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colorectal cancer, the development of which is associated with metabolic abnormalities (17), is accompanied by the overexpression of visfatin (18). Serum visfatin level is a good biomarker of colorectal malignant potential and stage progression (19). Visfatin stimulation increases cell proliferation in prostate and breast cancer cells (20, 21), whereas the use of visfatin inhibitor exerts an antitumor effect by inducing apoptosis (22). These findings suggest that visfatin is one of the key adipocytokines that links obesity and tumorigenesis and thus may be an effective target for the inhibition of obesity-related carcinogenesis. However, no detailed studies of the relationship between visfatin and HCCs have yet been conducted.

Branched-chain amino acids (BCAA; leucine, isoleucine, and valine) are used in patients with liver cirrhosis to improve protein malnutrition (23). Recent clinical trials have shown that oral supplementation with BCAA prevents progressive hepatic failure, improves event-free survival in patients with chronic liver diseases, and reduces the risk of HCCs in these patients who are obese (body mass index $\geq$ 25; refs. 4, 24). BCAA supplementation also prevents obesity-related carcinogenesis in both the liver and the colorectum of diabetic mice (25, 26). In the present study, we measured serum visfatin concentration in patients with HCCs and examined whether it was correlated with stage progression and tumor enlargement. We also examined in detail the effects of visfatin on the acceleration of HCC cell proliferation, focusing on the activation of signaling pathways, and investigated whether BCAA suppresses visfatin-induced growth of HCC cells.

Materials and Methods

Patients and measurement of serum visfatin concentration

Eighty-five primary HCC patients who underwent initial treatment at our hospital from January 2006 to December 2008 were enrolled in this study. Tumor stage was defined according to the staging system of the Liver Cancer Study Group of Japan (27). The greatest diameter of HCC was determined with dynamic computed tomography or magnetic resonance imaging. Fasting serum samples were collected at the time of diagnosis, and serum levels of visfatin were determined by ELISA (AdipoGen). The study protocol was approved by the Institutional Review Board for human research, and all patients gave written informed consents to enter the study.

Materials

Recombinant human visfatin was purchased from PeproTech Inc. BCAA (total amino acid content, 12.28 mmol/L), ΔBCAA (10.28 mmol/L), and neutral amino acid media (12.28 mmol/L) were obtained from Ajinomoto Pharmaceuticals Co. ΔBCAA serves as basal medium and contains 17 amino acids except BCAA. The concentrations of amino acids in the medium are as follows (in mmol/L): glycine, 0.40; alanine, 0.40; serine, 0.40; threonine, 0.80; cystine, 0.20; methionine, 0.20; glutamine, 4.00; asparagine, 0.40; glutamic acid, 0.40; aspartic acid, 0.40; phenylalanine, 0.40; tyrosine, 0.40; tryptophan, 0.08; lysine, 0.80; arginine, 0.40; histidine, 0.20; and proline, 0.40. BCAA medium was prepared by adding 2 mmol/L BCAAs (0.952 mmol/L leucine, 0.476 mmol/L isoleucine, and 0.572 mmol/L valine) to ΔBCAA medium. The composition of BCAA (2:1:1.2 = leucine:isoleucine:valine) was set at the clinical dosage used for the treatment of decompensated liver cirrhosis in Japan (4, 24). The neutral amino acid medium was prepared by adding 2 mmol/L neutral amino acids (0.667 mmol/L each of alanine, serine, and glycine) to the ΔBCAA medium and served as an amino acid content-matched control for BCAA medium. LY294002 was purchased from Cell Signaling Technology; PD98059, from Sigma; and CHIR99021, from Stemgent.

Cell lines and cultures

HepG2, Hep3B, and HuH7 human HCC cell lines were obtained from the Japanese Cancer Research Resources Bank and maintained in RPMI-1640 medium (Sigma) supplemented with 10% fetal calf serum. Hc human normal hepatocyte cell line was purchased from Cell Systems and maintained in a CS-S complete medium (Cell Systems). The cell lines have been characterized by each source, and any further authentication was not done in our laboratory. These cells were cultured in an incubator with humidified air with 5% CO_2 at 37°C.

Cell proliferation assay

Cell proliferation assays were conducted by a cell proliferation kit [2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2*H*-tetrazolium-5-carboxanilide (XTT); Roche)] according to the manufacturer's instructions. To examine the effects of visfatin on the proliferation of the HepG2, Hep3B, HuH7, and Hc cells, these cells were seeded on 96-well plates (1×10^4 cells per well). After 16 hours of serum starvation, the cells were treated with the indicated concentrations (0–400 ng/mL) of exogenous visfatin for 48 hours in the absence of serum. To investigate the effect of LY294002, PD98059, CHIR99021, and BCAA, HepG2 cells were treated with these agents in the absence and presence of visfatin (100 or 400 ng/mL) for 48 hours in serum-free medium. All assays were conducted in triplicate.

Protein extraction and Western blot analysis

Total cellular protein was extracted and equivalent amounts of protein were examined by Western blot analysis (28). The primary antibodies used to detect the respective protein bands have been described previously (28). An antibody to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a loading control. The intensities of the blots were quantified with NIH Image software, version 1.62.

