suggested that the cause of RLS in CLD is associated with impaired dopaminergic neurotransmission. However, we did not find any conclusive link between liver function, such as NH3 levels, and the frequency of RLS. In fact, some reports are also saying that there is no clear relationship between the severity of insomnia and liver disease.[41] Therefore, sleeplessness and RLS might be present from the early stages of liver disease.

We showed there were no significant differences in the morbidity rate of some diseases that could cause secondary RLS, such as anemia, HCV infection, diabetes, collagen disease and Parkinson's disease. However, it cannot be assured that all causal factors for secondary RLS were excluded, because so many conditions have been reported as causes of secondary RLS. However, in the present study, it was clear that RLS cases were highly complicated with Japanese CLD patients. It should be necessary to pay attention to the possible existence of RLS in CLD patients because many successful therapeutic agents for RLS have recently been reported, such as pramipexole, ropinirole, rotigotine (dopamine D2 agonist), gabapentin (voltage-dependent calcium channel $\alpha 2\delta$ subunit ligand).[38,42,43] In addition, these patients might benefit from such treatments. Future studies will be needed to clarify the mechanisms of the apparent association between RLS and liver disease.

In conclusion, the prevalence of RLS in Japanese CLD patients is much more frequent than that of the general population. In addition, RLS increases the incidence of poor sleep and decreases the quality of the life in CLD patients. This is the first report that clarifies the prevalence of RLS in Japanese CLD patients.

REFERENCES

1 Allen RP, Picchietti D, Hening WA, Trenkwalder C, Walters AS, Montplaisi J. Restless legs syndrome: diagnostic criteria, special considerations, and epidemiology. A report from the restless legs syndrome diagnosis and epidemiology workshop at the National Institutes of Health. *Sleep Med* 2003; 4: 101–19.

2 Berger K, Luedemann J, Trenkwalder C, John U, Kessler C. Sex and the risk of restless legs syndrome in the general population. *Arch Intern Med* 2004; **164**: 196–202.

3 Tison F, Crochard A, Leger D, Bouee S, Lainey E, El Hasnaoui A. Epidemiology of restless legs syndrome in French adults: a nationwide survey: the INSTANT Study. *Neurology* 2005; **65**: 239–46.

4 Lee HB, Hening WA, Allen RP, Earley CJ, Eaton WW, Lyketsos CG. Race and restless legs syndrome symptoms in an adult community sample in east Baltimore. *Sleep Med* 2006; **7**: 642–5.

5 Winkelman JW, Finn L, Young T. Prevalence and correlates of restless legs syndrome symptoms in the Wisconsin Sleep Cohort. *Sleep Med* 2006; **7**: 545–52.

6 Bjorvatn B, Leissner L, Ulfberg J *et al.* Prevalence, severity and risk factors of restless legs syndrome in the general adult population in two Scandinavian countries. *Sleep Med* 2005; **6**: 307–12.

7 Nomura T, Inoue Y, Kusumi M, Uemura Y, Nakashima K. Prevalence of restless legs syndrome in a rural community in Japan. *Mov Disord* 2008; **23**: 2363–9.

8 Tan EK, Seah A, See SJ, Lim E, Wong MC, Koh KK. Restless legs syndrome in an Asian population: a study in Singapore. *Mov Disord* 2001; **16**: 577–9.

9 Tsuboi Y, Imamura A, Sugimura M, Nakano S, Shirakawa S, Yamada T. Prevalence of restless legs syndrome in a Japanese elderly population. *Parkinsonism Relat Disord* 2009; **15**: 598–601.

10 Winkelman JW, Redline S, Baldwin CM, Resnick HE, Newman AB, Gottlieb DJ. Polysomnographic and health-related quality of life correlates of restless legs syndrome in the Sleep Heart Health Study. *Sleep* 2009; **32**: 772–8.

11 Hornyak M, Feige B, Voderholzer U, Philipsen A, Riemann D. Polysomnography findings in patients with restless legs syndrome and in healthy controls: a comparative observational study. *Sleep* 2007; **30**: 861–5.

12 Parrino L, Boselli M, Buccino GP, Spaggiari MC, Di Giovanni G, Terzano MG. The cyclic alternating pattern plays a gate-control on periodic limb movements during non-rapid eye movement sleep. *J Clin Neurophysiol* 1996; **13**: 314–23.

13 Karadeniz D, Ondze B, Besset A, Billiard M. EEG arousals and awakenings in relation with periodic leg movements during sleep. *J Sleep Res* 2000; **9**: 273–7.

14 Sforza E, Juony C, Ibanez V. Time-dependent variation in cerebral and autonomic activity during periodic leg movements in sleep: implications for arousal mechanisms. *Clin Neurophysiol* 2002; **113**: 883–91.

15 Guggisberg AG, Hess CW, Mathis J. The significance of the sympathetic nervous system in the pathophysiology of periodic leg movements in sleep. *Sleep* 2007; **30**: 755–66.

16 Kutner NG, Bliwise DL. Restless legs complaint in African-American and Caucasian hemodialysis patients. *Sleep Med* 2002; **3**: 497–500.

17 Gigli GL, Adorati M, Dolso P *et al.* Restless legs syndrome in end-stage renal disease. *Sleep Med* 2004; **5**: 309–15.

18 Bhowmik D, Bhatia M, Gupta S, Agarwal SK, Tiwari SC, Dash SC. Restless legs syndrome in hemodialysis patients in India: a case controlled study. *Sleep Med* 2003; **4**: 143–6.

19 Takaki J, Nishi T, Nangaku M *et al.* Clinical and psychological aspects of restless legs syndrome in uremic patients on hemodialysis. *Am J Kidney Dis* 2003; **41**: 833–9.

20 Taylor-Gjevre RM, Gjevre JA, Skomro R, Nair B. Restless legs syndrome in a rheumatoid arthritis patient cohort. *J Clin Rheumatol* 2009; **15**: 12–15.

21 Merlino G, Fratticci L, Valente M *et al.* Association of restless legs syndrome in type 2 diabetes: a case-control study. *Sleep* 2007; **30**: 866–71.
22 Weinstock LB, Bosworth BP, Scherl EJ *et al.* Crohn's disease is associated with restless legs syndrome. *Inflamm Bowel Dis* 2010; **16**: 275–9.
23 Franco RA, Ashwathnarayan R, Deshpandee A *et al.* The high prevalence of restless legs syndrome symptoms in liver disease in an academic-based hepatology practice. *J Clin Sleep Med* 2008; **4**: 45–9.
24 Cordoba J, Cabrera J, Lataif L, Penev P, Zee P, Blei AT. High prevalence of sleep disturbance in cirrhosis. *Hepatology* 1998; **27**: 339–45.
25 Cho SJ, Hong JP, Hahm BJ *et al.* Restless legs syndrome in a community sample of Korean adults: prevalence, impact on quality of life, and association with DSM-IV psychiatric disorders. *Sleep* 2009; **32**: 1069–76.
26 Pugh RN, Murray-Lyon IM, Dawson JL, Pietroni MC, Williams R. Transection of the oesophagus for bleeding oesophageal varices. *Br J Surg* 1973; **60**: 646–9.
27 DeMaeyer EM, Dallman P, Gurney JM, Hallberg L, Sood S, Srikantia S. *Preventing and Controlling Iron Deficiency Anaemia through Primary Health Care: A Guide for Health Administrators and Programme Managers; Preventing and Controlling Iron Deficiency Anaemia through Primary Health Care: A Guide for Health Administrators and Programme Managers.* Geneva: World Health Organization, 1989.
28 Doi Y, Minowa M, Uchiyama M *et al.* Psychometric assessment of subjective sleep quality using the Japanese version of the Pittsburgh Sleep Quality Index (PSQI-J) in psychiatric disordered and control subjects. *Psychiatry Res* 2000; **97**: 165–72.
29 Buysse DJ, Reynolds CF, 3rd, Monk TH, Berman SR, Kupfer DJ. The Pittsburgh Sleep Quality Index: a new instrument for psychiatric practice and research. *Psychiatry Res* 1989; **28**: 193–213.
30 Fukuhara S, Ware JE, Jr, Kosinski M, Wada S, Gandek B. Psychometric and clinical tests of validity of the Japanese SF-36 Health Survey. *J Clin Epidemiol* 1998; **51**: 1045–53.
31 McHorney CA, Ware JE, Jr, Raczek AE. The MOS 36-Item Short-Form Health Survey (SF-36): II. Psychometric and clinical tests of validity in measuring physical and mental health constructs. *Med Care* 1993; **31**: 247–63.
32 LaRochelle JS, Karp BI. Restless legs syndrome due to interferon-alpha. *Mov Disord* 2004; **19**: 730–1.
33 Bhowmik D, Bhatia M, Tiwari S *et al.* Low prevalence of restless legs syndrome in patients with advanced chronic renal failure in the Indian population: a case controlled study. *Ren Fail* 2004; **26**: 69–72.
34 Goffredo Filho GS, Gorini CC, Purysko AS, Silva HC, Elias IE. Restless legs syndrome in patients on chronic hemodialysis in a Brazilian city: frequency, biochemical findings and comorbidities. *Arq Neuropsiquiatr* 2003; **61**: 723–7.
35 Crespo J, Cifrian J, Pinto JA, Jimenez-Gomez A, Pons-Romero F. Sleep apnea obstructive syndrome: a new complication previously undescribed in cirrhotic patients with ascites. *Am J Gastroenterol* 2003; **98**: 2815–16.
36 Cervenka S, Palhagen SE, Comley RA *et al.* Support for dopaminergic hypoactivity in restless legs syndrome: a PET study on D2-receptor binding. *Brain* 2006; **129**: 2017–28.
37 Partinen M, Hirvonen K, Jama L *et al.* Efficacy and safety of pramipexole in idiopathic restless legs syndrome: a polysomnographic dose-finding study–the PRELUDE study. *Sleep Med* 2006; **7**: 407–17.
38 Trenkwalder C, Benes H, Poewe W *et al.* Efficacy of rotigotine for treatment of moderate-to-severe restless legs syndrome: a randomised, double-blind, placebo-controlled trial. *Lancet Neurol* 2008; **7**: 595–604.
39 Burkhard PR, Delavelle J, Du Pasquier R, Spahr L. Chronic parkinsonism associated with cirrhosis: a distinct subset of acquired hepatocerebral degeneration. *Arch Neurol* 2003; **60**: 521–8.
40 Kim JM, Kim JS, Jeong SH *et al.* Dopaminergic neuronal integrity in parkinsonism associated with liver cirrhosis. *Neurotoxicology* 2010; **31**: 351–5.
41 Mostacci B, Ferlisi M, Baldi Antognini A *et al.* Sleep disturbance and daytime sleepiness in patients with cirrhosis: a case control study. *Neurol Sci* 2008; **29**: 237–40.
42 Thorpe AJ, Clair A, Hochman S, Clemens S. Possible sites of therapeutic action in restless legs syndrome: focus on dopamine and alpha2delta ligands. *Eur Neurol* 2011; **66**: 18–29.
43 Jacques Montplaisir RPA, Walters A, Ferini-Strambi L. Restless Legs Syndrome and Periodic Limb Movements during Sleep. *Principles and Practice of Sleep Medicine.* 5 edn 2010; 1026–37.

