cell surface markers on human HCC cell lines

Huh-7, HepG2, and Hc cell lines were stained with fluorescence-conjugated primary antibodies against surface markers for hematopoietic stem cell (CD34, CD133), steel factor receptor (CD29, CD44), or stem cell factor receptor c-Kit (CD117), and were subjected to flow cytometry. HepG2 and Hc did not contain either $CD34^+$ or $CD133^+$ cells, but $CD133^+$ cells were detected in 46.7% of Huh-7 cells (Fig. 1). Only Hc cells were stained for CD44. Treatment with growth factors such as hepatocyte growth factor or epidermal growth factor did not affect the stains of the surface markers on either HepG2, Huh-7, or Hc cells (data not shown).

Characterization of $CD133^+$ HCC cells in vitro

To evaluate the difference in the *in vitro* proliferative activity and differentiation between $CD133^+$ cells and $CD133^-$ cells of Huh-7-origin, we determined the viable cell number

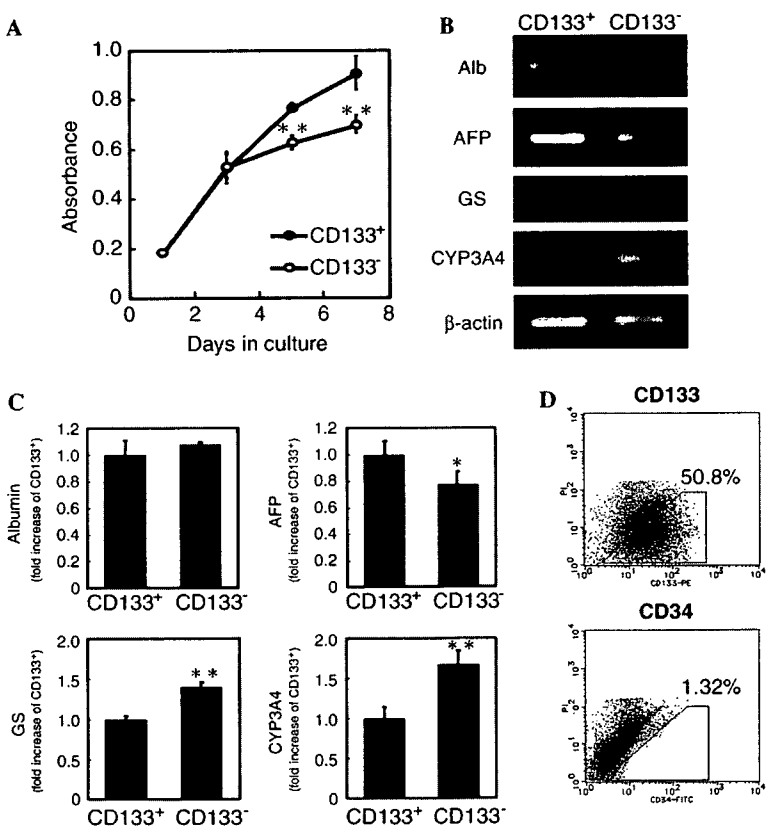

Fig. 2. (A) The proliferation was compared between $CD133^+$ (closed circle) and $CD133^-$ (open circle) subpopulations of Huh-7 using a cell proliferation assay. Huh-7 cells were sorted with anti-CD133 antibody and then subpopulations were plated to culture dishes. The optical density was determined at the indicated time points. The data are means ± SD from three independent experiments. $^{**}P < 0.01$ $CD133^+$ versus $CD133^-$ cells. (B) mRNA expressions of Alb, AFP, GS, CYP3A4, and β-actin were examined by RT-PCR. (C) A densitometric scan of the RT-PCR band was performed using the NIH Image program. The indicated mRNA/β-actin ratio is shown as a fold of $CD133^+$. The data are means ± SD from three independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$ compared with $CD133^+$ cells. (D) $CD133^+$ Huh-7 cells were cultured for 7 days. The expression levels of CD133 and CD34 were examined by FACS analysis. The results shown are representative of at least five independent experiments.

using a cell proliferation assay and gene expression using RT-PCR. $CD133^+$ cells showed higher proliferative activities than $CD133^-$ cells at 5 days after plating (Fig. 2A). $CD133^-$ cells also showed the mRNA expressions of hepatocyte-specific genes including GS and CYP3A4 to a greater extent than $CD133^+$ cells (Fig. 2B and C). On the other hand, the AFP mRNA level was significantly increased in the $CD133^+$ cells. These findings indicate that $CD133^+$ cells may therefore represent a less differentiated subpopulation.

To evaluate the self-renewal potential of $CD133^+$ cells, flow cytometry was performed to characterize the phenotype of $CD133^+$ Huh-7 cells that were cultured for 7 days. FACS analysis revealed that the cultured cells consisted of 50.8% $CD133^+$ cells and 49.2% $CD133^-$ cells (Fig. 2D). This proportion was consistent with the initial composition of Huh-7 cells.

Tumor formation and self-renewal of $CD133^+$ HCC cells in vivo

To determine whether $CD133^+$ Huh-7 cells were capable of tumor initiation *in vivo*, we compared the abilities of $CD133^+$ versus $CD133^-$ HCC cells to initiate tumor formation in SCID mice. $CD133^+$ cell injection could initiate significantly larger tumors ($n = 4$), which diameter was at least 10 mm (Fig. 3A), while $CD133^-$ cell injection ($n = 5$) showed small or no tumors ($P < 0.001$, Fig. 3B). A histological examination at 6 weeks showed a highly cellular mass below the $CD133^+$ cell injection site (Fig. 3C).

To elucidate whether $CD133^+$ cells self-renew and generate $CD133^-$ cells *in vivo*, a flow cytometry analysis of $CD133^+$ engraftment was thus conducted after dissociation (Fig. 3D). This analysis revealed that the tumor cells were consisted of 65.4% $CD133^+$ and 95.3% $CD34^-$ cells. The expression patterns of cell surface markers resembled those of the original Huh-7 cells and the $CD133^+$ Huh-7 cells cultured for 7 days *in vitro*.

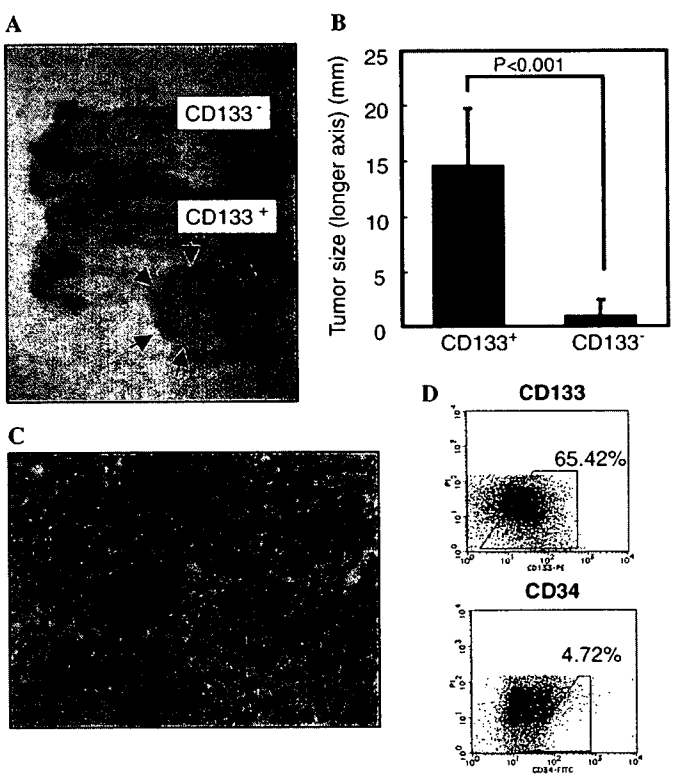

Fig. 3. $CD133^+$ and $CD133^-$ cells were isolated and injected into the subcutaneous space of SCID mice. (A) The photograph of the mice was taken at 6 weeks after cell transplantation. (B) Tumor size (longer axis) was compared between $CD133^+$ and $CD133^-$ groups at 6 weeks after injection. The data are means ± SD from for 4–5 mice. (C) The grafts of the $CD133^+$ Huh-7 cells were excised at 6 weeks after transplantation and then were stained with H.E. (original magnification 100×). (D) The grafts were treated with collagenase and the expression of CD133 and CD 34 of the isolated cells was examined by FACS analysis. The results shown are representative of five independent experiments.

Discussion

Stem cells are functionally defined as self-renewing and multipotent cells that exhibit multilineage differentiation [18,19]. Somatic stem/progenitor cells are thought to self-renew in order to generate all of the mature cell types of a particular tissue through differentiation. However, the rigorous identification and isolation of tissue-specific stem/progenitor cells has so far been accomplished in only a few organ systems [9,20], because a prospective such study on somatic stem cells has been previously limited by a lack of cell surface markers necessary for their isolation. In malignancies such as leukemia [10,21] multiple myeloma [22], brain tumors [16,17], and most recently breast cancer [23], rare cells were isolated with a remarkable potential for self-renewal, and these cells alone were found to drive the formation and growth of tumors. In this study, by cell sorting for the cell surface antigen CD133, we demonstrated that a functional hierarchy existed in the HCC cell population *in vitro* and that the $CD133^+$ cells were capable of tumor initiation *in vivo*.