Cell-cycle assays

Cell-cycle assays were conducted by a cell-cycle detection kit (Cayman) according to the manufacturer's instructions. HepG2 cells were treated with BCAA for 48 hours in the

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absence and presence of 100 ng/mL visfatin. After the harvested cells were fixed and stained, they were analyzed for DNA histograms and cell-cycle phase distribution with a FACScan flow cytometer (BD). The data were analyzed with the CellQuest computer program (BD) as described previously (28).

Apoptosis assays

The Annexin V-binding capacity of treated cells was examined with flow cytometry by the Annexin V-FITC Apoptosis Detection Kit I (BD) to evaluate the induction of apoptosis. HepG2 cells were treated with BCAA for 48 hours in the absence and presence of 100 ng/mL visfatin. After the cultured cells were washed with cold PBS, they were incubated in Annexin V-fluorescein isothiocyanate (FITC) and propidium iodide (PI) for 15 minutes on ice. Stained cells were analyzed within 1 hour. Annexin V-FITC–positive and PI-negative cells were counted as apoptotic cells as described previously (29).

Statistical analysis

The data are expressed as mean ± SD. The statistical significance of the difference in mean values was assessed with one-way ANOVA, followed by the Scheffe *t* test. Values of $P < 0.05$ were considered significant.

Results

Association of serum visfatin concentration with HCC clinical stage and tumor size

We initially analyzed the possible association of serum visfatin concentration with the clinical stage and tumor size (greatest diameter) of HCCs in 85 patients (54 men and 31 women, median age 73 years). The median serum visfatin concentration was 5.8 ng/mL (range: 1.2–42.0). We found that the progression of clinical stage was correlated with serum visfatin concentration; the level of this adipocytokine was significantly increased in stage IV patients compared with levels in those with stage I and II disease ($P < 0.05$; Fig. 1A). In 85 patients, the mean Pearson product-moment correlation coefficient (r) and the P value (P) of tumor size with serum visfatin concentration were 0.315 and 0.003, respectively (Fig. 1B). Moreover, similar results ($r = 0.326$ and $P = 0.01$) were obtained when patients with diabetes mellitus (HbA1c ≥ 6%) and/or obesity were excluded ($n = 53$, Fig. 1C), indicating a positive correlation between HCC tumor size and serum visfatin levels regardless of complications with obesity and diabetes.

Effects of visfatin on cell proliferation and phosphorylation of extracellular signal–regulated kinase, Akt, and GSK-3β proteins in human HCC cells

We next examined whether visfatin stimulates the proliferation of HCC cells by XTT assay. When series of HCC cells (i.e., HepG2, Hep3B, and HuH7 cells) were treated with visfatin (25–400 ng/mL) for 48 hours, cell proliferation was significantly stimulated in a dose-dependent

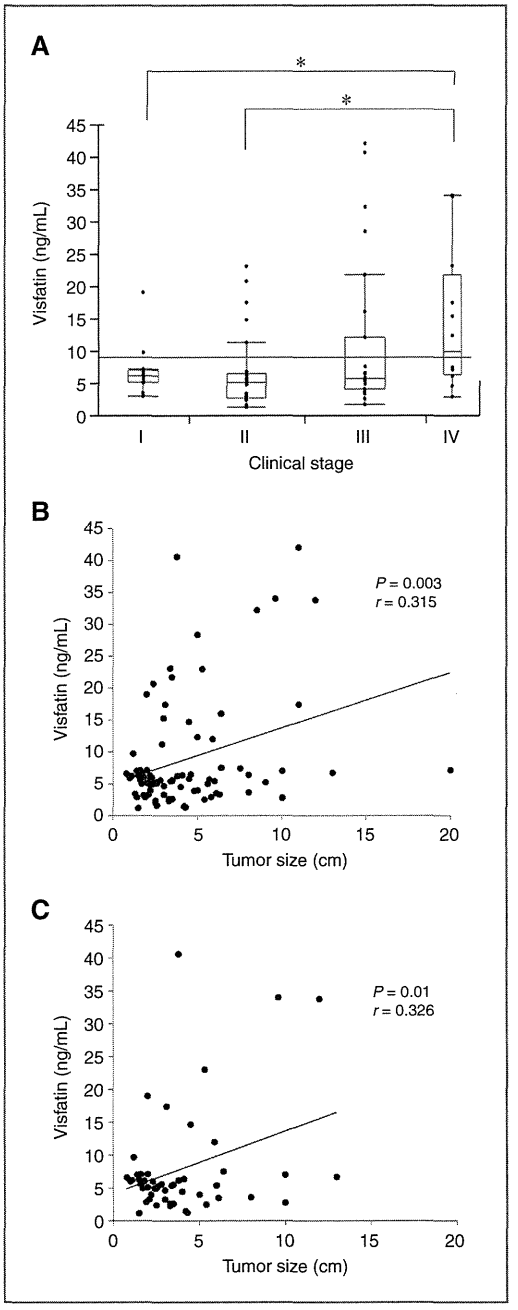

Figure 1. Correlation between serum visfatin concentrations and the clinical stage (A) and tumor size (B, C) of HCCs. A and B, the correlations were determined by analyzing 85 patients with primary HCCs. C, the correlation was determined by analyzing 53 HCC patients who are not obese and did not have diabetes mellitus. *, $P < 0.05$.