ARTICLE
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Branched-Chain Amino Acid Deficiency Stabilizes Insulin-Induced Vascular Endothelial Growth Factor mRNA in Hepatocellular Carcinoma Cells

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ABSTRACT

Abnormal sugar metabolism is closely related to chronic liver diseases, including hepatocellular carcinoma (HCC). We previously reported that fasting hyperinsulinemia is a poor prognostic factor for HCC patients. A recent large-scale study has shown that long-term administration of branched chain amino acids (BCAA) reduces the risk of HCC development in obese cirrhotic patients who have been diagnosed with diabetes mellitus, although the mechanism by which it does so is unclear. In this study, we analyzed the expression of vascular endothelial growth factor (VEGF) in HepG2 cells under high-insulin culture conditions, and examined the effect of BCAA on VEGF expression. VEGF secretion was significantly increased by 200 nM of insulin under BCAA deficient conditions, but it was repressed by the addition of BCAA. BCAA activated the mTOR pathway and increase HIF-1α expression under high-insulin culture conditions, however quantitative PCR analysis showed that insulin-induced expression of VEGF mRNAs (VEGF121 and VEGF165) decreased 2 h after the addition of BCAA. The half-lives of both VEGF121 and 165 mRNAs were shortened in the presence of BCAA compared to the absence of BCAA. Therefore it is thought that BCAA regulate VEGF expression mainly at the post-transcriptional level. We also examined which of the Valine, Leucine, and Isoleucine components of BCAA were essential for VEGF mRNA degradation. All three BCAA components were required for acceleration of insulin-induced VEGF mRNA degradation. These results suggest that administration of BCAA may downregulate VEGF expression in patients who have hyperinsulinemia and are in the process of developing HCC. J. Cell. Biochem. 113: 3113–3121, 2012.

KEY WORDS: BCAA; HCC; VEGF; HYPERINSULINEMIA; DECAY; STABILITY

Hepatocellular carcinoma (HCC) is the fifth most frequent malignant neoplasm in the world [Bosch et al., 2004]. The rising incidence of HCC has been extensively reported in the United States [El-Serag and Mason, 2000], Japan, and several other countries [Yu and Yuan, 2004]. In recent years, much interest has centered on the relationship between abnormal sugar metabolism and liver disease including HCC, because of its association with non-alcoholic fatty liver disease (NAFLD) including its severe form, non-alcoholic steatohepatitis (NASH) [Marchesini et al., 1999]. We have reported that the development of liver fibrosis is closely associated

Abbreviations: HCC, hepatocellular carcinoma; BCAA, branched chain amino acids; VEGF, vascular endothelial growth factor; NAFLD, non-alcoholic fatty liver disease; NASH, non-alcoholic steatohepatitis; HIF, hypoxia inducible factor; PI-3K, phosphoinositide 3-kinase; MAPK, mitogen-activated protein kinase; ROS, reactive oxygen species; FBS, fetal bovine serum; ELISA, enzyme linked immunosorbent assay; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; mTOR, mammalian target of rapamycin; qPCR, quantitative polymerase chain reaction; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; aa, amino acid.

Additional supporting information may be found in the online version of this article.

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with insulin resistance in HCV infected patients [Taura et al., 2006]. The combined data indicate that it is likely that insulin resistance in chronic liver disease triggers hyperinsulinemia and may modulate the biological characteristics of HCC cells. Indeed, Komura et al. [2007] have reported that insulin therapy for coexisting diabetes mellitus is an independent risk factor for HCC recurrence after a curative resection. To examine the relevance of hyperinsulinemia to the progression of HCC, we retrospectively studied a total of 140 patients, who were newly diagnosed with HCC at the Department of Gastroenterology and Hepatology in Nagasaki University Hospital [Miuma et al., 2009]. In that study, we reported that fasting hyperinsulinemia is a risk factor that is associated with a poor prognosis at the early stage of HCC and with a high-recurrence rate at the curative stage of HCC. Hyperinsulinemia alone is also thought to be a risk and a poor prognosis factor for patients with HCC.

A recent large-scale study has reported that administration of branched chain amino acids (BCAA) improves glucose intolerance and hyperinsulinemia in cirrhotic patients [Muto et al., 2005]. It has also been reported that in obese cirrhotic patients, who have been diagnosed with diabetes mellitus, the risk of developing HCC is significantly reduced following long-term administration of BCAA [Muto et al., 2006]. This report is very interesting, because it shows that a close association exists between insulin resistance due to hyperinsulinemia and BCAA, and that this association contributes to the progression of HCC in cirrhotic patients. There have been only a few reports to date regarding the suppression of liver cancer progression by BCAA. Murata and Moriyama [2007] showed that isoleucine prevents tumor growth in a mouse liver metastatic model of colon cancer through inhibition of vascular endothelial growth factor (VEGF). Yoshiji et al. [2010] reported that BCAA exerts a chemopreventive effect against HCC, which is associated with the suppression of VEGF expression and hepatic neovascularization in obese diabetic rats. Both of these reports suggest an anti-angiogenesis activity of BCAA or Isoleucine through suppression of VEGF expression. However, the mechanism by which BCAA administration suppresses VEGF expression remains unclear.

Angiogenesis is a necessary event for tumor growth and metastasis [Folkman et al., 1989; Weidner et al., 1991]. VEGF is one of the most potent of the angiogenic factors that have been identified [Ahmed et al., 2004; Underiner et al., 2004]. Furthermore previous studies [Ng et al., 2001; Poon et al., 2001] have reported that VEGF is a potent angiogenic factor leading to HCC invasiveness and metastasis. One trigger of VEGF expression is hypoxia caused by an imbalance in oxygen supply and consumption [Knighton et al., 1983; Shweiki et al., 1992]. Hypoxia-induced upregulation of VEGF is considered to be mediated primarily through hypoxia inducible factor (HIF), which is a heterodimeric basic helix–loop–helix transcription factor composed of two subunits, HIF-1α and HIF-1β [Wang et al., 1995; Sharp and Bernaudin, 2004]. Under hypoxic conditions, HIF-1α binds to HIF-1β and forms the HIF complex, which recognizes a consensus hypoxia response element in the VEGF promoter as well as in the promoters of a broad range of other HIF target genes [Hirota and Semenza, 2005]. Some reports suggest that the HIF-1 system is also induced by growth factors such as insulin under non-hypoxic conditions [Jiang et al., 2001; Laughner et al., 2001; Stiehl et al., 2002]. It has been reported that insulin induction of VEGF through HIF-1α mainly occurs through activation of the PI-3K pathway. However, VEGF induction by HIF-1α in HCC cells also involves a MAPK pathway and ROS production [Fukuda et al., 2002; Biswas et al., 2007].

Our aim was to determine the impact of BCAA on the development or progression of HCC in patients with hyperinsulinemia from the aspect of angiogenesis. Therefore, in the present study, we examined the effect of BCAA on VEGF expression in HCC cells cultured under high-insulin conditions.

MATERIALS AND METHODS

CELL CULTURE AND REAGENTS

Dulbecco's essential medium, fetal bovine serum (FBS) and a solution of human Insulin were obtained from Sigma Chemical Co. (St. Louis, MO). Each of the BCAA components; Valine, Leucine, and Isoleucine, as well as BCAA-free medium, which contained all amino acids except for the BCAA components, were obtained from Ajinomoto Pharmaceuticals Co. (Tokyo, Japan; Table I). Actinomycin D was obtained from Nacalai Tesque Co. (Kyoto, Japan). Human HCC cell lines, HepG2, Huh1, and Huh7 were obtained from the American Type Tissue Culture Collection (ATCC). They were maintained in Dulbecco's essential medium with low glucose containing 10% FBS, 100 mg/ml penicillin G, and 50 μg/ml streptomycin at 37°C in a humidified atmosphere containing 5% CO2.

QUANTIFICATION OF SECRETED VEGF PROTEIN BY ELISA

An equal number of HepG2, Huh-7, and Huh1 (5×10^4 cells per well) were plated in 96-well plates in DMEM containing 10% FBS. After the cells reached 70–80% confluency, the growth medium

TABLE I. Amino Acid Composition of BCAA Free Medium

Amino acid	Concentration (mM)	Amino acid	Concentration (mM)
Glycine	0.4	L-Asparagine	0.4
L-Alanine	0.4	L-Glutamic Acid	0.4
L-Serine	0.4	L-Aspartic Acid	0.4
L-Threonine	0.8	L-Valine	0
L-Cystine 2HCl	0.2	L-Leucine	0
L-Methionine	0.2	L-Isoleucine	0
L-Glutamine	4.0	L-Phenylalanine	0.4
L-Arginine-HCl	0.4	L-Tyrosine	0.4
L-Proline	0.4	L-Tryptophan	0.08
L-Lysine-HCl	0.8	L-HistidineHCl-H2O	0.2

was removed and was replaced with fresh BCAA-free medium without FBS. The cells were then incubated for 20 h, following which the medium was replaced with media with or without 200 nM insulin for 48 h. If BCAA was added, it was added at the concentrations indicated in the text, 30 min before insulin treatment. The medium was then harvested, filtered, and used for measurement of secreted VEGF. VEGF present in the medium was measured using the Quantikine Human VEGF ELISA kit (R&D Systems, Minneapolis, MN) according to the manufacturer's instructions. Cell viability was measured using the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS) assay. In brief, the cells were incubated in a 96-well plate as describe above, and 20 μl of CellTiter96® AQ_{ueous} One Solution Reagent (Promega, Madison, WI,) were added to each well. Following incubation for 2 h at 37°C, the color reaction was recorded at 490 nm using an automated plate reader (Bio-Rad, Melville, NY).

WESTERN BLOTTING

HepG2 cells were seeded in a 60 mm-diameter dish and grown to 80% confluence. The culture medium was then changed to BCAA-free medium without FBS and the cells were grown for a further 20 h. The cells were then treated with or without insulin for various times. If the components of BCAA were added, they were added at the concentrations indicated in the text 30 min before insulin treatment. The cells were then lysed by the addition of lysis buffer for 10 min at 4°C, and insoluble material was removed by centrifugation at 14,000 rpm for 30 min at 4°C. The same amount of protein from each lysate (each with 30 μg total protein, 20 μl/well) was subjected to 15% SDS-polyacrylamide gel electrophoresis. The proteins were transferred onto nitrocellulose membranes, which were then blocked for 1 h using 5% non-fat dried milk in PBS containing 0.1% Tween-20 (PBS-T). The membranes were washed with PBS-T and incubated at 4°C overnight in the presence of individual primary antibodies. The membranes were washed with PBS-T and incubated with sheep anti-mouse IgG or donkey anti-rabbit IgG coupled with horseradish peroxidase (Amersham Biosciences, Piscataway, NJ) for 1 h. The enhanced chemiluminescence system (SuperSignal West Pico Chemiluminescent Substrate; Pierce Chemicals, Rockford, IL) was used for signal detection.

Rabbit polyclonal anti-human phospho-mTOR, rabbit polyclonal anti-human phospho-p70S6K, rabbit monoclonal anti-human phospho-eIF4EBP1, rabbit polyclonal anti-human HIF-1α, and rabbit polyclonal anti-human β-Actin were obtained from Cell signaling Technology (San Diego, CA). All western blotting was performed at least in duplicate.

QUANTITATIVE PCR

The mRNAs of VEGF variants were quantified using quantitative polymerase chain reaction (qPCR). Total RNA was isolated from cell lines using the GenElute (TM) Mammalian Total RNA Miniprep Kit (Sigma Chemical Co.) according to the manufacturer's instructions. cDNA was synthesized from 1 μg of RNA using the QuantiTect Reverse Transcription kit (QIAGEN, Valencia, CA), and random hexamers. The cDNA was stored at −20°C until further analysis. Quantification of messenger RNA (mRNA) was performed using TaqMan or SYBR Green real-time PCR and the LightCycler (TM) 2.0 for Real-Time PCR system (Roche Applied Science, Indianapolis, IN). TaqMan qPCR of each VEGF isoform, without cross-reaction, was performed using the QuantiTect Probe PCR kit (QIAGEN). A common forward primer 5′-ATCTTCAAGCCATCCTGTGTGC-3′ and fluorescent hybridization probe 5′-AGTGTGTGCCCACTGAGGAGTCC-3′, both based on exon 3 sequences, were used. Each splice variant was amplified using specific reverse primers that spanned the variant specific exon boundaries: exon 5/8 for VEGF121 (5′-TGCGCTTGTCACATTTTTCTTG-3′), exon 5/7 for VEGF165 (5′-CAAGGCCCACAGGGATTTTC-3′), and exon 6/7 for VEGF189 (5′-CACAGGGAACGCTCCAGGAC-3′).