$CD133^+$ Huh-7 cells possess the characteristics suggesting that these cells are hepatoma progenitor/superior of hierarchy cells due to the following reasons: (a) they self-renew and proliferate; and (b) they differentiate to recapitulate the phenotype of the tumor from which they were derived. Most current studies on human brain tumors have demonstrated that $CD133^+$ cells isolated from human brain tumors represent the capacity for self-renewal, exact recapitulation of the original tumor, and tumorigenesis [17]. These cells can differentiate into cells with neural and glial phenotypes *in vitro* in proportions resembling the original tumor, and their proliferative capacity is also proportional to the aggressiveness of the original tumor. As few as 100 $CD133^+$ cells from medulloblastomas can produce tumors in NOD/SCID mice that were serially transplantable and identical to the original tumor [17]. Our findings suggest that CD133 may mark CSCs not only in brain tumors but also in HCC.

Chiba et al. have recently demonstrated that a minor population, detected as side population cells in HCC cells,

possesses extreme tumorigenic potential and provides heterogeneity to the cancer cell system [11]. It has also been currently reported that side population cells show some characteristics of stem cells [24]. Bonnet and Dick showed a small subset of acute myeloid leukemia, $CD34^{+}CD38^{-}$, to be capable of producing leukemic progenitors and leukemic blasts upon transplantation into immunodeficient mice [10]. In addition, CSCs have also been demonstrated in various solid tumors. In breast cancer, a subpopulation of cells with the surface antigen expression pattern of $ESA^{+}CD44^{+}CD24^{-/low}$ has also been shown to be capable of tumor formation [23]. It seems likely that CSCs exist in many tumors. The further characterization of a subset of CSCs in individual tumors may ultimately allow specific therapeutic targeting. In the present study, we demonstrated that CD133 could serve as a target to identify cancer stem/progenitor cells in a portion of HCCs.

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Synergistic growth inhibition by acyclic retinoid and vitamin K_2 in human hepatocellular carcinoma cells

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(Received August 23, 2006/Revised November 6, 2006/Accepted November 9, 2006/Online publication December 22, 2006)

Hepatocellular carcinoma (HCC) is one of the most prevalent cancers worldwide. However, effective chemopreventive and chemotherapeutic agents for this cancer have not yet been developed. In clinical trials acyclic retinoid (ACR) and vitamin K_2 (VK_2) decreased the recurrence rate of HCC. In the present study we examined the possible combined effects of ACR or another retinoid 9-*cis* retinoic acid (9cRA) plus VK_2 in the HuH7 human HCC cell line. We found that the combination of 1.0 μM ACR or 1.0 μM 9cRA plus 10 μM VK_2 synergistically inhibited the growth of HuH7 cells without affecting the growth of Hc normal human hepatocytes. The combined treatment with ACR plus VK_2 also acted synergistically to induce apoptosis in HuH7 cells. Treatment with VK_2 alone inhibited phosphorylation of the retinoid X receptor (RXR)α protein, which is regarded as a critical factor for liver carcinogenesis, through inhibition of Ras activation and extracellular signal-regulated kinase phosphorylation. Moreover, the inhibition of RXRα phosphorylation by VK_2 was enhanced when the cells were cotreated with ACR. The combination of retinoids plus VK_2 markedly increased both the retinoic acid receptor responsive element and retinoid X receptor responsive element promoter activities in HuH7 cells. Our results suggest that retinoids (especially ACR) and VK_2 cooperatively inhibit activation of the Ras/MAPK signaling pathway, subsequently inhibiting the phosphorylation of RXRα and the growth of HCC cells. This combination might therefore be effective for the chemoprevention and chemotherapy of HCC. (*Cancer Sci* 2007; 98: 431–437)

HCC is a major healthcare problem worldwide because it is the fifth leading cause of cancer mortality and the third most common cause of cancer-related death.[1] The development of HCC is frequently associated with chronic inflammation of the liver induced by a persistent infection with the hepatitis B virus or hepatitis C virus. Owing to the high incidence of recurrence, the prognosis for patients with HCC is poor. Therefore, even in early stage cases when surgical treatment might be expected to be curative, the incidence of recurrence in patients with underlying cirrhosis is approximately 20–25% every year.[2,3] In addition, at least, one-third of the secondary tumors are primary *de novo* cancers.[4] Therefore, strategies to prevent a second primary HCC are required to improve the prognosis for patients with HCC.[5] However, at the present time there are no established chemopreventive and chemotherapeutic agents for HCC.

Retinoids, a group of structural and functional analogs of vitamin A, exert fundamental effects on the regulation of epithelial cell growth, differentiation and development.[6,7] Retinoids exert their biological function primarily through two distinct nuclear receptors, RAR and RXR, both of which are composed of three subtypes (α, β and γ).[6,7] Nuclear retinoid receptors are ligand-dependent transcription factors that bind to RARE and RXRE, which are present in the promoter regions of retinoid responsive target genes.[6,7] Abnormalities in the expression and function of both RAR and RXR play an important role in influencing the growth of various cancers. Indeed, we previously found a malfunction of RXRα, due to post-translational modification by phosphorylation, to be associated with carcinogenesis in the liver.[8,9] In addition, we previously reported that ACR, a novel synthetic retinoid, inhibits experimental liver carcinogenesis and induces apoptosis in human HCC-derived cells.[10–12] A clinical trial demonstrated that the administration of ACR reduced the incidence of a post-therapeutic recurrence of HCC and improved the survival rate of patients, without causing significant adverse side-effects.[13–15] It is also of interest that ACR acts synergistically with interferon and OSI-461, a potent derivative of sulindac sulfone, in suppressing growth and inducing apoptosis in human HCC-derived cells.[16,17] Therefore, ACR may be a valuable agent in the chemoprevention and chemotherapy of HCC and its efficacy may be enhanced by combination with agents that target other signaling pathway in hepatoma cells.

Recent studies have reported that VK_2 can exert growth-inhibitory effects in a variety of human cancer cells, including HCC.[18–20] A recent clinical trial demonstrated that oral administration of VK_2 reduced the development and recurrence rates of HCC, thus improving the overall survival of patients.[21,22] Although the precise mechanism of how VK_2 exerts its growth-inhibitory effects on cancer cells has not yet been determined, there are some reports that the combined treatment of VK_2 plus retinoid exerts synergistic anticancer effects.[23,24] In view of these observations, there has been considerable interest in utilizing the combination of ACR and VK_2 for the prevention and treatment of HCC. The aim of the present study is to investigate whether the combination of retinoids, ACR or 9cRA, plus VK_2 exerts synergistic growth-inhibitory effects on human HCC cells and to examine possible mechanisms for such synergy. In the present study, we examined in detail the effects of such combined treatments on the inhibition of cell growth in HuH-7 human HCC cells, which express high levels of phosphorylated forms of p-RXRα with a focus on the Ras/MAPK signaling pathway.[8]

Materials and Methods

Materials. ACR (NIK333) was supplied by Kowa Pharmaceutical Company (Tokyo, Japan). 9cRA was purchased from Sigma Chemical Co. (St Louis, MO, USA). VK_2 was from Eisai Co. (Tokyo, Japan). Polyclonal anti-RXRα (DN197) antibody was from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Polyclonal anti-ERK antibody and polyclonal anti-p-ERK antibody were from Cell Signaling Technology (Beverly, MA, USA).

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Abbreviations: 9cRA, 9-*cis*-retinoic acid; ACR, acyclic retinoid; ERK, extracellular signal-regulated kinase; FCS, fetal calf serum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HCC, hepatocellular carcinoma; MAPK, mitogen-activated protein kinase; p-ERK, phospho-ERK; PKA, protein kinase A; p-RXRα, retinoid X receptor protein; RAR, retinoic acid receptor; RARE, retinoic acid receptor responsive element; RXR, retinoid X receptor; RXRE, retinoid X receptor responsive element; SXR, steroid and xenobiotic receptor; TUNEL, terminal deoxynucleotidyl transferase-meditated dUTP nick-end labeling; VK_2, vitamin K_2.

doi: 10.1111/j.1349-7006.2006.00384.x

Monoclonal antibody against GAPDH was from Chemicon International (Temecula, CA, USA). RPMI-1640 media and FCS were both from Invitrogen (Carlsbad, CA, USA). CS-C complete medium was from CellSystems Biotechnologie Vertrieb (St Katharinen, Germany).

Cell lines and cell culture. The HuH7 human HCC cell line was obtained from the Japanese Cancer Research Resources Bank (Tokyo, Japan) and was maintained in RPMI-1640 medium supplemented with 10% FCS. The Hc human normal hepatocyte cell line was purchased from Applied Cell Biology Research Institute (Kirkland, WA, USA) and was maintained in CS-C complete medium. Cells were cultured in an incubator with humidified air with 5% CO_2 at 37°C.