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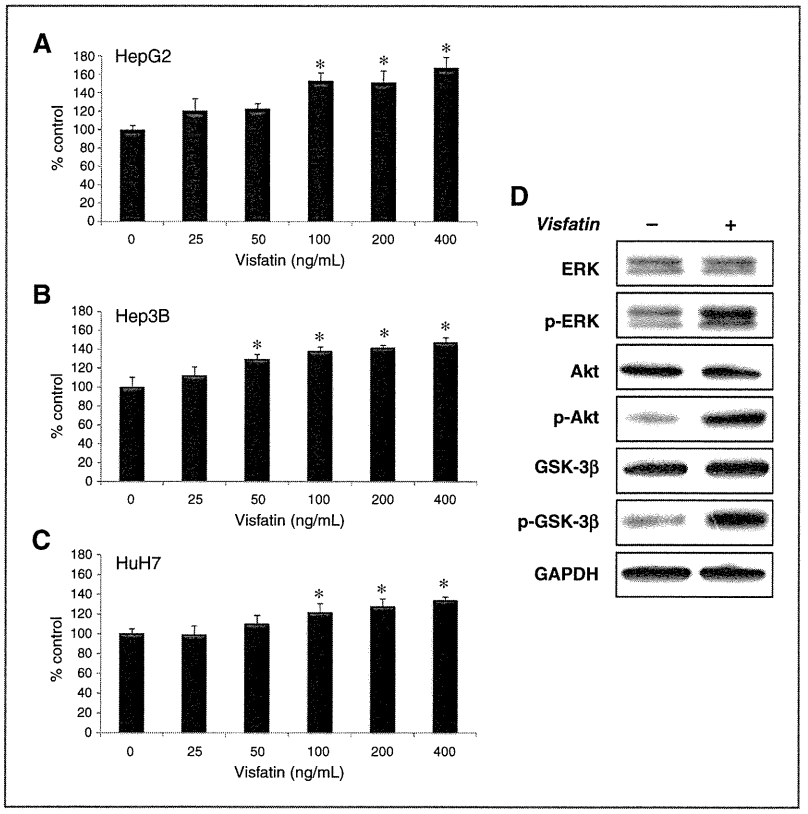

Figure 2. Effects of visfatin on the cell proliferation and phosphorylation of ERK, Akt, and GSK-3β proteins in HCC cells. HepG2 (A), Hep3B (B), and HuH7 (C) cells were treated with the indicated concentration of visfatin for 48 hours in serum-free medium. Cell proliferation was evaluated by an XTT assay. Results were expressed as a percentage of the control value. Bars, SD of triplicate assays. *, $P < 0.05$. D, HepG2 cells were treated with and without 100 ng/mL visfatin for 30 minutes, and cell lysates were prepared. The cell lysates were then analyzed with a Western blot using respective antibodies. Equal protein loading was verified by the detection of GAPDH. Repeated Western blotting yielded similar results. p-ERK, phosphorylated ERK; p-Akt, phosphorylated Akt; p-GSK-3β, phosphorylated GSK-3β.

manner ($P < 0.05$; Fig. 2A–C). In addition, treatment of HepG2 cells with 100 ng/mL of visfatin for 30 minutes caused a marked phosphorylation of extracellular signal–regulated kinase (ERK), Akt, and GSK-3β proteins (Fig. 2D), suggesting that visfatin might induce cell proliferation in HCC cells by activating PI3K/Akt and MAPK/ERK signaling pathways.

Effects of phosphoinositide-3-kinase, MAP/ERK 1 kinase, and GSK-3β inhibitors on visfatin-induced proliferation of HepG2 cells

We next examined whether pharmacologic inhibitors of phosphoinositide-3-kinase (PI3K; LY294002), MAP/ERK 1 kinase (MEK1; PD98059), and GSK-3β (CHIR99021) suppress visfatin-induced proliferation in HepG2 cells because the activation of PI3K/Akt and MAPK/ERK pathways might be involved in this proliferation (Fig. 2). As shown in Fig. 3, treatment with LY294002 (Fig. 3A), PD98059 (Fig. 3B), and CHIR99021 (Fig. 3C) significantly inhibited HepG2 cell proliferation both in the absence and presence of visfatin stimulation (100 and 400 ng/mL; $P < 0.05$). These findings suggest that PI3K and MAPK pathways could be effective targets for the inhibition of visfatin-induced proliferation in HepG2 cells.