SYBR Green real-time PCR was performed for human GAPDH (forward primer 5′-TGAAGGTCGGAGTCAACGGATTTGGTCGTA-3′, reverse primer 5′-ATCTCGCTCCTGGAAGATGGTGATGGGATT-3′. To confirm specific amplification by the SYBR Green PCR, a dissociation curve analysis was performed for each primer pair, and both non-RT negative controls and water controls were used for these analyses. The amounts of loaded cDNA were normalized using GAPDH as an endogenous control. Differential gene expression was calculated by evaluation of the threshold cycle (Ct), and relative quantification was calculated using the comparative Ct method. All experiments were performed at least in duplicate.

RNA KINETICS

After HepG2 cells were treated with actinomycin-D (5 μg/ml) they were then incubated with or without 0.8 mM BCAA and 200 nM insulin. VEGF121 and VEGF165 mRNA were prepared at 0, 1, 2, 4, 6, and 8 h after actinomycin-D treatment and their mRNA levels were quantified using qPCR. The RNA quantities are expressed as a percentage of the mRNA level at the time point when actinomycin-D was added (0 h), and are referred to as the "percentage of RNA remaining." Degradation curves were estimated for these mRNAs using GraphPad Software (San Diego, CA).

STATISTICAL ANALYSIS

Values are expressed as means ± SD. Multiple comparisons were done using one-way ANOVA. Intergroup comparisons were done using Bonferroni's correction for multiple comparisons. A level of $P < 0.05$ was considered statistically significant.

RESULTS

BCAA SUPPRESSES INSULIN-INDUCED VEGF EXPRESSION IN HCC CELL LINES

HepG2, Huh7, and Huh1 cells were exposed to 200 nM of insulin in BCAA-free medium with or without 0.8 mM of BCAA for 48 h. VEGF secreted from the cells was then measured using an ELISA. In BCAA-free medium, insulin increased VEGF secretion in all HCC cell lines 1.4- to 2.3-fold compared to VEGF secretion in the absence of insulin (Fig. 1A). When 0.8 mM of BCAA was added to the BCAA-free medium, insulin-induced VEGF secretion was significantly suppressed in the HepG2 cells, but was only slightly suppressed in the Huh7 and Huh1 cells (Fig. 1A). We next assayed the effect of exposure of HepG2 cells to various concentrations of BCAA, in the presence or absence of insulin for 48 h, on VEGF secretion and on

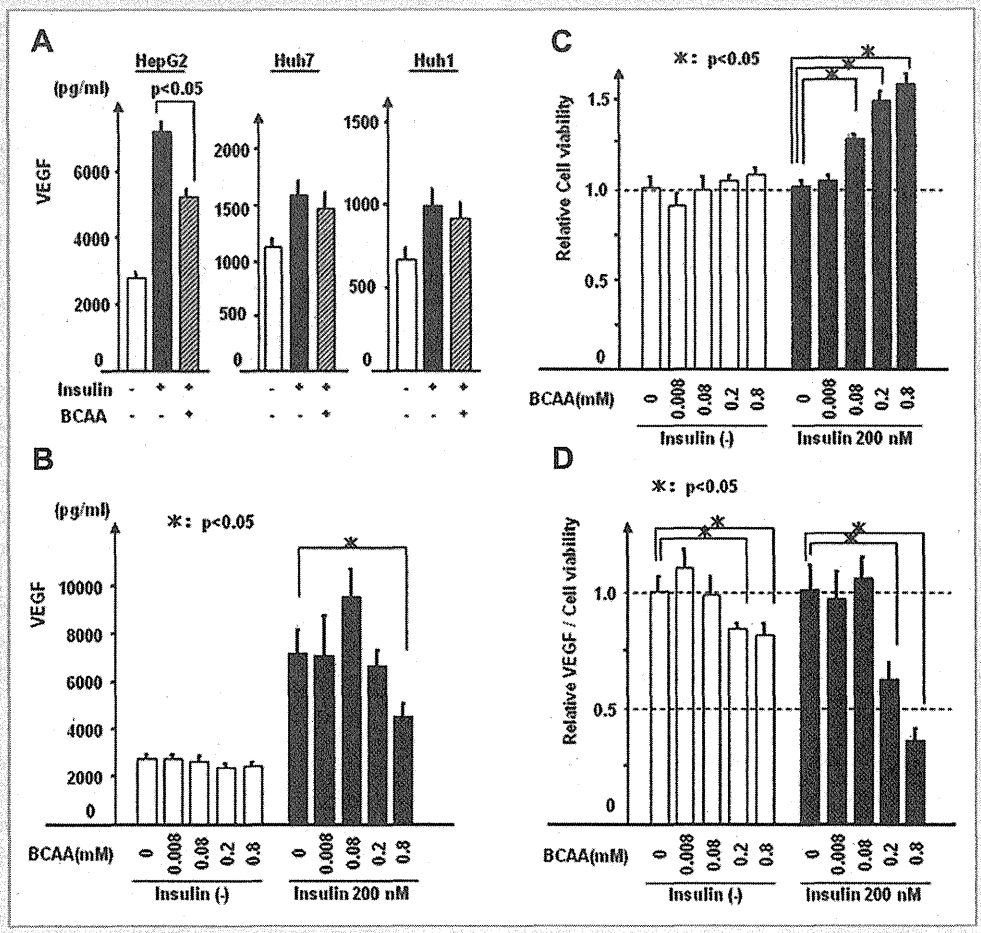

Fig. 1. Vascular endothelial growth factor (VEGF) secretion from HepG2, Huh-7, and Huh-1 cells after insulin treatment with or without BCAA. A: HepG2, Huh7, and Huh1 cells were exposed to BCAA-free medium with or without insulin (200 nM), or with insulin plus BCAA (0.8 mM) for 48 h, and VEGF secretion was analyzed by ELISA. Data represent the mean ± SD for four separate experiments. B,C: HepG2 cells were exposed to various BCAA concentrations with or without insulin for 48 h. VEGF secretion (B), and cell viability (C), were analyzed using an ELISA and an MTS assay, respectively. Data represent the mean ± SD for four separate experiments. D: The ratio of VEGF secretion to cell viability was then calculated. Data represent the ratio of VEGF secretion to cell viability over control value (the ratio in BCAA free medium), which was arbitrarily set to one with or without insulin, respectively, and represent the mean ± SD for four separate experiments.

cell viability. As the BCAA concentration was increased, insulin-induced VEGF secretion was significantly suppressed (Fig. 1B). In contrast, cell viability was significantly increased with increasing BCAA concentrations under high-insulin culture conditions (Fig. 1C). The ratio of VEGF secretion to cell viability was significantly decreased as the concentration of BCAA increased, either in the presence or absence of insulin. However, the decrease in this ratio was stronger in the presence of insulin (Fig. 1D).

BOTH BCAA AND INSULIN ACTIVATE THE mTOR PATHWAY AND INCREASE THE EXPRESSION OF HIF-1α

Previous reports showed that insulin-induced VEGF expression under non-hypoxic conditions involves activation of the PI-3K pathway, which is followed by activation of mTOR, P70S6K, and eIF4BP1 and results in the expression of HIF-1α [Fukuda et al., 2002; Stiehl et al., 2002]. It has also been shown that BCAA stimulates mTOR and activates signals that regulate protein translation and synthesis [Ijichi et al., 2003]. To determine the involvement of these signaling pathways in the above BCAA/insulin effects on HepG2 cells, we therefore examined the phosphorylation of PI-3K/m-TOR signaling proteins at 2 h, and the expression of HIF-1α at 6 h after BCAA and/or insulin treatment of HepG2 cells, using western blotting. As shown in Figure 2, the expression of phosphorylated mTOR, phosphorylated P70S6K, phosphorylated eIF4BP1, and HIF-1α was upregulated by BCAA or insulin treatment alone. Additionally, these changes were enhanced by combined treatment with both BCAA and insulin. These results indicate that BCAA and insulin synergistically activate intracellular signaling pathways that induce HIF-1α protein expression, although they had opposing effects on VEGF expression (Fig. 1).

A HIGH-INSULIN CONCENTRATION AUGMENTS, WHEREAS BCAA SUPPRESSES, THE EXPRESSION OF THREE MAJOR VARIANT VEGF mRNAs

We next analyzed the effect of BCAA and/or insulin treatment on the mRNA expression level of VEGF using qPCR. It is known that

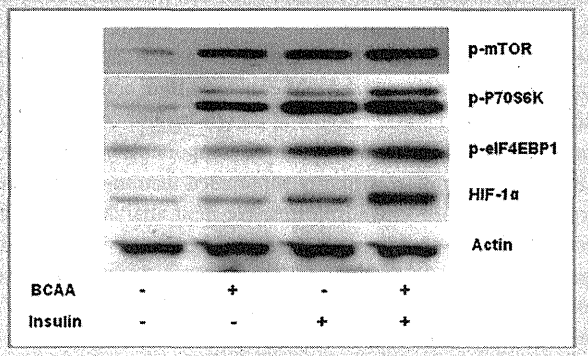

Fig. 2. Effects of BCAA and insulin on the expression of phosphorylated mTOR, phosphorylated p70S6K, phosphorylated eIF4EBP-1, and HIF-1α. HepG2 cells were exposed to BCAA-free medium with or without BCAA (0.8 mM) and/or insulin (200 nM). The expression of phosphorylated mTOR, phosphorylated p70S6K, and phosphorylated eIF4EBP-1 in cell lysates was analyzed after 2 h incubation by Western blotting using the appropriate antibodies. The expression of HIF-1α was similarly analyzed following 6 h incubation. Actin was blotted as a loading control.

there are many VEGF isoforms, that are derived from a single gene by alternative splicing. The three major isoforms of VEGF are VEGF121 (121 aa), VEGF165 (165 aa), and VEGF189 (189 aa) [Park et al., 1993; Neufeld et al., 1994; Takahashi and Shibuya, 2005]. It has been reported that the majority of HCC abundantly express VEGF121 and VEGF165, and that high-VEGF165 expression is related to poor prognosis of HCC patients [Jeng et al., 2004a]. Prior to analysis of VEGF mRNA expression in cells, we confirmed the reliability of the specific primer pairs and probes designed to assay VEGF121, VEGF165, and VEGF189 mRNA expression (Supplementary Figure). HepG2 cells were then incubated for various times with 200 nM of insulin in BCAA-free medium with or without 0.8 mM of BCAA, and the expression of VEGF mRNAs in the cells was then analyzed using qPCR. As shown in Figure 3, VEGF165 mRNA was the most abundant, and VEGF121 mRNA was the second-most abundant VEGF mRNA in HepG2 cells. VEGF189 mRNA was only weakly expressed. Insulin-induced expression of all three VEGF mRNAs decreased significantly 2h after BCAA treatment and remained low over the next 14 h. Since it takes 6 h to induce detectable levels of HIF-1α following BCAA treatment it is likely that the suppression of VEGF mRNA expression by BCAA is independent of the HIF-1 system.