Cell proliferation assays. Two thousand HuH7 or Hc cells were seeded into 96-well plates in RPMI-1640 medium supplemented with 1% FCS or CS-C complete medium, respectively, and 72 h later they were treated with 10 μM 9cRA or 5 μM ACR in the presence or absence of 10 μM VK_2. As an untreated solvent control, the cells were treated with ethanol (Sigma Chemical Co.) at a final concentration of 0.05%. The numbers of viable cells in replica plates were then counted using the Trypan Blue dye exclusion method, as described previously.[16] All assays were carried out in triplicate, and mean values were plotted. To determine whether the combined effects of retinoids plus VK_2 were synergistic, HuH7 cells were treated with ACR alone, 9cRA alone, VK_2 alone, or a combination of the indicated concentrations of these agents for 72 h, and the combination index-isobologram was then calculated and used in the drug combination assays.[17,25]

TUNEL assays. HuH7 cells were treated with 1.0 μM 9cRA or 1.0 μM ACR in the presence or absence of 10 μM VK_2 for 48 h on coverslips. The cells were fixed with 3.7% formaldehyde at room temperature for 10 min, permeabilized with 0.3% Triton X-100 in Tris-buffered saline (pH 7.4), and then stained using a TUNEL method with the *In Situ* Cell Death Detection Kit, Fluorescein (Roche Diagnostics, Mannheim, Germany), as described previously.[16]

Protein extraction and western blot analysis. Total cellular protein was extracted and equivalent amounts of protein were examined by western blot analysis using specific antibodies, as described previously.[8] The protein concentrations in the samples were determined using the BCA Protein Assay Reagent Kit (Pierce, Rockford, IL, USA). For detection of its expression levels, p-RXRα was affinity purified from the total cell extracts using anti-RXRα antibody-immobilized Sepharose beads and was then subjected to western blot analysis using an antiphosphoserine antibody.[8] An antibody to GAPDH was used as a loading control. Each membrane was developed using an ECL™ Western blotting detection reagents (Amersham Biosciences, Piscataway, NJ, USA). The intensities of the blots were quantified using the NIH image software package version 1.61.

Ras activation assay. The ras activities were determined using a Ras activation assay kit (Upstate Biotechnology, Lake Placid, NY, USA) according to the manufacturer's instructions. Ras was precipitated in equivalent amounts of cell extract (15 μg) using Raf-1/Ras-binding domain-immobilized agarose and was then subjected to western blot analysis using an anti-Ras antibody.[8]

RARE and RXRE reporter assays. Reporter assays were carried out as described previously.[8] The RXRE–luciferase reporter plasmid tk-CRBP II-RXRE-Luc and the RARE–luciferase reporter plasmid tk-CRBP II-RARE-Luc were kindly provided by the late Dr K. Umesono (Kyoto University, Kyoto, Japan). The HuH7 cells were transfected with the RXRE or RARE reporter plasmids (750 ng/35 mm-dish), along with p*RL*-CMV (*Renilla* luciferase, 100 ng/35 mm-dish; Promega, Madison, WI, USA) as an internal standard to normalize the transfection efficiency. Transfections were carried out using the UniFector reagent (B-Bridge, Sunnyvale, CA, USA) according to the manufacturer's protocol. After exposure of the cells to the transfection mixture for 24 h, the cells were treated with vehicle, 1.0 μM 9cRA, or 1.0 μM ACR in the presence or absence of 10 μM VK_2 for 24 h. Thereafter, the cell lysates were prepared and the luciferase activity of each cell lysate was determined using a dual-luciferase reporter assay system (Promega), as described previously.[8] Changes in the firefly luciferase activity were calculated and plotted after normalization with changes in the *Renilla* luciferase activity in the same sample.

Statistical analysis. The data are expressed as the mean values ± SD. The statistical significance of differences in the mean values was assessed using one-way ANOVA, followed by Sheffe's *t*-test.

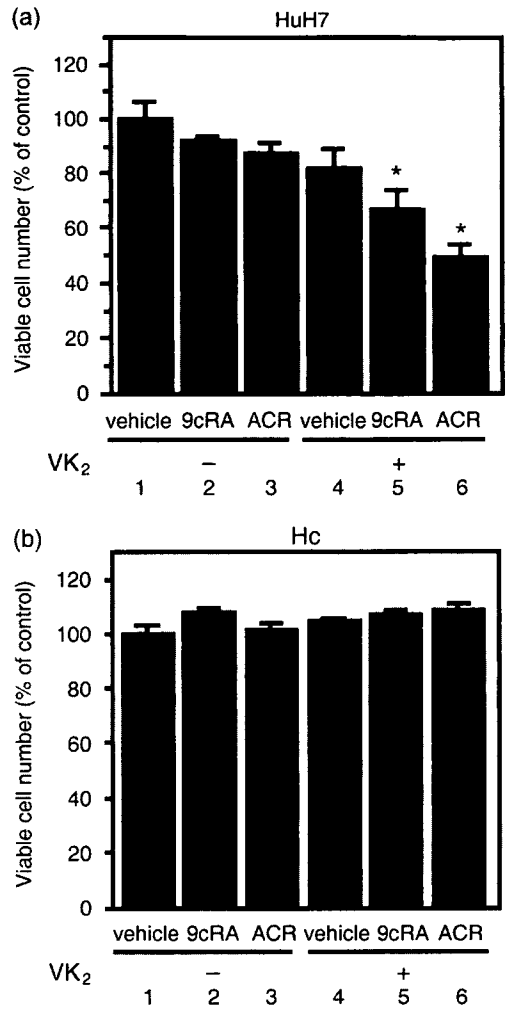

Fig. 1. Effect of retinoids and vitamin K_2 (VK_2) on the proliferation of (a) HuH7 human hepatocellular carcinoma cells and (b) Hc human normal hepatocytes. HuH7 cells and Hc cells were cultured for 72 h in the presence of vehicle (column 1), 10 μM 9-*cis*-retinoic acid (9cRA) (column 2), 5 μM acyclic retinoid (ACR) (column 3), 10 μM VK_2 (column 4), the combination of 10 μM VK_2 plus 10 μM 9cRA (column 5), or the combination of 10 μM VK_2 plus 5 μM ACR (column 6). Cell number was determined using the Trypan Blue dye exclusion method and expressed as a percentage vehicle. Values are the mean ± SD ($n = 5$). An asterisk represents significant differences ($P < 0.01$) compared to vehicle (column 1). Similar results were obtained in a repeat experiment.

Results

Combined treatment of retinoids plus VK_2 preferentially inhibits the growth of HuH7 human HCC cells. In our initial study we examined the growth-inhibitory effects of the combination of retinoids

doi: 10.1111/j.1349-7006.2006.00384.x

plus VK_2 in the HuH7 human HCC cell line and Hc normal human hepatocyte cell line (Fig. 1). We found that although 9cRA alone (10 μM), ACR alone (5 μM) or VK_2 alone (10 μM) could not inhibit the growth of HuH7 cells, the combination of ACR or 9cRA plus VK_2 significantly inhibited the growth of these HCC cells (Fig. 1a, columns 5 and 6). However, growth of the Hc cell line was not affected by treatment with 9cRA, ACR, VK_2 or the combination of these agents (Fig. 1b).

Retinoids plus VK_2 synergistically inhibits the proliferation of HuH7 cells. We then examined the effects of combined treatment with a range of concentrations of 9cRA or ACR plus VK_2 on the growth of HuH7 cells (Fig. 2a–c). We initially found that VK_2, ACR and 9cRA inhibited growth of the HuH7 cells with IC_{50} values of approximately 80 (Fig. 2a), 9.4 (Fig. 2b) and 33 μM (Fig. 2c), respectively, when the cells were grown in RPMI-1640 medium supplemented with 10% FCS. We also found that the combination of as little as 1.0 μM ACR or 1.0 μM 9cRA plus 10 μM VK_2 exerted synergistic growth inhibition. Therefore, when analyzed by the isobologram method,[17,25] the combination index for 1.0 μM ACR plus 10 μM VK_2 or for 1.0 μM 9cRA plus 10 μM VK_2 was less than 0.9, which indicates that there is a synergy between these combinations (data not shown).

We next examined whether retinoids might enhance the growth-inhibitive effect of VK_2 or, alternatively, whether VK_2 might amplify the antiproliferative effect of the retinoids (Fig. 2d). HuH7 cells were first exposed to either 1.0 μM retinoids or 10 μM VK_2 for 24 h, followed by incubation with the second respective agent for another 24 h. As shown in Fig. 2d, a significant reduction in the number of viable cells was found only when retinoids followed the VK_2 treatment (columns 5 and 8), but treatment with these agents in the reverse order did not yield a synergistic effect (columns 4 and 7). From these results, we speculated that VK_2 might enhance the sensitivity of the cancer cells to retinoids.

VK_2 plus retinoids act synergistically to induce apoptosis in HuH7 cells. We then examined whether the synergistic growth inhibition by the combined treatment of VK_2 plus retinoids (Figs 1,2) was associated with the induction of apoptosis. We counted TUNEL-positive cells, which indicate DNA fragmentation, and found that the treatment of HuH7 cells with either 1.0 μM ACR alone or the combination of 1.0 μM 9cRA plus 10 μM VK_2 induced apoptosis in 15% of the total remaining cells (Fig. 3, columns 3 and 5), whereas no significant changes were observed in 1.0 μM 9cRA alone or 10 μM VK_2 alone (Fig. 3, columns 2 and 4). Moreover, the combination of ACR plus VK_2 markedly enhanced the induction of apoptosis to 33% (Fig. 3, column 6). These results strongly suggest synergism in inducing apoptosis by the combination of retinoids and VK_2.