Effects of BCAA on visfatin-induced proliferation of HepG2 cells

BCAA is reported to suppress obesity-related liver carcinogenesis (4, 25). Therefore, we next examined whether BCAA inhibits visfatin-stimulated proliferation of HepG2 cells because this adipocytokine, which is increased in obese individuals (11, 12), might play a role in the progression of HCCs (Fig. 1). As shown in Fig. 4A, the proliferation of HepG2 cells was significantly inhibited when the cells were treated in BCAA medium; meanwhile, this inhibition did not occur in neutral amino acid medium, which was served as an amino acid content-matched control for BCAA medium ($P < 0.05$). This finding possibly indicates that BCAA itself is specific in inhibiting the growth of HCC cells. In addition, a marked potentiation in the proliferative activity of HepG2 cells occurred after stimulation with 100 and 400 ng/mL visfatin, whereas BCAA treatment inhibited such proliferation in a dose-dependent manner regardless of visfatin stimulation ($P < 0.05$). The inhibition of proliferation with 2 mmol/L BCAA was greater (65% reduction) when the cells were cultured at higher concentration of visfatin (400 ng/mL) than that in the absence of the adipocytokine (41% reduction; Fig. 4B). In contrast, cell proliferation was not induced when Hc normal hepatocytes

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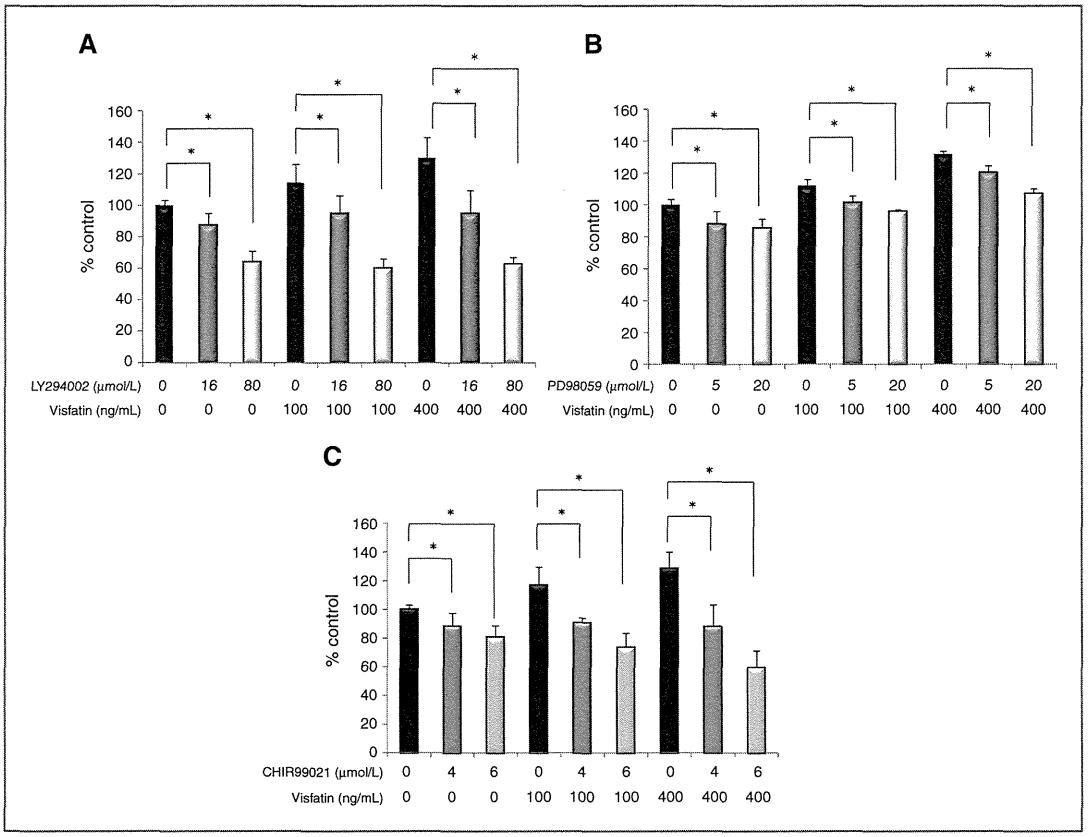

Figure 3. Effects of inhibitors of PI3K, MEK1, and GSK-3β on visfatin-induced proliferation in HepG2 cells. HepG2 cells were treated with LY294002, a PI3K inhibitor (A), PD98059, an MEK1 inhibitor (B), or CHIR99021, a GSK-3β inhibitor (C), in the absence or presence of visfatin (100 or 400 ng/mL) for 48 hours. Cell proliferation was evaluated by an XTT assay. Results were expressed as a percentage of the control value. Bars, SD of triplicate assays. *, $P < 0.05$.

were treated with similar concentrations of visfatin. BCAA also exerted no significant effect on the proliferation of Hc cells regardless of visfatin stimulation (Fig. 4C).

Effects of BCAA on visfatin-induced phosphorylation of ERK, Akt, and GSK-3β proteins in HepG2 cells

We next examined whether BCAA affected the phosphorylation of ERK, Akt, and GSK-3β proteins caused by visfatin in HepG2 cells. When the cells were stimulated by visfatin, the expression levels of phosphorylated (p)-GSK-3β protein were significantly decreased by BCAA treatment ($P < 0.05$; Fig. 5).