BCAA DECREASE THE STABILITY OF INSULIN-INDUCED VEGF mRNA

The above data suggested that BCAA and insulin modulate intracellular signaling that regulates HIF-1α expression in a coordinated manner, but that BCAA antagonizes the effect of insulin on induction of the expression of VEGF mRNA. We therefore further analyzed the effects of BCAA and insulin on the post transcriptional regulation of VEGF mRNAs in these cells by determination of their effect on the half-life of VEGF mRNAs following inhibition of transcription using actinomycin-D. HepG2 cells were treated with actinomycin-D (5 μg/ml), and were incubated with insulin (200 nM) with or without BCAA (0.8 mM) for 0, 1, 2, 4, 6, and 8 h. The level of VEGF121 and VEGF165 mRNAs at these time points (termed "the remaining mRNA") was analyzed using q-PCR. Insulin treatment increased the stability of VEGF121 and VEGF165 mRNA in BCAA-free medium, respectively (Fig. 4). The addition of BCAA significantly decreased the stability of VEGF121 or VEGF165

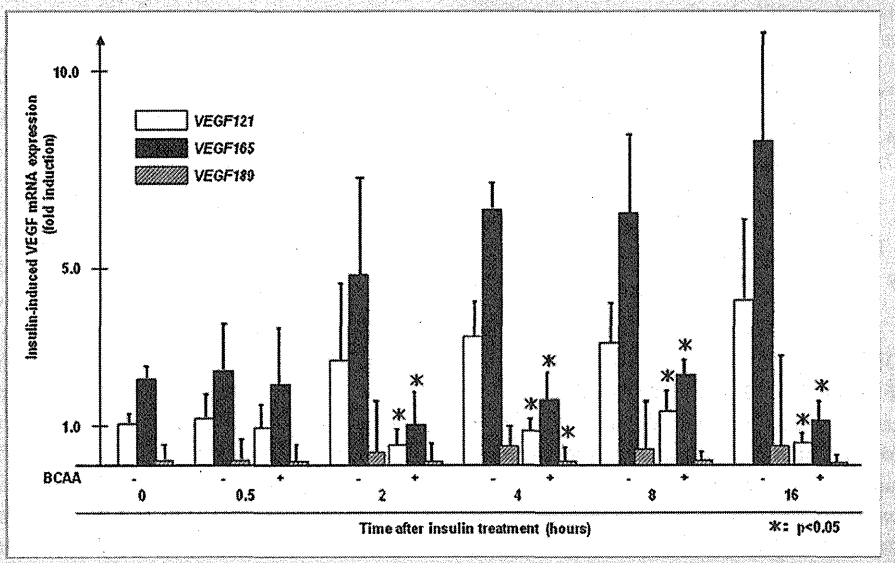

Fig. 3. Time course analysis of the mRNA expression of the three major isoforms of VEGF after insulin treatment with or without BCAA. HepG2 cells were exposed to insulin (200 nM) in BCAA free medium with or without BCAA (0.8 mM). The mRNA expression of VEGF121, VEGF165, and VEGF189 in the cells was then analyzed at the indicated times using quantitative PCR. Data represent fold induction of VEGF mRNA expression over control value (VEGF121 mRNA at 0 h), which was arbitrarily set to one, and represent the mean ± SD for four separate experiments. (*$P < 0.05$ for VEGF121, VEGF165, and VEGF189 mRNA level of BCAA(+)Insulin(+) group compared with BCAA(−)Insulin(+) group at each time).

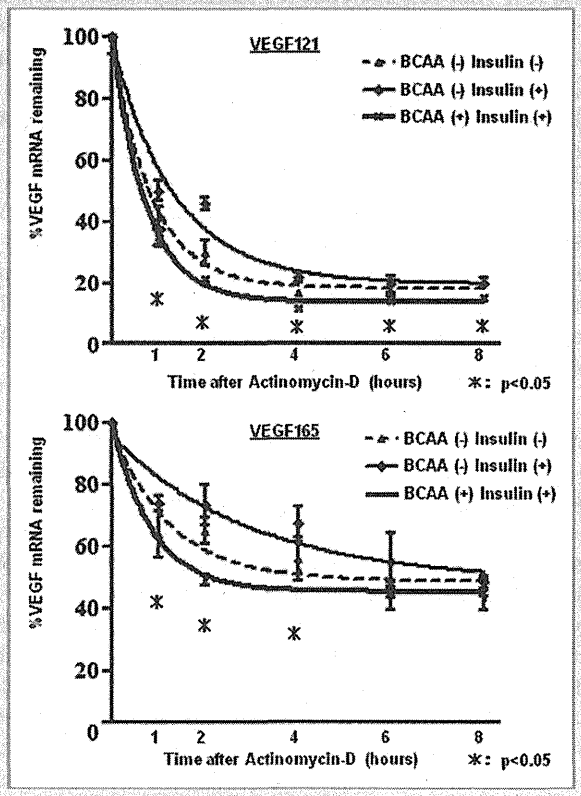

Fig. 4. VEGF121 and VEGF165 mRNA stability after actinomycin-D treatment with or without BCAA and insulin. HepG2 cells were incubated in BCAA-free medium for 20 h. The medium was then changed to fresh BCAA-free medium, or to medium with insulin (200 nM) with or without BCAA (0.8 mM), and actinomycin-D (5 μg/ml) was added to all cultures. mRNA was prepared 0, 1, 2, 4, 6, and 8 h after actinomycin-D treatment and the levels of VEGF121 and VEGF165 mRNA remaining at each time point were analyzed using quantitative PCR. Data represent mRNA expression over control value (VEGF 121 and VEGF 165 mRNA at 0 h), which was arbitrarily set to 100%, and represent the mean ± SD for four separate experiments. Degradation curves were constructed based on a model of exponential decay. (*$P < 0.05$ for VEGF121 and EGF165 mRNA level of BCAA(+)Insulin(+) group compared with BCAA(−)Insulin(+) group at each time).

mRNA, respectively. These data suggested that BCAA decreased VEGF mRNA stability at high-insulin concentrations.

A DEFICIT OF ONE COMPONENT OF BCAA ENHANCED THE EXPRESSION OF VEGF mRNA AT HIGH-INSULIN CONCENTRATIONS

BCAA consist of the three amino acids, Valine, Leucine, and Isoleucine. We examined which component, or combination of BCAA components, suppresses insulin-induced expression of VEGF mRNAs. HepG2 cells were exposed to insulin (200 nM) in BCAA-free medium containing Valine, Leucine, or Isoleucine at 0.8 or 2.4 mM concentrations. The concentration of 2.4 mM was used to adjust the Fischer ratio, which represents the ratio of branched-chain amino acids to aromatic amino acids. The cells were alternatively exposed to insulin (200 nM) and BCAA containing all three components, each at a concentration of 0.8 mM. Following incubation for 4 h, the mRNA expression of VEGF121 and VEGF165 was analyzed by qPCR. The results of this experiment are shown in Figure 5A. BCAA-free medium supplemented with individual amino acids at 0.8 mM concentration, or even at 2.4 mM concentration, could not suppress the expression of VEGF mRNAs at a high-insulin concentration. In contrast, the combination of the three components of BCAA, each at 0.8 mM concentration, strongly suppressed insulin-induced mRNA expression of both VEGF isoforms. We next analyzed the effect of combining two of the components of BCAA on insulin-induced upregulation of VEGF mRNAs. The cells were therefore exposed to insulin (200 nM) in BCAA-free medium supplemented with Valine and Leucine, Valine and Isoleucine, or Leucine and Isoleucine, each at a concentration of 0.8 or 1.2 mM. The cells were also exposed to insulin (200 nM) in media containing the three components of BCAA, each at a concentration of 0.8 mM. As seen for the single supplementation, double supplementation using any combination of the three components of BCAA, with each component at either 0.8 or 1.2 mM, did not suppress insulin-induced expression of VEGF mRNAs (Fig. 5B). These data indicate that all three components of BCAA are needed to accelerate the degradation of VEGF mRNAs at a high-insulin concentration, and thus, a deficiency in any of the components of BCAA stabilizes VEGF mRNA induced by a high-insulin concentration.

DISCUSSION

In this study, we demonstrated that treatment of HepG2 cells with BCAA suppresses insulin-induced VEGF secretion particularly at high-insulin concentrations. Individually BCAA and insulin similarly activate the mTOR pathway and increase the expression of HIF-1α. However, BCAA inhibits insulin-induced expression of VEGF mRNA by decreasing the stability of insulin-induced VEGF mRNAs.

We found that all three of the components of BCAA are required for this inhibitory effect. Neither individual components, nor any combination of two components, are inhibitory. In our experiments with complete BCAA, the concentration of Valine, Leucine, and Isoleucine was set at 0.8 mM each, to yield a Fischer ratio of 3.0. It has been reported that the mean value of the Fischer ratio is 3.5 in healthy controls, 2.7–3.0 in chronic hepatitis, 1.5 in compensated cirrhosis, and 1.1 in decompensated cirrhosis [Kano et al., 1991]. Based on these data we consider that the concentration of BCAA used in our experiments is appropriate for analysis of HCC cell metabolism. Indeed, the suppressive effect of BCAA on insulin-induced VEGF secretion was strengthened as the BCAA concentration was increased to give a Fischer ratio of 3.0 (Fig. 1D).

Several studies [Poon et al., 2001; Kaseb et al., 2009; Schoenleber et al., 2009] have reported a close association between the level of VEGF in tissue or serum and the development and progression of HCC, in which angiogenesis plays an important role. Park et al. [2000] have reported that the expression of VEGF gradually increases with the stepwise development of HCC. Therefore, BCAA administration has the potential to modulate the early stage of hepatocarcinogenesis, rather than the advanced stage of HCC, through the suppression of VEGF secretion.

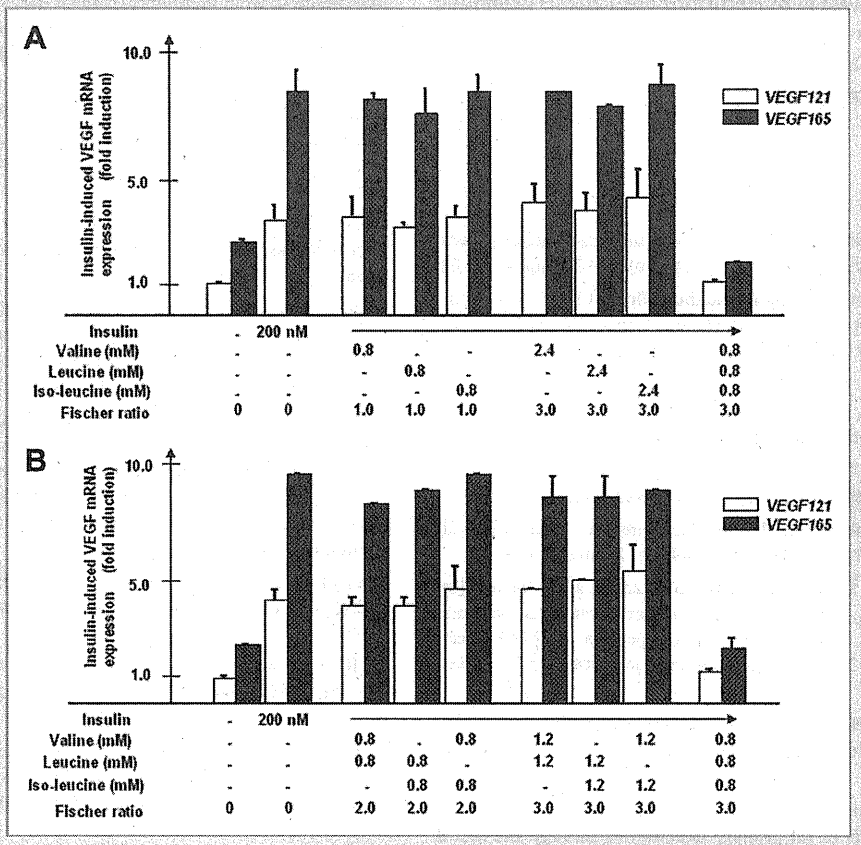

Fig. 5. The mRNA expression of VEGF 121 and VEGF 165 after insulin treatment with or without Valine, Leucine, and/or Isoleucine. A. HepG2 cells were exposed to BCAA-free medium with or without insulin (200 nM) in the presence or absence of individual amino acids, Valine, Leucine, or Isoleucine each at a concentration of 0.8 or 2.4 mM. The cells were alternatively exposed to insulin (200 nM) with BCAA (containing 0.8 mM each of all three amino acids). After 4 h incubation the mRNA expression of VEGF121 and VEGF165 was analyzed using quantitative PCR. B. HepG2 cells were exposed to BCAA free medium with or without insulin (200 nM) in the presence or absence of two amino acids, either Valine and Leucine, Valine and Isoleucine, or Leucine and Isoleucine, each at a concentration of 0.8 or 1.2 mM. The cells were alternatively exposed to insulin (200 nM) with BCAA (containing 0.8 mM each of all three amino acids). After 4 h incubation the mRNA expression of VEGF121 and VEGF165 was analyzed using quantitative PCR. The Fischer ratio, (ratio of branched-chain amino acids to aromatic amino acids) is shown below. Data represent fold induction of VEGF mRNA expression over control value (VEGF121 mRNA in BCAA free medium without insulin), which was arbitrarily set to one, and represent the mean ± SD for four separate experiments.