Retinoids and VK_2 inhibit activation of ras and the phosphorylation of ERK and RXRα proteins in HuH7 cells. Our previous work suggested that a malfunction of RXRα due to aberrant phosphorylation is associated with liver carcinogenesis.[8,9] Phosphorylation of p-RXRα is caused mainly by activation of the Ras/MAPK signaling

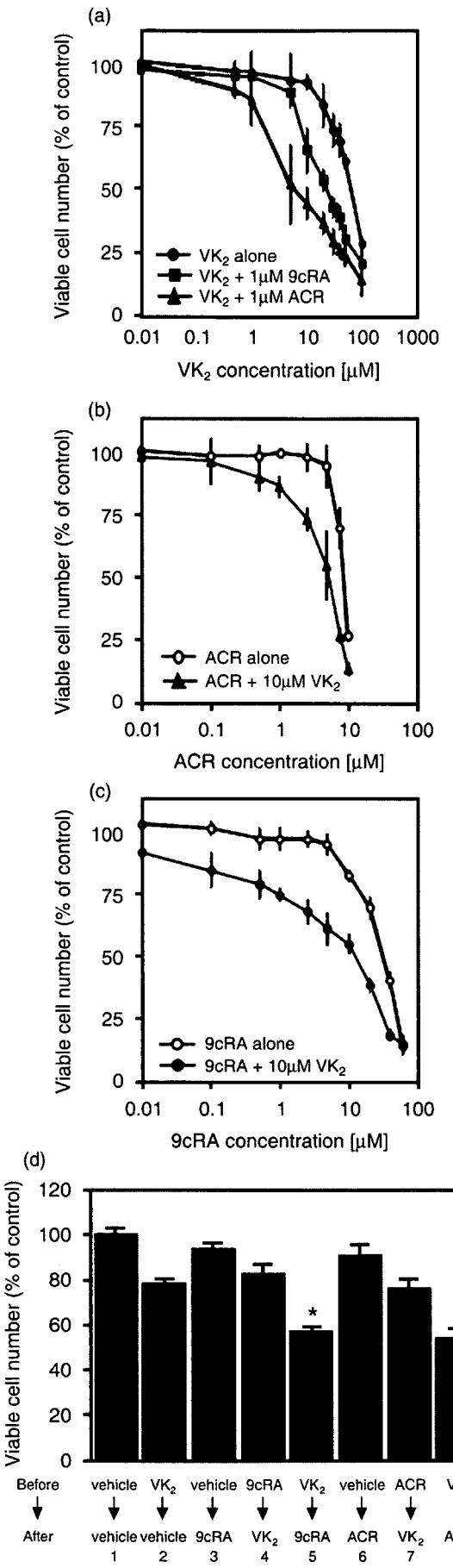

Fig. 2. Inhibition of cell growth by retinoids, vitamin K_2 (VK_2) and the combination of these agents in HuH7 cells. (a) The cells were treated with the indicated concentrations of VK_2 in the presence or absence of 1.0 μM acyclic retinoid (ACR) or 1.0 μM 9-*cis*-retinoic acid (9cRA) in 96-well plates for 72 h. (b) The cells were treated with the indicated concentrations of (b) ACR or (c) 9cRA in the presence or absence of 10 μM VK_2 in 96-well plates for 72 h. (d) Effects of sequential treatment with VK_2 and retinoids on the proliferation of HuH7 cells. The cells were cultured for the initial 24 h in the presence of vehicle, 1.0 μM 9cRA, 1.0 μM ACR or 10 μM VK_2, and subsequently incubated for another 24 h with one of these agents, as indicated. Cell number was then determined using the Trypan Blue dye exclusion method and expressed as a percentage vehicle. Values are the mean ± SD ($n = 5$). Similar results were obtained in a repeat experiment. Asterisks represent significant difference ($P < 0.05$) compared to column 1.

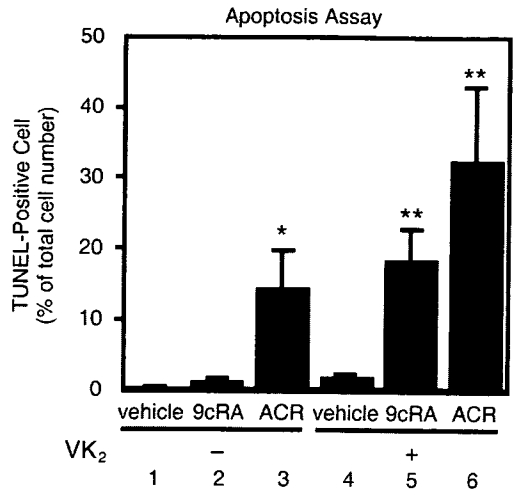

Fig. 3. Effects of the combination of retinoids plus vitamin K_2 (VK_2) on the induction of apoptosis in HuH7 cells. The cells were treated for 48 h with vehicle (column 1), 1.0 μM 9-*cis*-retinoic acid (9cRA) (column 2), 1.0 μM acyclic retinoid (ACR) (column 3), 10 μM VK_2 (column 4), the combination of 10 μM VK_2 plus 1.0 μM 9cRA (column 5), or the combination of 10 μM VK_2 plus 1.0 μM ACR (column 6). The cells were then stained using a terminal deoxynucleotidyl transferase-meditated dUTP nick-end labeling (TUNEL) method, and TUNEL-positive cells were counted and expressed as a percentage of total cell numbers (500 cells were counted in each flask). Values are the mean ± SD ($n = 5$). Asterisks represent significant difference ($P < 0.05$) compared to column 1. Representative results from three independent experiments with similar results are shown.

pathway in HCC cells.[8,9] Therefore, we examined whether the combined treatment of retinoids plus VK_2 downregulates the Ras/MAPK signaling pathway and inhibits phosphorylation of p-RXRα in HuH7 cells. As shown in Fig. 4a, Raf-1-bound Ras activities were significantly inhibited when the cells were treated with 10 μM VK_2 alone and with the combination of VK_2 plus 1.0 μM 9cRA or 1.0 μM retinoids (lanes and columns 4–6). The expression levels of phosphorylated (i.e. activated) forms of the ERK protein were also decreased when the cells were treated with ACR alone, VK_2 alone, or the combination of VK_2 plus retinoids (Fig. 4b, lanes and columns 3–6). Moreover, treatment of the cells with VK_2 alone or the combination of VK_2 plus retinoids caused a marked decrease in the expression level of p-RXRα (Fig. 4c, lanes and columns 4–6). This decrease was most apparent when the cells were treated with a combination of 1.0 μM ACR plus 10 μM VK_2 (Fig. 4c, lane and column 6).

VK_2 enhances the stimulation of both RARE and RXRE promoter activities produced by retinoids. RAR and RXR modulate the expression of target genes by interacting with RARE or RXRE located

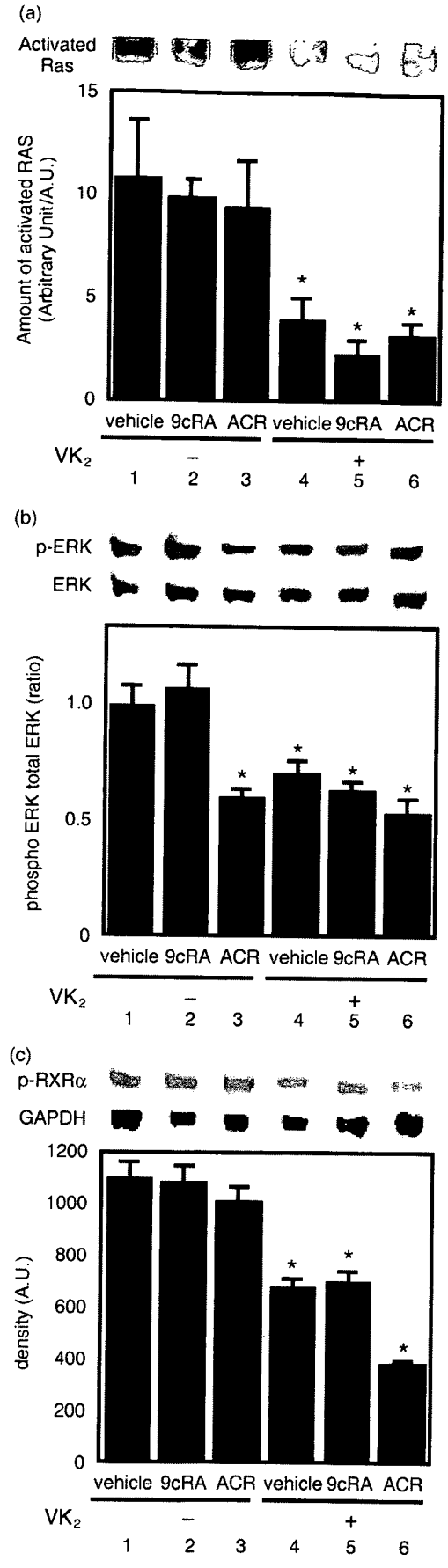

Fig. 4. Inhibition of Ras activation and phosphorylation of the extracellular signal-regulated kinase (ERK) and retinoid X receptor (RXR)α proteins by the combination of vitamin K_2 (VK_2) plus retinoids in HuH7 cells. After the cells were treated for 72 h with the indicated concentrations and combinations of retinoids plus VK_2 as described in the legend of Fig. 3, whole cell lysates were then prepared. (a) Ras was precipitated from the cell lysates using Raf/Ras binding domain-immobilized agarose and then subjected to western blot analysis using an anti-Ras specific antibody. Relative intensity of the bands was quantitated by densitometry and is displayed in the lower panel. (b,c) Western blot analysis for the phospho-ERK (p-ERK) and RXRα proteins. The cell extracts were examined by western blot analysis, and the results for (b) p-ERK and ERK or for (c) RXRα protein and glyceraldehyde-3-phosphate dehydrogenase were quantitated by densitometry, and the ratios of these proteins are displayed in the lower panel. Values are the mean ± SD ($n = 5$). Asterisks represent significant differences ($P < 0.01$) compared with vehicle-treated cells. Representative results from three independent experiments with similar results are shown.