Effect of BCAA on cell-cycle progression, $p21^{CIP1}$ expression, and apoptosis induction in HepG2 cells in the presence and absence of visfatin

To determine whether the suppression of cell proliferation caused by BCAA (Fig. 4A and B) was associated with specific changes in cell-cycle distribution, we conducted cell-cycle analysis with DNA flow cytometry. When HepG2 cells were stimulated by visfatin for 48 hours, the percentage of cells in G_2/M phase (38%) was increased compared with that of cells not stimulated by visfatin (18%). Furthermore, regardless of visfatin stimulation, BCAA treatment increased the percentage of cells in G_0/G_1 phase; the percentage of cells in this phase was increased from 59% to 71% in the unstimulated cells and from 48% to 70% in the stimulated cells (Fig. 6A). Expression levels of $p21^{CIP1}$ protein, which suppresses tumors by promoting cell-cycle arrest (30), were also increased by BCAA treatment regardless of visfatin stimulation ($P < 0.05$; Fig. 6B). In addition, BCAA induced apoptosis in HepG2 cells because the percentage of Annexin V–positive cells was increased by the addition of BCAA in both the absence (2%–27%) and the presence (2%–10%) of visfatin stimulation (Fig. 6C).

Discussion

Obesity and related metabolic abnormalities are significant risk factors for the development of HCCs (1–5).

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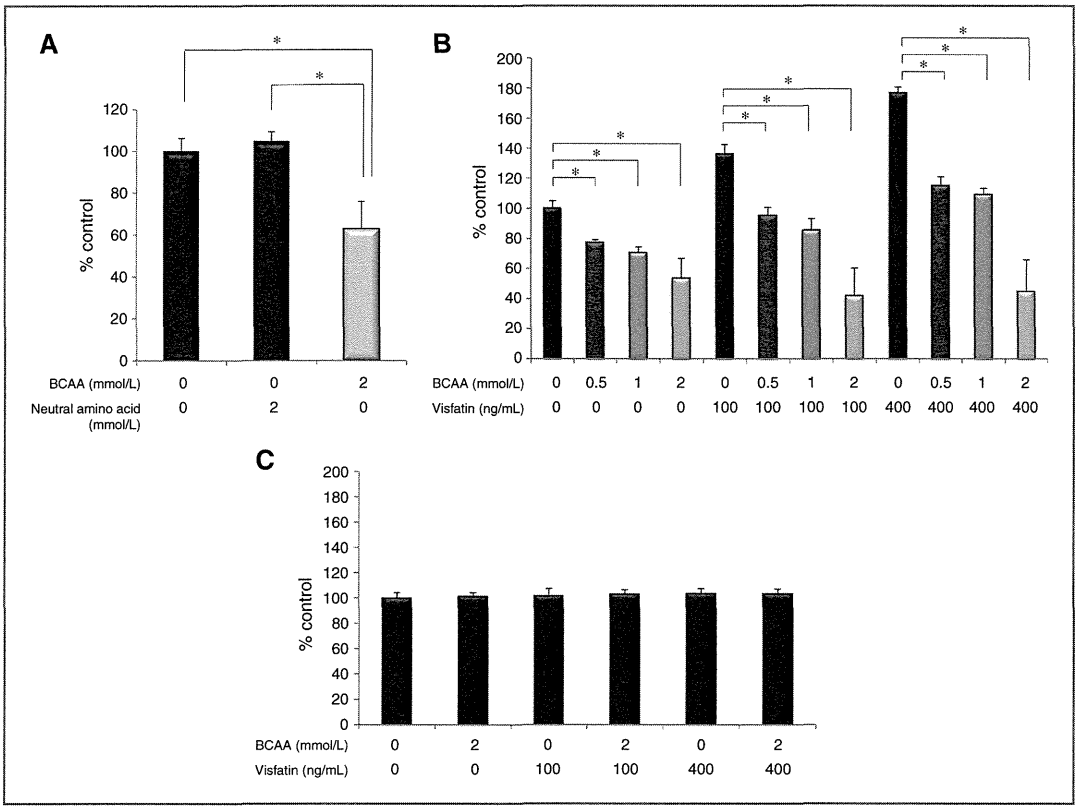

Figure 4. Effects of BCAA on visfatin-induced cell proliferation in HepG2 cells. A, HepG2 cells were treated in 2 mmol/L BCAA or 2 mmol/L neutral amino acid medium for 48 hours. Cell proliferation was evaluated by an XTT assay. HepG2 (B) and Hc (C) cells were treated with or without BCAA (0, 0.5, 1, and 2 mmol/L) in the absence or presence of visfatin (100 or 400 ng/mL) for 48 hours. Cell proliferation was evaluated by an XTT assay. Results were expressed as a percentage of the control value. Bars indicate SD values of triplicate assays. *, $P < 0.05$.