In our study, BCAA suppressed insulin-induced expression of all isoforms of VEGF mRNAs in a similar manner, including that of VEGF165 mRNA, whose expression has been reported to be linked to poor prognosis of HCC patients [Jeng et al., 2004b]. These results indicate that the process of alternative splicing of the VEGF gene is unaffected by BCAA.

It is a well-known fact that a major transcriptional activator of the VEGF gene is HIF1α [Semenza, 2002]. In this study, HIF1α expression was enhanced by combined treatment with both BCAA and insulin. It is likely that BCAA, which was reported to activate mTOR pathways [Ijichi et al., 2003], contribute to upregulate HIF1α expression with additive effect. Nevertheless, secretory VEGF expression was suppressed through the decrease of insulin-induced VEGF mRNA stability by BCAA. This result raises the possibility that the regulation of VEGF mRNA at post-transcriptional level plays an important role in secretory VEGF expression. Regulation of VEGF expression by modulation of its mRNA stability has also been reported to occur during induction of VEGF by hypoxia [von Marschall et al., 2001; Yoo et al., 2006].

Contrary to our expectations, all three components of BCAA are a prerequisite for the decay of VEGF mRNAs. Neither individual components, nor any combination of two components had this inhibitory effect. Interestingly, BCAA suppression was stronger at high-insulin concentrations. This phenomenon is in agreement with the results of a recent large-scale study, which reported that the administration of BCAA reduced the development of HCC in obese cirrhotic patients who had been diagnosed with diabetes mellitus [Muto et al., 2006]. This phenomenon may simply reflect an improvement of a BCAA deficiency rather than a direct effect of added BCAA. In other words, if there is a deficit in even one component of BCAA, VEGF mRNA is not degraded. It is likely that a BCAA deficit is a critical condition for HCC cell survival and that, even in the presence of a growth factor (such as insulin), HCC cells will give priority to the synthesis of proteins that are essential for survival.

In conclusion, we have demonstrated that a deficit of BCAA prevents the degradation of insulin-induced VEGF mRNA. This effect is repressed by supplementation with BCAA. These results

suggest that administration of BCAA to cirrhotic patients exhibiting both hyperinsulinemia and a decreased Fischer ratio has the potential to decrease HCC development or progression through suppression of VEGF expression.

REFERENCES

Ahmed SI, Thomas AL, Steward WP. 2004. Vascular endothelial growth factor (VEGF) inhibition by small molecules. J Chemother 16(Suppl 4):59–63.

Biswas S, Gupta MK, Chattopadhyay D, Mukhopadhyay CK. 2007. Insulin-induced activation of hypoxia-inducible factor-1 requires generation of reactive oxygen species by NADPH oxidase. Am J Physiol Heart Circ Physiol 292:H758–H766.

Bosch FX, Ribes J, Diaz M, Cleries R. 2004. Primary liver cancer: Worldwide incidence and trends. Gastroenterology 127:S5–S16.

El-Serag HB, Mason AC. 2000. Risk factors for the rising rates of primary liver cancer in the United States. Arch Intern Med 160:3227–3230.

Folkman J, Watson K, Ingber D, Hanahan D. 1989. Induction of angiogenesis during the transition from hyperplasia to neoplasia. Nature 339:58–61.

Fukuda R, Hirota K, Fan F, Jung YD, Ellis LM, Semenza GL. 2002. Insulin-like growth factor 1 induces hypoxia-inducible factor 1-mediated vascular endothelial growth factor expression, which is dependent on MAP kinase and phosphatidylinositol 3-kinase signaling in colon cancer cells. J Biol Chem 277:38205–38211.

Hirota K, Semenza GL. 2005. Regulation of hypoxia-inducible factor 1 by prolyl and asparaginyl hydroxylases. Biochem Biophys Res Commun 338: 610–616.

Ijichi C, Matsumura T, Tsuji T, Eto Y. 2003. Branched-chain amino acids promote albumin synthesis in rat primary hepatocytes through the mTOR signal transduction system. Biochem Biophys Res Commun 303:59–64.

Jeng KS, Sheen IS, Wang YC, Gu SL, Chu CM, Shih SC, Wang PC, Chang WH, Wang HY. 2004. Is the vascular endothelial growth factor messenger RNA expression in resectable hepatocellular carcinoma of prognostic value after resection? World J Gastroenterol 10:676–681.

Jeng KS, Sheen IS, Wang YC, Gu SL, Chu CM, Shih SC, Wang PC, Chang WH, Wang HY. 2004. Prognostic significance of preoperative circulating vascular endothelial growth factor messenger RNA expression in resectable hepatocellular carcinoma: A prospective study. World J Gastroenterol 10: 643–648.

Jiang BH, Jiang G, Zheng JZ, Lu Z, Hunter T, Vogt PK. 2001. Phosphatidylinositol 3-kinase signaling controls levels of hypoxia-inducible factor 1. Cell Growth Differ 12:363–369.

Kano T, Nagaki M, Takahashi T, Ohnishi H, Saitoh K, Kimura K, Muto Y. 1991. Plasma free amino acid pattern in chronic hepatitis as a sensitive and prognostic index. Gastroenterol Jpn 26:344–349.

Kaseb AO, Hanbali A, Cotant M, Hassan MM, Wollner I, Philip PA. 2009. Vascular endothelial growth factor in the management of hepatocellular carcinoma: A review of literature. Cancer 115:4895–4906.

Knighton DR, Hunt TK, Scheuenstuhl H, Halliday BJ, Werb Z, Banda MJ. 1983. Oxygen tension regulates the expression of angiogenesis factor by macrophages. Science 221:1283–1285.

Komura T, Mizukoshi E, Kita Y, Sakurai M, Takata Y, Arai K, Yamashita T, Ohta T, Shimizu K, Nakamoto Y, Honda M, Takamura T, Kaneko S. 2007. Impact of diabetes on recurrence of hepatocellular carcinoma after surgical treatment in patients with viral hepatitis. Am J Gastroenterol 102:1939–1946.

Laughner E, Taghavi P, Chiles K, Mahon PC, Semenza GL. 2001. HER2 (neu) signaling increases the rate of hypoxia-inducible factor 1alpha (HIF-1alpha) synthesis: Novel mechanism for HIF-1-mediated vascular endothelial growth factor expression. Mol Cell Biol 21:3995–4004.

Marchesini G, Brizi M, Morselli-Labate AM, Bianchi G, Bugianesi E, McCullough AJ, Forlani G, Melchionda N. 1999. Association of nonalcoholic fatty liver disease with insulin resistance. Am J Med 107:450–455.

Miuma S, Ichikawa T, Taura N, Shibata H, Takeshita S, Akiyama M, Motoyoshi Y, Ozawa E, Fujimoto M, Kawashimo H, Miyaaki H, Eguchi K, Nakao K. 2009. The level of fasting serum insulin, but not adiponectin, is associated with the prognosis of early stage hepatocellular carcinoma. Oncol Rep 22:1415–1424.

Murata K, Moriyama M. 2007. Isoleucine, an essential amino acid, prevents liver metastases of colon cancer by antiangiogenesis. Cancer Res 67:3263–3268.

Muto Y, Sato S, Watanabe A, Moriwaki H, Suzuki K, Kato A, Kato M, Nakamura T, Higuchi K, Nishiguchi S, Kumada H. 2005. Effects of oral branched-chain amino acid granules on event-free survival in patients with liver cirrhosis. Clin Gastroenterol Hepatol 3:705–713.

Muto Y, Sato S, Watanabe A, Moriwaki H, Suzuki K, Kato A, Kato M, Nakamura T, Higuchi K, Nishiguchi S, Kumada H, Ohashi Y. 2006. Overweight and obesity increase the risk for liver cancer in patients with liver cirrhosis and long-term oral supplementation with branched-chain amino acid granules inhibits liver carcinogenesis in heavier patients with liver cirrhosis. Hepatol Res 35:204–214.

Neufeld G, Tessler S, Gitay-Goren H, Cohen T, Levi BZ. 1994. Vascular endothelial growth factor and its receptors. Prog Growth Factor Res 5: 89–97.

Ng IO, Poon RT, Lee JM, Fan ST, Ng M, Tso WK. 2001. Microvessel density, vascular endothelial growth factor and its receptors Flt-1 and Flk-1/KDR in hepatocellular carcinoma. Am J Clin Pathol 116:838–845.

Park JE, Keller GA, Ferrara N. 1993. The vascular endothelial growth factor (VEGF) isoforms: Differential deposition into the subepithelial extracellular matrix and bioactivity of extracellular matrix-bound VEGF. Mol Biol Cell 4:1317–1326.

Park YN, Kim YB, Yang KM, Park C. 2000. Increased expression of vascular endothelial growth factor and angiogenesis in the early stage of multistep hepatocarcinogenesis. Arch Pathol Lab Med 124:1061–1065.

Poon RT, Ng IO, Lau C, Zhu LX, Yu WC, Lo CM, Fan ST, Wong J. 2001. Serum vascular endothelial growth factor predicts venous invasion in hepatocellular carcinoma: A prospective study. Ann Surg 233:227–235.

Schoenleber SJ, Kurtz DM, Talwalkar JA, Roberts LR, Gores GJ. 2009. Prognostic role of vascular endothelial growth factor in hepatocellular carcinoma: Systematic review and meta-analysis. Br J Cancer 100:1385–1392.

Semenza GL. 2002. HIF-1 and tumor progression: Pathophysiology and therapeutics. Trends Mol Med 8:S62–S67.

Sharp FR, Bernaudin M. 2004. HIF1 and oxygen sensing in the brain. Nat Rev Neurosci 5:437–448.

Shweiki D, Itin A, Soffer D, Keshet E. 1992. Vascular endothelial growth factor induced by hypoxia may mediate hypoxia-initiated angiogenesis. Nature 359:843–845.

Stiehl DP, Jelkmann W, Wenger RH, Hellwig-Burgel T. 2002. Normoxic induction of the hypoxia-inducible factor 1alpha by insulin and interleukin-1beta involves the phosphatidylinositol 3-kinase pathway. FEBS Lett 512: 157–162.

Takahashi H, Shibuya M. 2005. The vascular endothelial growth factor (VEGF)/VEGF receptor system and its role under physiological and pathological conditions. Clin Sci (Lond) 109:227–241.

Taura N, Ichikawa T, Hamasaki K, Nakao K, Nishimura D, Goto T, Fukuta M, Kawashimo H, Fujimoto M, Kusumoto K, Motoyoshi Y, Shibata H, Abiru N, Yamasaki H, Eguchi K. 2006. Association between liver fibrosis and insulin sensitivity in chronic hepatitis C patients. Am J Gastroenterol 101:2752–2759.

Underiner TL, Ruggeri B, Gingrich DE. 2004. Development of vascular endothelial growth factor receptor (VEGFR) kinase inhibitors as

anti-angiogenic agents in cancer therapy. Curr Med Chem 11:731–745.

von Marschall Z, Cramer T, Hocker M, Finkenzeller G, Wiedenmann B, Rosewicz S. 2001. Dual mechanism of vascular endothelial growth factor upregulation by hypoxia in human hepatocellular carcinoma. Gut 48:87–96.

Wang GL, Jiang BH, Rue EA, Semenza GL. 1995. Hypoxia-inducible factor 1 is a basic-helix-loop-helix-PAS heterodimer regulated by cellular O2 tension. Proc Natl Acad Sci USA 92:5510–5514.

Weidner N, Semple JP, Welch WR, Folkman J. 1991. Tumor angiogenesis and metastasis-correlation in invasive breast carcinoma. N Engl J Med 324:1–8.

Yoo PS, Mulkeen AL, Cha CH. 2006. Post-transcriptional regulation of vascular endothelial growth factor: Implications for tumor angiogenesis. World J Gastroenterol 12:4937–4942.

Yoshiji H, Noguchi R, Kaji K, Ikenaka Y, Shirai Y, Namisaki T, Kitade M, Tsujimoto T, Kawaratani H, Fukui H. 2010. Attenuation of insulin-resistance-based hepatocarcinogenesis and angiogenesis by combined treatment with branched-chain amino acids and angiotensin-converting enzyme inhibitor in obese diabetic rats. J Gastroenterol 45:443–450.