doi: 10.1111/j.1349-7006.2006.00384.x

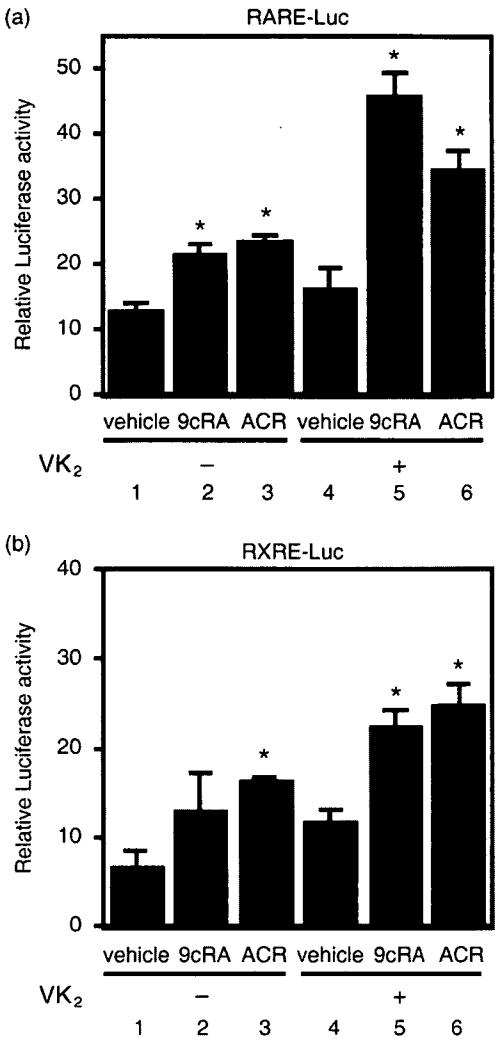

Fig. 5. Effects of the combination of retinoids plus vitamin K_2 (VK_2) on transcriptional activity of retinoid X receptor responsive element (RXRE) and RXRE promoters in HuH7 cells. The cells were cotransfected with vectors expressing the (a) retinoic acid receptor responsive element (RARE)–luciferase reporter gene or (b) RXRE–luciferase reporter gene, along with pRL-CMV as an internal standard using lipofection. The transfected cells were then treated for 24 h with the indicated concentrations and combinations of retinoids plus VK_2 as described in the legend of Fig. 3. Thereafter, the cell lysates were prepared and the relative luciferase activity of each cell lysate was measured and plotted as fold induction compared with the activity in vehicle after normalization to *Renilla* luciferase activity. Values are the mean ± SD ($n = 5$). Asterisks represent significant differences ($P < 0.01$) compared with vehicle-treated cells. Representative results from three independent experiments with similar results are shown.

in the promoter regions of target genes.[6,7] Therefore, we next examined whether VK_2 might enhance the transcriptional activity of the RARE or RXRE promoters using transient transfection luciferase reporter assays. We found that 1.0 μM 9cRA alone caused an increase in RARE reporter activity (Fig. 5a, column 2) and 1.0 μM ACR alone caused an increase in both RARE and RXRE reporter activities (Fig. 5, columns 3). VK_2 alone (10 μM) did not have an effect on the transcriptional activity of these responsive elements (Fig. 5, columns 4). However, when VK_2 was combined with these retinoids, there was a synergistic increase in the transcriptional activity of these luciferase reporter activities (Fig. 5, columns 5 and 6).

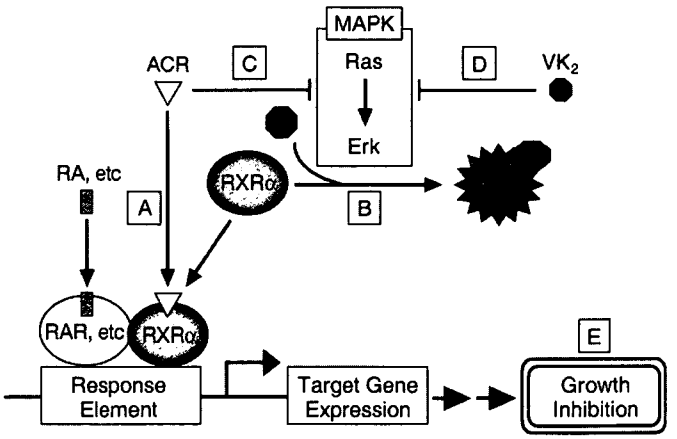

Fig. 6. Schematic representation of the effect of vitamin K_2 (VK_2) and acyclic retinoid (ACR) on retinoid X receptor (RXR)α phosphorylation in hepatocellular carcinoma cells. (a) In normal hepatocytes, ACR binds to RXRα and transactivates downstream genes via retinoid X receptor responsive element (RXRE), which may regulate cell proliferation. (b) In hepatocellular carcinoma (HCC) cells, the Ras/mitogen-activated protein kinase (MAPK) pathway is highly activated and phosphorylates RXRα at serine residues, thus impairing the functions of the receptor. (c) ACR and (d) VK_2 cooperatively suppress the Ras/MAPK signaling pathway, inhibit phosphorylation of RXRα, restore the function of the receptor, and thus subsequently activate the transcriptional activity of the responsive element, including RXRE. (e) These effects may contribute to growth inhibition of the hepatoma cells.

Discussion

In the present study we found that the combination of retinoids (especially ACR) plus VK_2 caused the synergistic inhibition of growth in human hepatoma HuH7 cells (Figs 1,2) and that this was associated with the induction of apoptosis (Fig. 3). These findings are consistent with recent published results showing that retinoids have a synergistic growth-inhibitory effect when combined with VK_2 in leukemia cells, with an enhanced therapeutic benefit.[23,24] However, the precise mechanisms of how the combination of retinoids plus VK_2 could cause this preferable effect have not yet been determined. A hypothetical scheme that addresses this question is shown in Fig. 6. This scheme emphasizes cooperative inhibition of the Ras/MAPK signaling pathway by retinoids plus VK_2 in HCC cells. In HCC cells, but not in normal hepatocytes, the Ras/MAPK signaling pathway is highly activated and phosphorylates RXRα, thus impairing the functions of this receptor.[8,9] These findings indicate that p-RXRα may be a useful target for inhibiting the growth of HCC cells. As shown in our present (Fig. 4) and previous studies,[8,26] retinoids can inhibit Raf-1-bound Ras activity and phosphorylation of the ERK protein. We found in this study that VK_2 itself also inhibits the Ras/MAPK signaling pathway via inhibition of Ras activation, thus causing a decrease in the levels of p-ERK and p-RXRα (Fig. 4). In a previous study we found that the accumulation of non-functional p-RXRα interfered with the function of remaining normal p-RXRα in a dominant-negative manner, thereby promoting the growth of hepatoma cells.[8] Therefore, our finding in the present study that the combination of retinoids plus VK_2 synergistically inhibits the phosphorylation of p-RXRα seems to be very significant in the inhibition of HCC growth, without affecting the growth of normal hepatocytes (Fig. 1).

We also found in the present study that VK_2 enhances binding of retinoids to the RARE and RXRE promoters, thereby enhancing transcriptional activity (Fig. 5). As previously noted, the restoration

of the function of RXR as a master regulator for nuclear receptors, by inhibiting the Ras/MAPK signaling pathway (Fig. 4), was thus considered to possibly play a role in these promoter activities. However, there are reports that VK_2 itself also works as a transcriptional regulator in addition to its role as an enzyme cofactor. For instance, VK_2 inhibits growth and invasiveness of HCC cells via the activation of protein kinase A, which modulates the activation of several transcriptional factors.[19] Tabb *et al.* reported that VK_2 binds directly to the nuclear receptor SXR, which can dimerize with RXR and regulate the transcription of target genes, thus transcriptionally activating this receptor in a dose-dependent manner.[27] Moreover, RXR serves as an active partner of SXR and retinoids can activate the RXR/SXR-mediated pathway and regulate the expression of target genes.[28] Further studies are required to clarify whether both retinoids and VK_2 synergistically exert their growth-inhibitory effects in cancer cells at the level of transcription, especially focusing on the regulation of transcription factors and the expression of target genes located in the RXR/SXR-mediated downstream signaling pathway.