Among obesity-related metabolic disorders, adipocytokine dysbalance is considered to play a role in liver carcinogenesis (7–9); however, the detailed relationship remains unclear. The results of the present study provide the first evidence that higher levels of serum visfatin, which are frequently found in obese individuals (11, 12), are

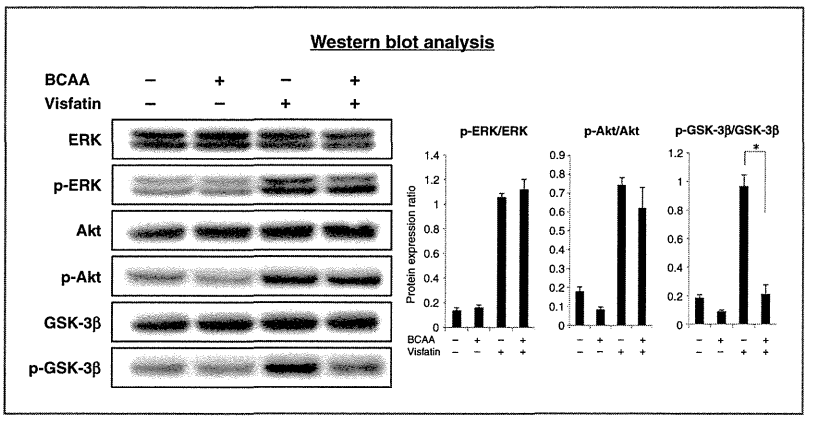

Figure 5. Effects of BCAA on visfatin-induced phosphorylation of ERK, Akt, and GSK-3β proteins in HepG2 cells. HepG2 cells were treated with or without BCAA in the absence or presence of 100 ng/mL visfatin for 30 minutes, and cell lysates were prepared. The cell lysates were then analyzed by Western blotting using corresponding antibodies (left). The intensities of the blots were quantified with densitometry. Columns and lines indicate mean ± SD (right). Repeated Western blotting produced similar results. *, $P < 0.05$. p-ERK, phosphorylated ERK; p-Akt, phosphorylated Akt; p-GSK-3β, phosphorylated GSK-3β.

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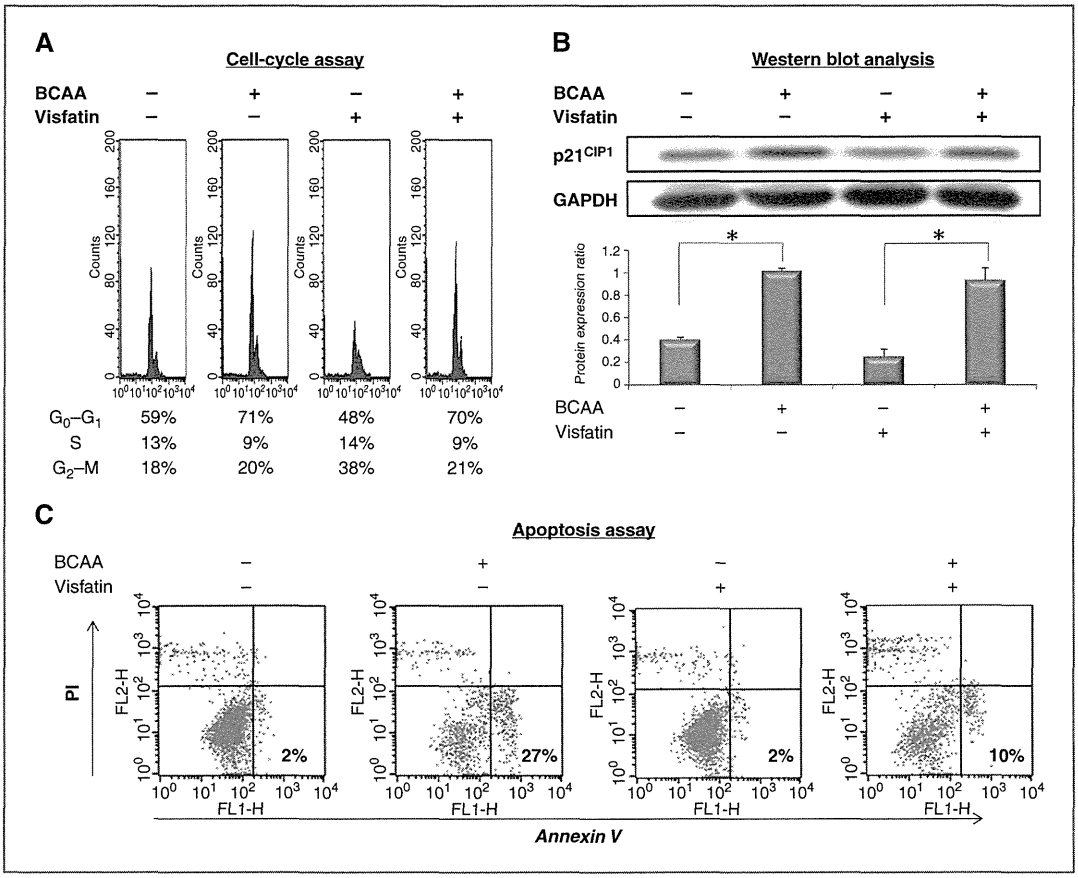