Yu MC, Yuan JM. 2004. Environmental factors and risk for hepatocellular carcinoma. Gastroenterology 127:S72–S78.

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ORIGINAL ARTICLE

Human T-cell leukemia virus type 1 infection worsens prognosis of hepatitis C virus-related living donor liver transplantation

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Keywords
donor age, HCV, HTLV-1, liver transplantation.

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Conflicts of interest
None.

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Summary

Severe and life-threatening donor-transmitted human T-cell leukemia virus type 1 (HTLV-1) infections after solid organ transplantation have been reported. However, in HTLV-1-infected recipients, graft and patient survival were not fully evaluated. A total of 140 patients underwent living donor liver transplantation (LDLT). Of these, 47 of 126 adult recipients showed indications of hepatitis C virus (HCV)-related liver disease. The HTLV-1 prevalence rate was 10 of 140 recipients (7.14%) and three of 140 donors (0.02%). In HCV-related LDLT, graft and patient survival was worsened by HTLV-1 infection in recipients (seven cases). The 1-, 3-, and 5-year survival rates in the HCV/HTLV-1-co-infected group were 67%, 32%, and 15%, respectively, and the corresponding rates in the HCV-mono-infected group were 80%, 67%, and 67%, respectively. Only the 5-year survival rates were statistically significant ($P = 0.04$, log-rank method). HTLV-1 infection in recipients is also an important factor in predicting survival in HTLV-1 endemic areas.

Introduction

Human T-cell leukemia virus type 1 (HTLV-1) is highly endemic in the southwestern area of Japan, including Nagasaki as well as in Saharan Africa, South America, the Caribbean islands, and aboriginal Australia [1,2]. However, HTLV-1 infects approximately 15–25 million people worldwide [3] and is associated with adult T-cell leukemia/lymphoma (ATL), HTLV-1-associated myelopathy (HAM), uvetitis, sialadenitis-like Sjögren syndrome (SjS), and a wide variety lymphocyte-mediated disorders [2,4,5]. Severe and life-threatening donor-transmitted HTLV-1 infections after solid organ transplantation have been reported [6–8]. However, in HTLV-1-infected recipients, graft and patient survival has not been fully evaluated. The development of three ATL cases in eight HTLV-1 infected recipients after living donor liver transplantation (LDLT) was reported in Japan [9]. We also reported the development of HAM [10] and sialadenitis-like SjS [11] resulting from HTLV-1 in LDLT recipients. Previous reports state that HTLV-1 infection is associated with a nonresponse to interferon (IFN) monotherapy for chronic hepatitis C (CHC) [12] and flare-up of alanine aminotransferase in hepatitis C virus (HCV)-RNA carriers [13]. HTLV-1/HCV co-infection may affect the course of HCV-associated liver disease and liver cancer [14].

Additionally, HTLV-1 interferes with intracellular signaling by type 1 IFN and upregulates HCV replication [14–16]. However, the influence of HTLV-1 in recipients on the grafted liver has not been explored. The effect of HTLV-1/HCV co-infections in recipient compared with HCV mono-infections has similarly not been explored.

Between 1988 and 2000, 0.027% of donors reporting to the United Network for Organ Sharing (UNOS) were diagnosed with HTLV-1 infections [6]. However, the prevalence of anti-HTLV-1 antibodies in patients visiting Nagasaki University Hospital between 2000 and 2007 was 13.57% [2], indicating that HTLV-1 carriers are clustered in Nagasaki. To prevent vertical transmission of HTLV-1, the ATL Prevention Program, which is a prefecture-wide breastfeeding intervention study for HTLV-1 carrier mothers, was initiated in Nagasaki in 1987 [17]. As a result, age-specific rates of HTLV-1 among residents in Nagasaki have annually declined (Seropositive rate, 14.5% in 2000; 12.7% in 2007) [2]. The prevalence of anti-HCV antibody increased with age and was higher in populations in the southwestern area of Japan (including Nagasaki) [18]. In endemic areas of HTLV-1 infection, HTLV-1/HCV co-infected patients are frequently observed and increase the probability a person will have a liver transplantation.

The HTLV-1 infection rate is lower in Western countries; however, the influence of HTLV-1 on HCV infection after transplantation has not been examined. It is necessary to evaluate HTLV-1 infection rates in HTLV-1 endemic areas. We examined whether HTLV-1 infection influences patient and graft survival in cases of liver transplantation in endemic areas of HTLV-1 infection in Nagasaki.

Patients and methods

Patients

In total, 126 consecutive adult LDLT patients, 47 of who were HCV-infected, were enrolled in this study. This retrospective cohort study of LDLT recipients included a comparative analysis of HTLV-1-positive and HTLV-1-negative recipients to determine graft and patient survival. In particular, we evaluated whether HTLV-1 infection influenced HCV-related LDLT. Anti-HTLV-1 antibody was detected using an enzyme immunoassay (EIA). In addition, in HTLV-1-positive patients, we used polymerase chain reaction (PCR) analysis to evaluate HTLV-1 proviral DNA in the peripheral blood mononuclear cells. We diagnosed patients with the anti-HTLV-1 antibody and proviral DNA as being HTLV-1 positive. In our hospital, HTLV-1-positive grafts are not used for negative recipients, but are used for positive recipients. Recipient characteristics such as age, gender, body mass index, Child-Pugh score and medical model for end-stage liver disease (MELD) score at the time of transplantation, presence or absence diabetes mellitus (DM), and presence of hepatocellular carcinoma (HCC) were also analyzed. Surgical factors examined included blood type matching, bleeding volume, (ml), and surgery time (min). Donor age was categorized into those less than 50 years old and 50 years old and older. Additional donor characters, such as donor gender, donor BMI, and donor HTLV-1 status were analyzed. HCV factors included genotype (1b or non-1b), titer in 1b, core amino acid mutation in 1b, and IL28B SNP. The HCV-RNA high group (100 000 IU/ml or more in the serum) of patients was analyzed using real-time PCR.

Primary outcomes evaluated included recipient and graft survival. The cause of death was determined using various factors together with biopsy and necropsy. Liver biopsy was performed each year and at exacerbation of liver function.

Methods

The study design, which also included the collection of data from medical records from the associated hospitals mentioned above, was approved by the Ethics Review Board of our hospital.

In this study, 3 *IL28B* SNPs, i.e., rs8099917, rs12979860, and rs12980275, were examined (Nagasaki University Institutional Review Board approval number: 100511184). SNPs were detected using pyrosequence analysis. The sense, antisense, and pyrosequence primers were **B**-5′-TCCTCCTTTTGTTTTCCTTTCTG-3′, 5′-AAAAAG CCAGCTACCAAACTGT-3′, and 5′-TGGTTCCAATTT GGG-3′ for rs8099917, 5′-GTCGTGCCTGTCGTGTAC TGA-3′, 5′-**B**-GGAGCGCGGAGTGCAATT-3′, and 5′-GG AGCTCCCCGAAGG-3′ for rs12979860, and 5′-GCTG TATGATTCCCCCTACATG-3′, 5′-**B**-TACATTGTTCGG CAAGCAATCT-3′, and 5′-AGAAGTCAAATTCCTAG AAA-3′ for rs12980275, respectively. "**B**" in the primer sequences indicates that the primer is biotin-labeled.

Statistical analysis

Data were processed on a personal computer and analyzed using StatView 5.0 (SAS Institute, Inc., Cary, NC, USA).Graft and patient survival was determined using the Kaplan–Meier method and survival curves were compared using a log-rank test. A cox proportional hazard model was used to determine risk factors for graft and patient survival. Differences between each laboratory data were analyzed using the Mann–Whitney *U*-test and χ^2 test. *P*-values < 0.05 were considered statistically significant.

Results

We evaluated the impact of HTLV-1 on general graft and patient survival in HCV-infected patients. Of the 140 patients who had undergone LDLT at the Nagasaki University Hospital between 1997 and January 2011, 47 of 126 adult recipients showed indications of HCV-related liver disease. The HTLV-1 prevalence rate was 7.8% (11/140) in the recipients and 2% (3/140) in the donors. Fourteen of the 140 recipients were pediatric recipients. HCV-related LDLT was observed only in adults. All HTLV-1 infected recipients were adult cases. First, we evaluated impact of HTLV-1 for LDLT in adult cases. In HCV-related LDLTs (Fig. 1a), graft and patients survival was worsened by the presence of HTLV-1 infection of recipients. The 1-, 3-, and 5-year survival rates in the HCV/HTLV-1-co-infected group were 67%, 32%, and 15%, respectively, and the corresponding rates in the HCV-mono-infected group were 80%, 67%, and 67%, respectively. Only the 5-year survival rate was found to be statistically significant ($P = 0.04$, log-rank method). However, adult recipients without HCV infection did not develop graft loss and patient death (Fig. 1b). In HCV-related LDLTs, clinical and demographic characteristics in HTLV-1-positive and HTLV-1-negative recipients did not differ between groups, except for donor age (Table 1). We attempted to clarify the factors of graft and patient survival in HCV-infected recipients by univariate analysis. MELD score and donor age at transplantation in the HTLV-1-infected recipients were shown to be significant factors. However, according to multivariate analysis, only donor age was a factor in worsening prognosis ($P < 0.05$; Relative risk 1.048). Three types of IL28B SNPs were not associated with graft and patient survival in HCV infected recipients according to a log rank test and univariate analysis of a Cox proportion hazard test.

Second, we analyzed stratification by donor age. Clinical characteristics, shown in Table 1, in the recipients who tested positive and negative for HTLV-1 did not differ between groups. In HCV-related LDLT recipients from old age donor group (age, 50 years and more; co-infected, 3 cases; HCV mono-infected, 13 cases), graft and patient survival was not worsened by recipient HTLV-1 infection (log-rank test, not significant). However, in the young age donor group (age less than 50 years; co-infected, 4 cases; HCV mono-infected, 34 cases), graft and patient survival was significantly worsened by recipient HTLV-1 infection (log-rank test, $P < 0.05$). However, graft and patient survival in HCV/HTLV-1-co-infected patients did not differ between the old and young donors, and the outcomes of HCV-mono-infected patients differed between the old and young donors according to the log-rank test. On the basis of multivariate analysis using a Cox proportional hazard test, HTLV-1 infection in HCV-infected recipients who received the transplant from younger donors was the only factor contributing to a worsened prognosis ($P = 0.03$; relative risk, 0.207).

Finally, we present the profile of seven cases of HCV/HTLV-1 co-infected recipients (Table 2). In the HCV/HTLV-1 co-infected group, chronic rejection (CR) developed in 3 patients, cases 60, 80, and 117, during peg-interferon/ribavirin treatment. CR did not develop in HCV-mono-infected patients. However, the CR rate was not statistically significant between the HCV/HTLV-1-co-infected group and the HCV-mono-infected group. Patients with CR did not have a prior history of acute rejection and used cyclosporine as an immunosuppressant. HCV-RNA disappearance during peg-interferon combination treatment with ribavirin was not observed in 3 CR cases. The period of peg-interferon combination treatment with ribavirin is 47, 23, and 2 months for HTLV-1/HCV co-infected CR patients. The treatment regimen of the

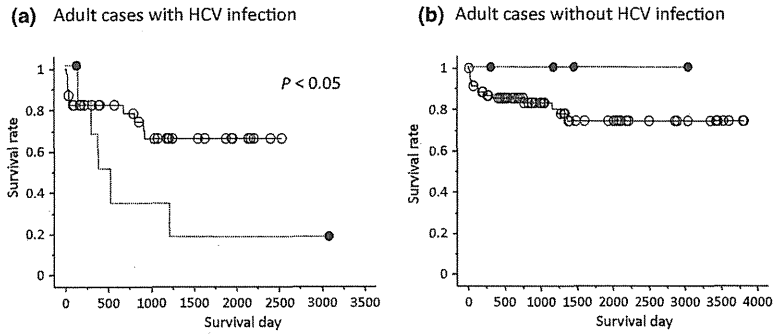

Figure 1 Kaplan–Meier curves for graft and recipient survival in adult transplant cases. A Kaplan–Meier curve revealed adult recipients with HCV infection (a) and without HCV infection (b). Black circle and dot line indicate HTLV-1 positive recipients and white circle and solid line indicate HTLV-1 negative recipients. In adult case with HCV-related LDLT, graft and patient survival of HTLV-1-infected recipients significantly decreased (a, $P < 0.05$).