Although clinical studies suggest that VK_2 has a suppressive effect on the development and recurrence of HCC,[21,22] the precise mechanism by which VK_2 causes these antiproliferative effects in HCC cells remains to be determined. However, the structure of VK_2 may play a role in the induction of apoptosis because geranylgeraniol, which is a side chain of VK_2, strongly induces apoptosis in tumor cells.[29] Such a report seems to be of interest as ACR also has geranylgeraniol in its side chain, thus suggesting that the synergistic induction of apoptosis caused by ACR plus VK_2 (Fig. 3) may be associated with their similar structure. In addition, VK_2 can cause an increase of cells in the G_1 phase of the cell cycle in HCC cells[30,31] and similar effects are also caused by ACR.[17,32] These findings suggest that the combination of these agents might synergistically induce antitumor effects in HCC cells via the induction of cell cycle arrest. Therefore, in future studies it will be of interest to further examine the effects of ACR plus VK_2 on cell cycle progression and on the expression of cell cycle control molecules including cyclin D1 or $p21^{CIP1}$, which are target molecules of ACR.[17,32]

The chemoprevention of HCC must improve the prognosis for patients[5] because this cancer has a high frequency of recurrence even after patients undergo curative therapy.[2,3] Safety is critical when agents are applied as chemopreventive drugs in clinical use. A clinical trial demonstrated that the administration of both ACR and VK_2 reduced the incidence of post-therapeutic recurrence of HCC and improved the survival rate of patients without causing any untoward effects.[13–15,22] Pharmacokinetic studies in clinical trials also indicate that the plasma concentrations of ACR and VK_2 are approximately the same as the dosages that we used in the present study.[13,33] In addition, our finding that the combination of appropriate dosages of ACR and VK_2 can inhibit the growth of human HCC cells without affecting the growth of normal hepatocytes (Fig. 1) should encourage further clinical studies using these materials to investigate HCC prevention and treatment. The safety and efficacy for the combination of VK_2 plus other agents were also demonstrated in an *in vivo* study. The antitumor activity of VK_2 was enhanced when it was combined with perindooril, the antihypertensive drug, in a rat chemical-induced liver carcinogenesis model without causing any toxicities.[20] In conclusion, these reports, together with the results of our *in vitro* mechanistic studies on HCC cells as described in the present paper, suggest that combining specific retinoids (especially ACR) with VK_2 might hold promise as a clinical modality for the prevention and treatment of HCC, due to their synergistic effects.

Acknowledgments

This work was supported in part by a Grant-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.1349-7006.2006.00384.x

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Nutrition 23 (2007) 113–120

NUTRITION

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Applied nutritional investigation

BCAA-enriched snack improves nutritional state of cirrhosis

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Manuscript received May 17, 2006; accepted October 26, 2006.

Abstract

Objective: A late evening snack improves the catabolic state in patients with advanced liver cirrhosis. We tested whether long-term (3 mo) late evening snacking that included a branched-chain amino acid (BCAA)–enriched nutrient mixture produces a better nutritional state and better quality of life than ordinary food in patients with hepatitis C virus–positive liver cirrhosis.

Methods: In a multicenter, randomized study, 48 patients with liver cirrhosis received late-evening supplementation with the BCAA-enriched nutrient mixture or ordinary food, such as a rice ball or bread, for 3 mo. During the study period, each patient was instructed on energy and protein intake. Blood biochemical data, nitrogen balance, respiratory quotient, and health-related quality of life (Short Form 36 questionnaire) were evaluated at baseline and at the end of the study.

Results: Total and late-evening energy intakes were similar in the two groups at 3 mo. Serum albumin level, nitrogen balance, and respiratory quotient were significantly improved by the BCAA mixture but not by ordinary food. The parameters of the Short Form 36 did not statistically significantly improve over 3 mo in either group.

Conclusion: Long-term oral supplementation with a BCAA mixture is better than ordinary food in a late evening snack at improving the serum albumin level and the energy metabolism in patients with cirrhosis.

Keywords: Liver cirrhosis; Late evening snack; Branched-chain amino acid; Nutritional therapy

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0899-9007/07/$ – see front matter
doi:10.1016/j.nut.2006.10.008

Introduction

The liver plays an important role in energy metabolism. Patients with cirrhosis lack adequate glycogen stores because of liver atrophy and therefore develop a severe catabolic state after fasting. Owen et al. [1] reported that, after an overnight fast, patients with cirrhosis have a marked decrease in glucose oxidation, with enhanced fat and protein catabolism similar to that observed in healthy subjects after 2 to 3 d of starvation. To avoid such nocturnal starvation, energy supplements have been developed [2–5] and are recommended as late evening snacks (LESs) in current American Society for Parenteral and Enterel Nutrition [6] and European Society for Clinical Nutrition and Metabolism [7] guidelines. Carbohydrate-rich snacks have been used previously in LESs, but some recent studies have indicated that a LES with a branched-chain amino acid (BCAA)–enriched nutrient mixture (BCAA mixture) can also improve the oxidation of aberrant substrates in the early morning in patients with cirrhosis [8,9]. However, these studies have focused solely on the short-term effect of LESs on energy metabolism, and there have been no studies on the long-term outcome of LESs. Another important question that has not been answered is whether or not LESs should accompany BCAA supplementation.

Supplementation with BCAAs has mainly been attempted as a nutritional intervention for decompensated liver cirrhosis. Clinical evidence about the efficacy of this therapy has accumulated particularly in the past 3 y [10–13] and indicates that BCAA supplementation raises the serum albumin level and improves the quality of life (QOL) and survival of patients with decompensated liver cirrhosis. A laboratory study also supports the view that BCAA regulates albumin synthesis at a subcellular level [14]. However, there are also many criticisms against BCAA administration in cirrhosis [15]. To confirm its efficacy, if any, supplementation conditions, including dose, time, and use of energy, might be further optimized. In this context, it is relevant that BCAA supplementation at night improved the nitrogen balance compared with daytime administration [16].

Therefore, we investigated which would be better to improve energy malnutrition, an ordinary LES or a BCAA-enriched LES.

Materials and methods

Patients

Forty-eight patients with liver cirrhosis were recruited from 16 centers between April 2003 and June 2004. The study protocol was approved by the ethics committee of each hospital, and informed consent was obtained from each subject. Liver cirrhosis was diagnosed by documented laboratory data and/or histology. The patients were graded according to the Child-Pugh classification. Eligibility criteria were 1) positive test results for the hepatitis C virus antibody and 2) a serum albumin level <3.5 g/dL. Exclusion criteria were the presence of overt hepatic encephalopathy, uncontrolled esophageal varices, refractory ascites, reduced renal function, or previous poor compliance with pharmacologic treatment or nutritional counseling. Those who used antidiabetic drugs or intravenous albumin regularly were also excluded. Those who had been treated for hepatocellular carcinoma were included in this study if ultrasound had not detected current local lesions and the α-fetoprotein levels were not elevated.

After dynamic balancing of the treatment groups for age, Child-Pugh score, and serum albumin level, patients were randomly assigned to one of two groups: LESs with BCAA mixture (Aminoleban EN, Otsuka Pharmaceutical Co. Ltd., Tokyo, Japan; BCAA group, $n = 25$) and LESs with ordinary food (snack group, $n = 23$).

Study protocol

The intervention schedule is presented in Figure 1. After randomization, patients had a standard diet for 2 wk and had LESs for the next 3 mo. During the standard diet period, patients were instructed to maintain a diet containing 30–35 kcal and 1.2–1.3 g of protein per kilogram of ideal body weight per day. In the LES period, the patients were educated to adjust their total energy intake by subtracting 210 kcal from the standard meals. As a LES food, the BCAA mixture contained 210 kcal of energy, 13.5 g of protein, 3.5 g of fat, and trace minerals and vitamins [17]. The control snack provided 210 kcal of energy with 9 g of protein and 5 g of fat. Dietitians showed examples of ordinary LESs, such as rice balls, bread, and cookies.

Nutritional education was presented to all patients once every month during the study period by dietitians. The patients were told to completely record food intake for a 72-h period at home. Food consumption was calculated from these records at baseline and at the end of the study. Standard therapies such as diuretics were allowed but dos-

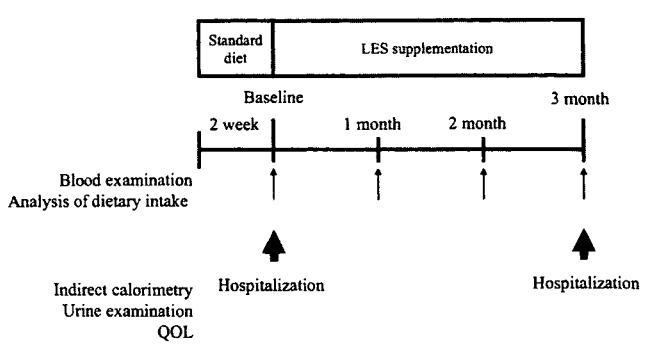

Fig. 1. Study protocol. After randomization, the patients spent 2 wk on a standard diet and the next 3 mo with late evening snack supplementation. For detailed contents of the standard diet and late evening snack, see STUDY PROTOCOL. QOL, quality of life measured by the Short Form 36 questionnaire and subjective symptoms.

ages were fixed during the study. Compliance with treatment was assessed at each outpatient visit by interview.

Nutritional parameters

The baseline assessment included routine physical and laboratory examinations, anthropometric measurements, and self-administered questionnaires to evaluate dietary intake, subjective symptoms, and health-related QOL.