Figure 6. Effect of BCAA on the progression of cell cycle, expression of $p21^{CIP1}$, and induction of apoptosis in HepG2 cells in the presence and absence of visfatin. After treatment with and without BCAA in the presence and absence of 100 ng/mL visfatin for 48 hours, the cells were corrected and then used for cell-cycle assay (A), Western blot analysis (B), and apoptosis assay (C). A, the cells were stained with PI to analyze cell-cycle progression. B, total proteins were extracted from the cells, and the cell extracts were analyzed with a Western blot using anti-$p21^{CIP1}$ and GAPDH antibodies (top). The intensities of the blots were quantitated with densitometry. Columns and lines indicate mean and SD (bottom). *, $P < 0.05$. C, the cells were incubated with Annexin V–FITC to evaluate induction of apoptosis. Annexin V–FITC–positive and PI-negative cells were counted as apoptotic cells.

positively involved in stage progression and tumor enlargement in HCCs. On the other hand, the serum levels of other adipocytokines, including leptin, adiponectin, and resistin, are not associated with the stage progression of this malignancy (data not shown). Furthermore, visfatin stimulation strongly induced proliferation in a series of human HCC cells but not in Hc normal human hepatocytes. These findings suggest that visfatin, which might act as a growth factor in HCC cells, is one of the key adipocytokines that links obesity and the progression of HCCs. In addition, this study revealed that serum visfatin levels are significantly correlated with tumor enlargement of HCCs in patients who are not obese and do not have diabetes mellitus. A recent report has shown that visfatin is constitutively released from human HCC cells (31). This finding raises the possibility that visfatin is produced by HCC tissue itself, which might also explain the positive correlation between tumor size and serum visfatin levels observed in the present study. Therefore, our findings and the results of a previous report (31) together suggest that visfatin-dependent autocrine or paracrine loops contribute to abnormal proliferation in HCC cells.

The present study showed that visfatin induced cell proliferation in HepG2 cells by activating PI3K and MAPK signaling pathways because visfatin stimulation significantly increased phosphorylation of Akt, ERK, and GSK-3β proteins in these cells. These findings are consistent with previous reports that visfatin regulates a variety of signaling pathways, including PI3K/Akt, MAPK/ERK, and Stat3 (20, 32, 33). Visfatin stimulation also increases cell proliferation and ERK activity in prostate cancer cells (20). Moreover, recent experimental studies have shown that the activation of PI3K/Akt, MAPK/ERK, and Stat3 pathways is

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significantly associated with the development of liver tumors in obese mice, and that inhibiting the activation of these signaling pathways is critical to the prevention of obesity-related liver tumorigenesis (34, 35). These reports (34, 35), together with the present findings that specific inhibitors of PI3K, MEK1, and GSK-3β significantly suppress visfatin-induced proliferation in HCC cells, suggest that visfatin and its related signaling pathways might be effective targets for inhibiting obesity-related liver carcinogenesis.

BCAA, which was originally developed to improve protein malnutrition in patients with liver cirrhosis (23), produces improvements in metabolic abnormalities, especially insulin resistance and glucose tolerance (36, 37). BCAA supplementation also reduces the weights of white adipose tissue and improves liver steatosis in mice fed with a high-fat diet (38). In addition, long-term oral supplementation with BCAA is associated with a reduced frequency of HCCs in obese individuals (4). In rodent models, BCAA prevents obesity-related liver and colorectal carcinogenesis, and their beneficial effects are involved in the amelioration of insulin resistance and reduction of serum leptin levels (25, 26, 39). In the present study, BCAA significantly inhibited the proliferation of HCC cells stimulated by visfatin without affecting that of normal hepatocytes. This mechanism is a new one of BCAA that might explain the suppressive effects of this agent on obesity-related tumorigenesis. Therefore, the evidences in the present and previous studies (4, 25, 39) strongly support the active administration of BCAA as an HCC chemopreventive agent in patients with liver cirrhosis, especially obese patients who are at an increased risk for this malignancy. We are currently trying to gather evidence that BCAA prevents obesity-related liver carcinogenesis by targeting visfatin, in an ongoing animal study.

GSK-3β phosphorylation plays a critical role in cell survival, prevention of apoptosis, and progression of cell cycle in tumors (40). Therefore, the results of the present study suggest that BCAA might have inhibited visfatin-induced proliferation in HCC cells by, at least in part, inhibiting the phosphorylation of GSK-3β protein, which induces apoptosis and cell-cycle arrest in the G_0/G_1 phase in HepG2 cells. These findings are significant when considering the possibility of BCAA as a chemopreventive agent for HCCs because GSK-3β phosphorylation is closely associated with liver carcinogenesis (41). Phosphorylation of GSK-3β is also involved in the development of liver tumors in obese mice, and inhibition of this kinase effectively suppresses obesity-related liver tumorigenesis (35). Conversely, a recent study has shown that visfatin exerts antiapoptotic effects in HCC cells, and this might be associated with the enzymatic synthesis of NAD^+ (15). FK866, a visfatin inhibitor, effectively inhibited cell growth and induced apoptosis in human HCC cells by reducing cellular levels of NAD^+ (22). Further studies are required to clarify the effects of BCAA on the synthesis and regulation of NAD^+ and their relevance to the chemopreventive characteristics of this agent.