Table 1. Clinical characteristics in patients with HCV infection.

	HTLV-1 positive	HTLV-1 negative	*P*-value
Number	7	40	
Age (years)	57.6 (7.16)	58.5 (6.48)	NS
Gender M/F	6/1	20/20	NS
BMI	25.6 (5.17)	25.0 (3.08)	NS
CP-score	10.6 (3.25)	9.85 (2.27)	NS
MELD	11.8 (8.38)	13.6 (8.16)	NS
DM +/-	2/5	20/20	NS
HCC +/-	4/3	27/13	NS
Donor age	48 (14.5)	37.6 (11.8)	0.04
Donor gender M/F	6/1	18/22	NS
Donor BMI	21.9 (2.42)	22.0 (2.62)	NS
Matching +/-	7/0	32/8	NS
Bleeding volume (ml)	5222 (3840)	14 182 (21 230)	NS
Surgery time (min)	902 (171)	934 (215)	NS
HCV GT1/non GT1	6/1	30/10	NS
HCV high titer in GT1	5	28	NS
IL28B SNP Major/Minor	1/2	24/6	NS
SVR rate	1/7	9/40	NS

Data are shown as means (standard deviation) and numbers, with statistical analysis assessed using a Mann–Whitney test for means and χ^2 test for numbers. Statistically significant difference between HTLV-1 positive and negative groups is $P < 0.05$. CP-score, Child–Pugh score; HCV GT, HCV genotype; Matching, Blood type matching; IL28B SNP Major, TT of rs8099917 in recipient and donor; Minor, TG or GG of rs8099917 in recipient and/or donor. BMI, body weight (kg)/height (m)/height (m). SVR, sustained viral response.

Table 2. Clinical characteristics in recipients with HCV and HTLV-1 co-infection.

Case number	20	59	60	80	112	117	132
Age (years)	58	50	68	65	48	54	58
Gender	M	M	M	F	M	M	M
Survival	+	+	–	–	–	–	–
Survival time (day)	3086	1210	528	378	139	301	132
Cause of death	–	HCC	CR	CR	Infect.	CR	–
IFN	+	–	+	+	–	+	–
Viral response	–	–	–	–		–	
IFN period (month)	48		47	23		2	
HCV GT1b	1b	1b	1b	1b	1b	N	1b
BMI	19.3	31.1	23.2	30.5	19.2	30.2	25.9
HCC	–	outside	Milan	Milan	–	Milan	–
MELD	8.1	8.5	4.4	7.3	29.3	15.2	9.9
DM	–	+	+	–	+	+	+
Donor	Sister	Brother	Child	Brother	Brother	Uncle	Child
Donor age	56	45	41	61	46	65	22
Donor HTLV-1	+	–	–	–	–	–	–

Viral response is the disappearance of HCV-RNA in patients under peg-IFN/ribavirin treatment. IFN period is treatment length (month) of peg-IFN/ribavirin treatment. Infect., infection; AIH, autoimmune hepatitis; BA, biliary atresia; LCN, cryptogenic cirrhosis; LCB, hepatitis B virus infected liver cirrhosis. Milan, HCC within Milan criteria, Outside, HCC without Milan criteria.

peg-interferon combination with ribavirin was performed under the rules of our hospital and was the same as was conducted for other HCV-related transplanted patients [19]. Hence, as an immunosuppressive therapy, tacrolimus was used for all HCV-infected patients as an induction therapy combined with steroid tapering; subsequently, tacrolimus treatment was intentionally replaced with cyclosporine treatment to facilitate interferon therapy [20,21] except in case 20. Case 20, which involved an HTLV-1 infected donor, suffered an onset of HAM and sialadenitis under the tacrolimus immunosuppressant regime [11]. Five cases of death occurred in the co-infected group. Causes of death in patients with HTLV-1/HCV co-infection included hepatoma recurrence, infection, and CR. ATL was not observed in this study. Progression of HCV and/or HTLV-1 infection was not always related to death. In particular, all CR cases developed during interferon treatment. Poor survival of HTLV-1/HCV-co-infected patients may have been caused by CR. HCV-RNA levels decreased in the CR cases when the length of peg-IFN/ribavirin treatment was less than 1 year.

Discussion

In this study, we clarified that HTLV-1 infection in HCV-infected recipients is an exacerbation factor involved in survival of both the graft and the patient. Particularly, young donors suffer detrimental effects caused by HTLV-1 infection. Survival of HCV-infected recipients is affected by donor age, MELD score, and HTLV-1 infection. Donor age is the most significant factor in graft and patient survival, and HTLV-1 infection in recipients is the second most important factor in survival in HTLV-1 endemic areas. Donors of advanced age and high MELD scores have been reported as complicating factors [22,23]. We report the impact of HTLV-1 infection on graft and patient survival for the first time.

The presence of HTLV-1 infection as a complicating factor in recipients was revealed after adjusting for age. As HCV/HTLV-1 co-infection occurred in three cases in older donors and four cases in younger donors, it was necessary to determine the role of donor age in HCV/HTLV-1-co-infected recipients. In the HTLV-1 infection-negative group, graft and patient survival was shorter in older donors than in younger donors, but in the HCV/HTLV-1-co-infected group, graft and patient survival did not differ between the old and young donors. Donor age is a complicating factor for graft and patient survival regardless of HTLV-1 infection [23]. The survival rate of the young donor group may initially be high, but survival rate decreases in the presence of HTLV-1 infection. HTLV-I possesses a unique and innate (or acquired) capacity to preserve cellular immunity, such as IL-2 and

IL-2-receptor induction [24]. HTLV-1 infection may lead to a stronger immune response in recipients when the donor is young than when the donor is old.

The relationship among IFN, HCV, and CR at post-liver transplantation has been previously studied. It is reported that peg-IFN/ribavirin treatment for HCV may trigger rapid CR in patients with therapeutic immunosuppressant trough levels, with or without first inducing acute cellular rejection [25]. Other reports state that the use of cyclosporine, ribavirin discontinuation, a peg-IFN treatment duration of over 1 year, and HCV infection elimination for IFN treatment appear to be associated with CR [26,27]. We suspect that HTLV-1-infected recipients under peg-IFN/ribavirin treatment may be associated with CR for young graft donors and have different immunological mechanisms than HTLV-1 negative recipients.

Recently, the relationship of IL28B SNP and HCV infection has been studied [28]. It has been reported that IL28B SNP is not only related to the effect of IFN treatment, but also to the natural course of HCV infection [29]. We conducted an analysis of IL28B SNP in only 33 pairs of donors and recipients who had obtained agreement in 47 cases of HCV-related liver transplantation. In this study, Il28B SNP was not related to graft and patient survival. However, upon analysis of three types of IL28B SNPs, the survival rate was the same for all three SNPs. Previous reports state that there are no statistical differences in overall graft survival according to recipient and donor IL28B SNPs [30,31].Since it is reported that IL28B SNPs in both recipients and donors is associated with IFN response [30–32], differences in long-term survival between IL28B SNP groups has been examined.

Due to the low prevalence HTLV-1 infection in western countries, the association of liver disease and HTLV-1 infection has not been evaluated. In this study, performed in an HTLV-1 endemic area, we determined that HTLV-1 increases mortality after HCV-related LDLT. Presently, to improve mortality rates, the presence of CR should be determined when HCV/HTLV-1 co-infected transplanted patients are treated using IFN/ribavirin. However, as CR treatment has not been fully evaluated, the mechanism of HTLV-1 infected T cells in HCV-infected graft liver patients under peg-IFN/ribavirin treatment should be determined. The follow-up period of the seven HCV/HTLV-1-co-infected patients was 132–3086 days. As our study population was small and follow-up periods were short, we will extend the follow-up period to validate our results.

Authorship

TI, NT, HM, TM, MO, SE, MT, AS, MH, SO, TU, SM: performed study. SK, TK and KN: designed the study. TI: wrote the manuscript.

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References

1. Verdonck K, Gonzalez E, Van DoorenS, Vandamme AM, Vanham G, Gotuzzo E. Human T-lymphotropic virus 1: recent knowledge about an ancient infection. *Lancet Infect Dis* 2007; **7**: 266.
2. Koga Y, Iwanaga M, Soda M, *et al.* Trends in HTLV-1 prevalence and incidence of adult T-cell leukemia/lymphoma in Nagasaki, Japan. *J Med Virol* 2010; **82**: 668.
3. Gessain A, Mahieux R. A virus called HTLV-1. Epidemiological aspects. *Presse Med* 2000; **29**: 2233.
4. Goncalves DU, Proietti FA, Ribas JG, *et al.* Epidemiology, treatment, and prevention of human T-cell leukemia virus type 1-associated diseases. *Clin Microbiol Rev* 2010; **23**: 577.
5. Terada K, Katamine S, Eguchi K, *et al.* Prevalence of serum and salivary antibodies to HTLV-1 in Sjogren's syndrome. *Lancet* 1994; **344**: 1116.
6. Kusne S. Regarding unexpected severe and life-threatening donor-transmitted viral infections and use of high-risk behavior donors. *Liver Transpl* 2008; **14**: 1564.
7. Toro C, Rodes B, Poveda E, Soriano V. Rapid development of subacute myelopathy in three organ transplant recipients after transmission of human T-cell lymphotropic virus type I from a single donor. *Transplantation* 2003; **75**: 102.
8. Hoshida Y, Li T, Dong Z, *et al.* Lymphoproliferative disorders in renal transplant patients in Japan. *Int J Cancer* 2001; **91**: 869.
9. Kawano N, Shimoda K, Ishikawa F, *et al.* Adult T-cell leukemia development from a human T-cell leukemia virus type I carrier after a living-donor liver transplantation. *transplantation* 2006; **82**: 840.
10. Soyama A, Eguchi S, Takatsuki M, *et al.* Human T-cell leukemia virus type I-associated myelopathy following living-donor liver transplantation. *liver transpl* 2008; **14**: 647.
11. Nakamura H, Ichikawa T, Nakamura T, *et al.* Macrophage-dominant sialadenitis in human T-cell leukemia virus type I-associated myelopathy after living-donor liver transplantation. *Transplant Proc* 2010; **42**: 2797.
12. Kishihara Y, Furusyo N, Kashiwagi K, Mitsutake A, Kashiwagi S, Hayashi J. Human T lymphotropic virus type 1 infection influences hepatitis C virus clearance. *J Infect Dis* 2001; **184**: 1114.
13. Tsuji K, Yamasaki K, Yamanishi M, Kawakami M, Shirahama S. Risk of alanine aminotransferase flare-up among asymptomatic hepatitis C virus RNA carriers: a 10-year follow-up study. *J Gastroenterol Hepatol* 2001; **16**: 536.
14. Boschi-Pinto C, Stuver S, Okayama A, *et al.* A follow-up study of morbidity and mortality associated with hepatitis C virus infection and its interaction with human