Laboratory tests were performed in individual centers according to standard techniques. The BCAA-to-tyrosine ratio (BTR) was measured to assess the intake of BCAAs. The BTR was measured by an enzymatic method (Ono Pharmaceutical Co. Ltd., Osaka, Japan). The clinical significance of the results obtained corresponds to Fischer's ratio (molar ratio of BCAAs and aromatic amino acids) [18]. At baseline, when patients were in the hospital and after 3 mo of the study, urine was collected and the nitrogen balance and urinary excretion of 3-methylhistidine normalized by creatinine were measured. The nitrogen balance was calculated from the intake of dietary protein and excreted urinary nitrogen using the following equation:

$$\text{nitrogen balance (g)} \frac{\text{protein intake (g)}}{6.25} \times \text{urinary urea nitrogen (g)} - 4$$

The anthropometric measurements included the midarm circumference and triceps skinfold thickness. The midarm muscle circumference and midarm fat area were calculated subsequently.

At baseline and at 3 mo, indirect calorimetry was performed to measure oxygen and carbon dioxide consumption per unit time after overnight fasting, and the non-protein respiratory quotient (npRQ) was estimated. The equipment for measurement of respiratory parameters differed in each institution, i.e., Deltatrac II respiratory gas analyzer (Datex Ohmeda Inc., Helsinki, Finland), Calorie Scale (Cest MI, Tokyo, Japan), and Aeromonitor AE-300S (Minato Medical Science, Osaka, Japan), although each patient was measured with the same equipment both times.

QOL questionnaire

Health-related QOL was measured using the Short Form 36 (SF-36) questionnaire [19–21]. The SF-36 contains 36 questions that provide a quantitative evaluation on each of eight subscales: physical function, mental health, role physical, role emotional, bodily pain, general health, vitality, and social function. Subjective symptoms possibly related to liver cirrhosis, such as weakness, easy fatigability, pruritus, muscle cramp, bleeding, and abdominal fullness, were also surveyed with yes/no questions.

Outcome markers

The main outcome markers were serum albumin level, nitrogen balance, and QOL measurements. The npRQ served as an intermediate biomarker to evaluate directly the effect of late evening energy supplementation and BTR to evaluate BCAA supplementation.

Statistical analysis

Data analysis was performed using JMP 5.1J (SAS Institute Japan Ltd., Chuo-ku, Tokyo, Japan). The differences between groups in baseline characteristics were analyzed by chi-square test or Student's *t* test. The effects of LESs were analyzed by repeated measures analysis of variance between groups and by the Wilcoxon signed-rank test within each group. The subjective symptoms were analyzed by chi-square test between groups and by McNemar's test within each group. All data in the text and tables are presented as mean ± SD.

Results

Clinical course

Forty-eight patients were initially enrolled and randomized (Fig. 2). However, one patient in the BCAA group opted for other treatments and declined. Therefore, the supplementation was initiated in 24 patients in the BCAA group and 23 patients in the snack group. During the study period of 3 mo, five patients in the BCAA group and four in

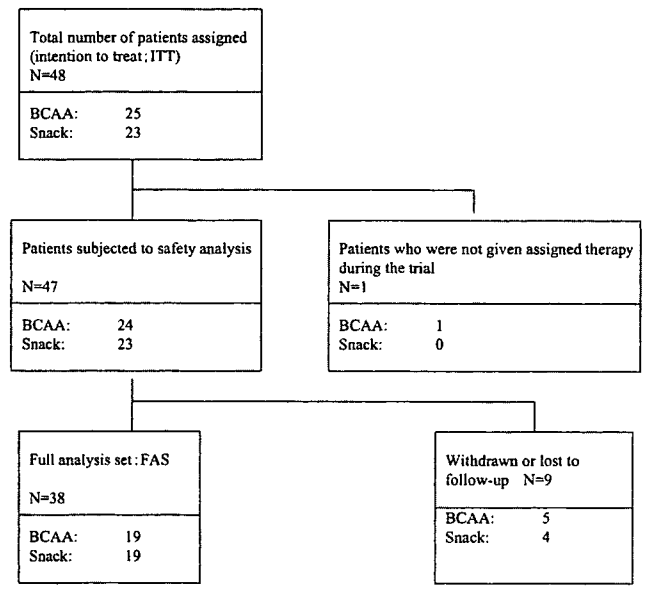

Fig. 2. Details of analysis. The BCAA group consumed a late evening snack with a BCAA-enriched nutrient mixture, and the snack group consumed a late evening snack with ordinary food. BCAA, branched-chain amino acid; ITT, intention to treat.

Table 1
Baseline characteristics of study patients*

	BCAA group (n = 19)	Snack group (n = 19)
Age (y)	67 ± 9	67 ± 8
Male/female	13/6	7/12
Severity of cirrhosis		
Child-Pugh group (A/B/C)	6/11/2	10/9/0
Child-Pugh score	7 (6–10)	7 (5–9)
History of HCC	8	6
Presence of ascites	3	4
Albumin (g/dL)	3.0 ± 0.4	3.0 ± 0.3
Hemoglobin (g/dL)	11.5 ± 1.5	11.8 ± 1.3
Total bilirubin (mg/dL)	1.2 ± 0.8	1.4 ± 0.7
Platelet count ($10^4/\mu L$)	8.4 ± 3.8	8.1 ± 4.6
Cholinesterase (IU/L)	204 ± 247	238 ± 351
Aspartate aminotransferase (IU/L)	68 ± 24	72 ± 41
Alanine aminotransferase (IU/L)	56 ± 29	57 ± 35
Height (m)	1.60 ± 0.09	1.56 ± 0.09
Body weight (kg)	58.6 ± 9.0	56.6 ± 7.7
Body mass index (kg/m^2)	22.9 ± 3.1	23.3 ± 2.6

BCAA, late evening snack with branched-chain amino acids; HCC, hepatocellular carcinoma; Snack, late evening snack with ordinary food
* Data are presented as number of patients, median (range), or mean ± SD. There was no significant difference between groups.

the snack group were lost to follow-up. One patient in the BCAA group died from a cause unrelated to liver disease (cerebral bleeding). Two patients in the BCAA group and one in the snack group were withdrawn from the study due to aggravation of liver failure (severe ascites and hepatic encephalopathy). One patient in the BCAA group and three in the snack group failed to make a routine visit. One patient in the BCAA group withdrew consent because of the bad taste of the mixture. Thus, 19 patients in the BCAA group and 19 in the snack group were subjected to full analysis (Fig. 2).

As to the possible adverse effects of the LES, one patient in the snack group complained of nausea and one in the BCAA group developed a high fever. However, they recovered spontaneously without any treatment and were able to complete the study. The 19 patients in the BCAA group tolerated the taste of the mixture well.

Baseline characteristics

Baseline demographic and clinical characteristics are listed in Table 1. There was no significant difference between the two groups in the distribution of randomization parameters of age, Child-Pugh score, and serum albumin. Although the snack group seemed to have more female patients, the difference did not reach statistical significance.

Dietary intake

Total energy intake and that from the LESs were not significantly different between the two groups throughout the study period (Table 2). Protein intake was significantly greater and fat intake was less at the end of the study than at baseline in the BCAA group but not in the snack group.

Nutritional parameters

Anthropometry. Body weight was slightly but significantly increased in the BCAA group (58.6 ± 9.0 to 59.4 ± 9.6 kg, $P = 0.008$), but not in the snack group. No significant improvement was shown in other anthropometric parameters in either group (data not shown). The change in nitrogen balance significantly correlated with that of the midarm muscle circumference ($r = 0.545$, $P < 0.01$), but not with the change in triceps skinfold thickness ($r = -0.031$, P = NS).

Blood biochemistry. The LES with the BCAA mixture had significantly increased the BTR, as expected, by the end of the study, but the ordinary food did not (Table 3). Serum albumin, total protein, and red blood cell count significantly increased in the BCAA group but not in the snack group.

The serum blood urea nitrogen level significantly rose in the BCAA group between baseline and the end of the study (Table 3), probably due to the increased nitrogen intake (Table 2). Fasting plasma glucose levels were not significantly different between the groups at baseline, and LESs did not affect them significantly in either group (Table 3).

Table 2
Total and LES energy intakes*

	BCAA group		Snack group		Statistical analysis
	Baseline	3 mo	Baseline	3 mo	
Total energy (kcal/d)	1829 ± 274	1848 ± 181	1714 ± 330	1714 ± 358	NS
Protein (g/d)	67.2 ± 14.5	75.8 ± 8.4	66.5 ± 15.8	68.1 ± 17.4	†
Carbohydrate (g/d)	274.9 ± 45.5	286.2 ± 39.2	268.7 ± 63.6	266.5 ± 66.7	NS
Fat (g/d)	48.1 ± 14.5	41.6 ± 9.9	40.0 ± 10.0	39.6 ± 9.6	†
	1 mo	3 mo	1 mo	3 mo	
LES energy (kcal/d)	212.7 ± 7.8	204.0 ± 32.0	198.2 ± 65.0	207.1 ± 73.8	NS

BCAA, late evening snack with branched-chain amino acids; LES, late evening snack; Snack, late evening snack with ordinary food
* Data are presented as mean ± SD.
† $P < 0.05$, baseline versus 3 mo in BCAA group.