T lymphotropic virus type I in Miyazaki, Japan. *J Infect Dis* 2000; **181**: 35.
15. Zhang J, Yamada O, Kawagishi K, *et al.* Up-regulation of hepatitis C virus replication by human T cell leukemia virus type I-encoded Tax protein. *Virology* 2007; **369**: 198.
16. Feng X, Ratner L. Human T-cell leukemia virus type 1 blunts signaling by interferon alpha. *Virology* 2008; **374**: 210.
17. Hino S, Katamine S, Miyata H, Tsuji Y, Yamabe T, Miyamoto T Primary prevention of HTLV-1 in Japan. *Leukemia* 1997; **11** Suppl 3: 57.
18. Tanaka J, Kumagai J, Katayama K, *et al.* Sex- and age-specific carriers of hepatitis B and C viruses in Japan estimated by the prevalence in the 3,485,648 first-time blood donors during 1995-2000. *Intervirology* 2004; **47**: 32.
19. Ichikawa T, Nakao K, Miyaaki H, *et al.* Hepatitis C virus kinetics during the first phase of pegylated interferon-alpha-2b with ribavirin therapy in patients with living donor liver transplantation. *Hepatol Res* 2009; **39**: 856.
20. Eguchi S, Takatsuki M, Soyama A, *et al.* Intentional conversion from tacrolimus to cyclosporine for HCV-positive patients on preemptive interferon therapy after living donor liver transplantation. *Ann Transplant* 2007; **12**: 11.
21. Hirano K, Ichikawa T, Nakao K, *et al.* Differential effects of calcineurin inhibitors, tacrolimus and cyclosporin a, on interferon-induced antiviral protein in human hepatocyte cells. *Liver Transpl* 2008; **14**: 292.
22. Ishigami M, Honda T, Okumura A, *et al.* Use of the Model for End-Stage Liver Disease (MELD) score to predict 1-year survival of Japanese patients with cirrhosis and to determine who will benefit from living donor liver transplantation. *J Gastroenterol* 2008; **43**: 363.
23. Iwamoto T, Yagi T, Umeda Y, *et al.* The impact of donor age on the outcome of adult living donor liver transplantation. *Transplantation* 2008; **85**: 1240.
24. Casseb J. Possible mechanism for positive interaction of human T cell leukemia type I on liver disease in a hepatitis C virus-infected Japanese cohort. *J Infect Dis* 2000; **182**: 379.
25. Selzner N, Guindi M, Renner EL, Berenguer M. Immune-mediated complications of the graft in interferon-treated Hepatitis C positive Liver transplant recipients. *J Hepatol* 2010; **55**: 207.
26. Stanca CM, Fiel MI, Kontorinis N, Agarwal K, Emre S, Schiano TD. Chronic ductopenic rejection in patients with recurrent hepatitis C virus treated with pegylated interferon alfa-2a and ribavirin. *Transplantation* 2007; **84**: 180.
27. Fernandez I, Ulloa E, Colina F, *et al.* Incidence, risk factors, and outcome of chronic rejection during antiviral therapy for posttransplant recurrent hepatitis C. *Liver Transpl* 2009; **15**: 948.
28. Tanaka Y, Nishida N, Sugiyama M, *et al.* Genome-wide association of IL28B with response to pegylated interferon-alpha and ribavirin therapy for chronic hepatitis C. *Nat Genet* 2009; **41**: 1105.
29. Thomas DL, Thio CL, Martin MP, *et al.* Genetic variation in IL28B and spontaneous clearance of hepatitis C virus. *Nature* 2009; **461**: 798.
30. Charlton MR, Thompson A, Veldt BJ, *et al.* Interleukin-28B polymorphisms are associated with histological recurrence and treatment response following liver transplantation in patients with hepatitis C virus infection. *Hepatology* 2011; **53**: 317.
31. Lange CM, Moradpour D, Doehring A, *et al.* Impact of donor and recipient IL28B rs12979860 genotypes on hepatitis C virus liver graft reinfection. *J Hepatol* 2011; **55**: 322.
32. Fukuhara T, Taketomi A, Motomura T, *et al.* Variants in IL28B in liver recipients and donors correlate with response to peg-interferon and ribavirin therapy for recurrent hepatitis C. *Gastroenterology* 2010; **139**: 1577.

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Is liver-targeted FOXp3 staining beneficial after living-donor liver transplantation?

S. Eguchi, M. Hidaka, A. Soyama, M. Takatsuki, H. Miyaaki, T. Ichikawa, K. Nakao, T. Kanematsu. Is liver-targeted FOXp3 staining beneficial after living-donor liver transplantation? Transpl Infect Dis 2012: 14: 156–162. All rights reserved.

Abstract: As treatments for acute cellular rejection (ACR) and recurrent hepatitis caused by hepatitis C virus (HCV) are dramatically different, making a precise diagnosis is considered to be essential in patients after liver transplantation. Therefore, we investigated whether immunohistochemical detection of FOXp3, a marker for regulatory T cells (CD4+ CD25+), could be used to differentiate between recurrent hepatitis C and ACR. From a group of 103 cases of living-donor liver transplantation (LDLT), 48 samples were taken via liver biopsy from 20 patients with HCV infection. An initial diagnosis was made based on hematoxylin and eosin staining, which was scored with the hepatitis activity index (HAI) grading, whereas ARC was scored with the rejection activity index (RAI). The FOXp3 immunohistochemical staining on serial specimens was retrospectively analyzed, scoring from 0 to III. The time after LDLT was a median of 270 (range: 14–2000) days, whereas the median number of biopsies per patient was 3 (range: 1–8). The HAI was significantly different between 0 vs. I, and II vs. III, in terms of the FOXp3 score. On the other hand, a significant difference in the RAI was only found between 0 vs. I. In conclusion, FOXp3 may represent a surrogate marker for recurrent HCV infection after LDLT.

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Key words: FOXp3; recurrent hepatitis C; acute cellular rejection; living-donor liver transplantation; CD25

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Recurrent hepatitis C virus (HCV) infection after liver transplantation (LT) remains a therapeutic challenge, especially in a setting with living donors, where the possibility of retransplantation is limited. To date, the best treatment for chronic HCV infection is interferon (IFN) combined with ribavirin, with a 50% sustained virologic response rate (1). In fact, diagnosis of recurrent hepatitis C is sometimes difficult to make, because the presentation, in the histologic view, has many similarities to rejection (2).

Regulatory T cells (Tregs) are supposed to regulate an over-reactive autoimmune response, and were detected to be CD4+ CD25+. Tregs are engaged in the maintenance of self-tolerance by suppressing the activation and expansion of self-reactive lymphocytes (3–5). Loss of this suppressing function may lead to chronic inflammation and/or autoimmunity (6–12). Currently, the best indicator of Tregs function is thought to be the intracellular expression of forkhead box P3 (FOXp3), which is also crucial for Tregs development (13). One of our co-authors (H.M.) previously reported the usefulness of examining liver-targeted Tregs as indicators of chronic hepatitis B virus and HCV infection (14).

In the setting of LT, it was also reported that needle biopsy could provide a source for determining FOXp3 messenger RNA (mRNA) expression after LT (15). In addition, it was reported that FOXp3 mRNA in peripheral blood is a useful marker for acute cellular rejection (ACR), whereas CD4+ CD25+ numbers in peripheral blood may be a marker to predict recurrence of HCV after LT (16, 17).

However, to the best of our knowledge, studies have not previously examined the use of FOXp3 in

Characteristics of liver transplant patients with HCV

Pt no.	Age/gender	Genotype	Days after LDLT	Histologic diagnosis		FOXp3	IFN
				HAI	RAI		
1	58/M	1b	1800	5	4	0	+
			2000	5	3	1	+
2	57/F	1b	540	0	0	0	+
			720	3	0	2	+
3	53/F	1b	30	3	3	0	–
			180	6	6	1	–
			540	3	7	0	+
			1020	3	0	2	+
4	52/M	II	20	1	8	1	–
			35	6	4	2	–
5	63/F	1b	150	3	4	1	+
			270	4	0	2	+
			510	5	0	2	+
			630	0	0	1	– (SVR)
6	57/M	1b	14	1	4	0	–
			180	6	2	2	+
			540	6	0	0	+
7	55/F	1b	180	3	6	1	–
			194	7	5	3	–
			208	6	4	2	–
			360	7	3	1	+
			1080	7	3	1	+
8	64/F	1b	21	2	3	2	–
			74	2	3	2	–
			180	6	3	3	+
9	61/F	1b	30	3	0	3	–
			720	4	2	1	–
			780	4	2	1	–
10	62/M	II	60	6	4	3	+
11	67/M	1b	14	7	7	1	–
			400	7	3	2	+
			720	3	5	1	–
12	58/M	1b	90	2	1	0	–
			360	3	6	1	+
			720	2	4	2	+ (SVR)
13	51/F	1b	450	10	5	3	–
			900	10	6	3	–
			1380	10	5	3	+
			1835	10	4	3	+
14	59/M	1b	360	2	0	0	+ (SVR)
15	54/M	1b	390	0	0	0	– (SVR)
16	68/F	1b	150	3	3	1	–
			300	6	3	1	+
17	59/F	1b	180	4	0	0	–
			360	5	0	0	+
			480	2	1	0	+
18	65/F	1b	30	4	4	1	–
			120	9	6	3	+
			135	3	3	2	+

Table 1 continued

Pt no.	Age/gender	Genotype	Days after LDLT	Histologic diagnosis			
				HAI	RAI	FOXp3	IFN
19	59/M	1b	21	4	3	0	–
			41	1	3	0	–
20	65/M	1b	480	4	0	2	+
			570	3	0	1	+

HCV, hepatitis C virus; Pt no., patient number; LDLT, living-donor liver transplantation; HAI, hepatitis activity index; RAI, rejection activity index; FOXp3, marker for regulatory T cells; IFN, interferon; M, male; F, female; SVR, sustained virologic response.

Table 1

liver infiltrating lymphocytes to differentiate recurrent HCV infection from ACR.

Patients and methods

Patients

Of 103 cases of living-donor LT (LDLT), 29 patients (mean age: 57.8 ± 10.6, male:female ratio: 17:12) were positive for anti-HCV antibodies. Fifty-eight samples were taken via liver biopsy from 20 patients (Table 1). Liver biopsy tissue specimens were taken by a needle puncture for diagnostic purposes. HCV serotype was type I in 18 of those patients, whereas it was type II in 2 patients. In all patients, IFN therapy was eventually attempted. Immunosuppression was based on our protocol using cyclosporine as previously reported (1).

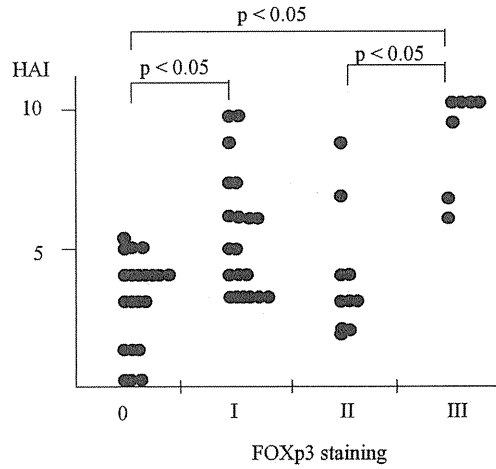

Fig. 1. The relationship between hepatitis activity index (HAI) grading and FOXp3 staining. Significant differences were seen between 0 and I, II and III, and 0 and III with regard to the FOXp3 staining.

Methods

All tissues were fixed in 10% neutral buffered formalin and were then embedded in paraffin, and 4-mm-thick serial sections were cut from each paraffin block. T cells were examined immunohistochemically using an anti-CD4 antibody (Novocastra, Newcastle, UK).

Initial diagnosis was made based on hematoxylin and eosin (H&E) staining, followed by FOXp3 immunohistochemical staining (eBioscience, San Diego, California, USA) on serial specimens. Among aggregated lymphocytes, the number of FOXp3-positive CD4+ lymphocytes was scored as 0 = none, I = 1–9 cells, II = 10–19 cells, and III = >20 cells, as in our previous report (14). The association of FOXp3 with hepatitis activity index (HAI) and/or rejection activity index (RAI) (median 3, range: 0–8) was investigated.

To classify the degree of hepatic inflammation (hepatic activity), we used the HAI score as described by Knodell et al. (18). Based on their criteria, the H&E-stained specimens of the non-cancerous liver tissues were examined and classified into 4 categories. ACR was scored based on the RAI according to the Banff schema (19, 20).

All data are expressed as the median values with ranges. The statistical analysis was performed using the Mann–Whitney *U*-test for continuous values, and the chi-squared test for categorical values. A significant difference was defined as a *P*-value of <0.05. The StatView 5.0 statistical software package (Abacus Concepts, Berkeley, California, USA) was used for all statistical analyses.