Table 3
Changes in laboratory parameters*

	BCAA group		Snack group		Statistical analysis
	Baseline	3 mo	Baseline	3 mo	
BTR	2.95 ± 0.92	3.61 ± 1.07	2.99 ± 1.17	2.89 ± 1.13	†‡
Albumin (g/dL)	3.0 ± 0.4	3.2 ± 0.4	3.0 ± 0.3	3.0 ± 0.4	‡
Total protein (g/dL)	6.9 ± 0.6	7.3 ± 0.7	6.9 ± 0.8	7.0 ± 0.9	†‡
RBC count ($\times 10^4$/uL)	355 ± 48	374 ± 40	353 ± 46	355 ± 44	‡
AST (IU/L)	68 ± 24	71 ± 27	72 ± 41	87 ± 48	NS
Total bilirubin (mg/dL)	1.2 ± 0.8	1.4 ± 1.0	1.4 ± 0.7	1.5 ± 0.7	†§
BUN (mg/dL)	14.4 ± 4.0	16.8 ± 4.7	14.3 ± 4.6	14.2 ± 4.7	‡
FPG (mg/dL)	107 ± 23	118 ± 39	99 ± 26	95 ± 10	NS
IRI (μU/mL)	16.2 ± 6.8	32.9 ± 34.5	21.3 ± 19.5	20.9 ± 14.4	NS

AST, aspartate aminotransferase; BCAA, late evening snack with branched-chain amino acids; BTR, molar ratio of branched-chain amino acid to tyrosine; BUN, serum urea nitrogen; FPG, fasting plasma glucose; IRI, immunoreactive insulin; RBC, red blood cell; Snack, late evening snack with ordinary food
* Data are presented as mean ± SD.
† $P < 0.05$, difference between groups for change over 3 mo.
‡ $P < 0.05$, baseline versus 3 mo in BCAA group.
§ $P < 0.05$, baseline versus 3 mo in snack group.

Urine analysis. The nitrogen balance had significantly increased in the BCAA group by the end of the study, but not in the snack group (Table 4). Neither LES type showed an effect on the urinary excretion of 3-methylhistidine normalized by creatinine (Table 4).

Health-related QOL

Neither the BCAA LES nor the ordinary snack improved the SF-36 scores within 3 mo (data not shown). Concerning other subjective symptoms for liver cirrhosis, both types of LES improved weakness (Fig. 3). Easy fatigability was improved significantly by the BCAA mixture but not by the ordinary food (Fig. 3).

Energy metabolism

Table 5 lists the changes in resting energy expenditure and npRQ at baseline and at the end of the study. Resting energy expenditure was not different within or between groups at baseline, and the npRQ was not different at baseline between groups. However, npRQ was significantly higher at the end of the study with the BCAA mixture than with the ordinary food. The difference between the two groups for the change of npRQ over 3 mo was significant by repeated measures analysis of variance ($P < 0.05$).

Discussion

This study was performed to test the feasibility of the BCAA mixture, in comparison with ordinary food, as a LES in patients with cirrhosis. The results indicate that the LES with the BCAA mixture, but not with ordinary food, significantly improved nutritional parameters. The BCAA mixture improved the catabolic state (low npRQ), as expected (Table 5). Further, the BCAA mixture raised the nitrogen balance (Table 4) and serum albumin level (Table 3) in patients with cirrhosis when compared with an equicaloric food. However, in each group the patients did not report any improvement in their QOL.

Patients with cirrhosis develop a catabolic state more rapidly than do normal subjects. Such a catabolic state brings about worse survival of patients with cirrhosis [22,23]. Chang et al. [2] reported that carbohydrate-rich supplementation taken before bedtime can shorten nocturnal fasting and effectively diminish fat and protein oxidation in patients with cirrhosis. An improvement in the nitrogen

Table 4
Nitrogen balance and 3-methylhistidine excretion*

	BCAA group		Snack group		Statistical analysis
	Baseline	3 mo	Baseline	3 mo	
Nitrogen balance (g/24h)	−0.02 ± 2.83	1.54 ± 1.92	0.04 ± 3.86	0.00 ± 2.86	†
3-methylhistidine/creatinine ratio (μmol/g creatine per day)	172.4 ± 71.4	196.9 ± 142.2	172.6 ± 62.1	154.6 ± 40.6	NS

BCAA, late evening snack with branched-chain amino acids; Snack, late evening snack with ordinary food
* Data are presented as mean ± SD.
† $P < 0.05$, baseline versus 3 mo in BCAA group.

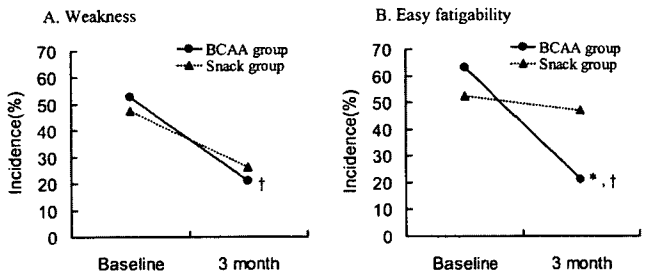

Fig. 3. Effects of a late evening snack on the incidence of subjective weakness (left) and easy fatigability (right) in patients with cirrhosis. The incidence of each symptom was compared between groups by chi-square test and within each group by McNemar's test. $^{*}P < 0.05$ versus S group; $^{\dagger}P < 0.05$ versus baseline incidence in B group. B, late evening snack with a branched-chain amino acid–enriched nutrient mixture; S, late evening snack with ordinary food.

balance, as a result of a LES, has also been reported in other studies [5]. In addition, carbohydrate-rich LESs correct increased protein metabolism and decrease ketone body levels in patients with liver cirrhosis [24]. Therefore, nocturnal energy supplementation may prevent the progression of malnutrition that costs body fat and protein. However, all these effects were observed within a couple of weeks of LES consumption, and long-term evaluation of LES has not been done. The present study elucidated the effects of LESs as described above in a 3-mo observation period.

Various nutrients have been used in LESs to improve the catabolic state in liver cirrhosis, such as a carbohydrate-rich snack [2–5] and commercially available liquid nutrients [8]. Nakaya et al. [9] reported that the improvement of the RQ with a BCAA mixture was similar to that with ordinary food with equal energy. An oral BCAA mixture contains not only BCAAs but alo glucose, lipids, and other nutrients, which by themselves are insufficient in liver cirrhosis. Therefore, the BCAA mixture seems to be a more favorable food for LESs, but there has been limited evidence on the benefits of the use of a BCAA mixture as compared with ordinary food. Our study indicated beneficial effects of BCAA on energy and nitrogen metabolism.

There are various opinions about the mechanism of the decreased levels of BCAA in cirrhosis. Hyperinsulinemia increases the uptake of BCAAs by skeletal muscle [24,25] and BCAAs are increasingly utilized as energy substrates [26]. In addition, BCAAs are used for ammonia degradation in skeletal muscles of cirrhotic patients. A low BCAA level impairs synthesis of proteins such as albumin [14] and destroys muscle proteins [27]. However, the benefits of oral BCAA supplements in cirrhosis have been controversial [10,11,13,17]. This might be due to differences in study design, study duration, and outcome markers. The most recent large-scale, multicenter, long-term trials have shown the effects of BCAAs on nutrition, survival, and QOL of patients with cirrhosis [11,13] by using granulated BCAAs over the course of 1 to 2 y.

The timing of administration of BCAA seems to be important. Fukushima et al. [16] reported that, when cirrhotic patients were given BCAA granules after each meal (12 g/d), their Fischer's ratio remained low. However, when they were given 8 g of BCAAs at bedtime and 4 g after breakfast, an increased Fischer's ratio, a significant recovery of the nitrogen balance and a significant improvement in the serum albumin levels were observed [16]. Nocturnal BCAA administration maintains Fischer's ratio high during the night-time and may then stimulate hepatic albumin synthesis [16]. When administered during the daytime to patients with cirrhosis, most BCAAs are oxidized for energy generation but not directed to protein synthesis [28]. In the present study, the patients in the BCAA group received supplemental 6.075 g of BCAAs (isoleucine 2.04 g, leucine 2.25 g, and valine 1.785 g/210 kcal) before bedtime. However, this small addition of BCAAs might have influenced not only serum BTR but also albumin (Table 3) and other nutritional parameters over 3 mo, thus supporting the importance of timing of BCAA supplementation.

Total energy intake and that by LES were not different between groups at the beginning of our study (Table 2). However, the BCAA group showed a greater consumption of food at the end of the study than did the snack group, although the difference was not significant (Table 2). Dietary instructions were given regularly and similarly to both groups by dietitians. These results suggest that BCAA supplementation improves anorexia, although we did not ask directly about anorexia. Similar effects were also reported in another liver cirrhosis survey [29] and in subjects with cancer-related anorexia [30]. The group receiving BCAAs,

Table 5
REE and npRQ*

	BCAA group		Snack group		Statistical analysis
	Baseline	3 mo	Baseline	3 mo	
REE	1192 ± 154	1232 ± 181	1187 ± 179	1220 ± 220	N.S.
npRQ	0.82 ± 0.04	0.85 ± 0.05	0.84 ± 0.11	0.81 ± 0.07	†‡

BCAA, late evening snack with branched-chain amino acids; npRQ, non-protein respiratory quotient; REE, resting energy expenditure; Snack, late evening snack with ordinary food

* Data are presented as mean ± SD.

† $P < 0.05$, difference between groups for change over 3 mo.

‡ $P < 0.05$, baseline versus 3 mo in BCAA group.