In summary, our data explained, for the first time, the molecular mechanisms responsible for HCC cell proliferation induced by visfatin, establishing a direct association between obesity and HCC progression. Because the evaluation of obesity-related metabolic disorders such as insulin resistance and hyperleptinemia are useful for predicting the risk of recurrence in HCCs (8, 42), we presume that, along with these metabolic abnormalities, measurement of serum visfatin levels might also have the potential to become a valuable biomarker for HCC development and progression. The results of the present study also indicate that targeting visfatin and related signaling pathways might be a promising strategy for the prevention or treatment of HCCs in obese patients with chronic liver disease. BCAA is potentially effective and critical candidate for this purpose because it can inhibit visfatin-mediated cell proliferation and activation of intracellular signaling pathways.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Article

C57BL/KsJ-*db/db*-*Apc*$^{Min/+}$ Mice Exhibit an Increased Incidence of Intestinal Neoplasms

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Abstract: The numbers of obese people and diabetic patients are ever increasing. Obesity and diabetes are high-risk conditions for chronic diseases, including certain types of cancer, such as colorectal cancer (CRC). The aim of this study was to develop a novel animal model in order to clarify the pathobiology of CRC development in obese and diabetic patients. We developed an animal model of obesity and colorectal cancer by breeding the C57BL/KsJ-*db/db* (db/db) mouse, an animal model of obesity and type II diabetes, and the C57BL/6J-*Apc*$^{Min/+}$ (Min/+) mouse, a model of familial adenomatous polyposis. At 15 weeks of age, the N9 backcross generation of C57BL/KsJ-*db/db*-*Apc*$^{Min/+}$ (db/db-Min/+) mice developed an increased incidence and multiplicity of adenomas in the intestinal tract when compared to the db/m-Min/+ and m/m-Min/+ mice. Blood biochemical profile showed significant increases in insulin (8.3-fold to 11.7-fold), cholesterol (1.2-fold to 1.7-fold), and triglyceride (1.2-fold to 1.3-fold) in the db/db-Min/+ mice, when compared to those of the

db/m-Min/+ and m/m-Min/+ mice. Increases (1.4-fold to 2.6-fold) in RNA levels of insulin-like growth factor (IGF)-1, IRF-1R, and IGF-2 were also observed in the db/db-Min/+ mice. These results suggested that the IGFs, as well as hyperlipidemia and hyperinsulinemia, promoted adenoma formation in the db/db-Min/+ mice. Our results thus suggested that the db/db-Min/+ mice should be invaluable for studies on the pathogenesis of CRC in obese and diabetes patients and the therapy and prevention of CRC in these patients.

Keywords: C57BL/KsJ-db/db; C57BL/6J-Apc$^{Min/+}$; Type 2 diabetes mellitus; colon carcinogenesis; animal model

1. Introduction

Epidemiological studies have shown that obesity and diabetes mellitus may be one of the risk factors for colorectal cancer (CRC) development [1,2]. To date the underlying mechanisms of how obesity and diabetes promote colon carcinogenesis remain unknown, although insulin-resistance and hyperinsulinemia are proposed to be responsible for the risk factor [3]. Excess body weight is a major determinant for the development of insulin resistance with associated hyperinsulinaemia and hyperglycemia, and further leads to CRC development [4]. Certainly, insulin resistance and hyperinsulinaemia are key biological mechanisms underlying the relationship between adiposity and tumor development [5]. Recently, the anti-diabetic drug, metformin, in addition to reduction of insulin resistance has shown anti-tumor properties [6], and is considered as a drug to prevent and treat obesity-related cancers, including CRC [7]. The pathophysiological and biological mechanisms underpinning the associations between excess body weight/obesity, type 2 diabetes, and CRC are proposed, and insulin resistance is at the heart of the matter [8]. However, there are several other candidate systems, including insulin-like growth factors (IGF), adipocytokines, and inflammatory cytokines [9]. One such hypotheses is the role of insulin-IGF axis, where chronic hyperinsulinemia is associated with decreased concentrations of IGF-binding protein1 (IGFBP-1) and IGFBP-2, leading to increased availability of IGF-I and concomitant changes in the cellular environment that favor tumor development. Indeed, inhibition of the activation of the IGF/IGF-1R axis resulted in suppression of colonic premalignant lesions in an obesity-associated colon cancer model, which was also associated with hyperlipidemia, hyperinsulinemia, and hyperleptinemia [10]. However, hyperinsulinemia is also associated with alterations in related molecular systems (sex steroid hormones and adipocytokines). In this context, novel researches using appropriate animal models are needed to investigate the detailed mechanisms. We previously reported that C57BL/KsJ-*db/db* mice with hyperleptinemia and hyperinsulinemia are highly susceptible to azoxymethane (AOM)-induced colon carcinogenesis. C57BL/KsJ-*db/db* mice received AOM developed high frequency of premalignant lesions [11,12], and the mice are useful for studies for identifying chemopreventive agents against obesity/diabetes-assosiated colon carcinogenesis [13,14]. Min/+ mice [15], known to develop a number of adenomas by the same mechanism or process as seen in humans, have frequently been used as an animal model of familial adenomatous polyposis (FAP) for investigation of carcinogenesis, prevention, and therapy of CRC [16–21].

In the current study, we aimed to develop a spontaneous animal model of obesity with adenoma formation in the intestinal tract in order to investigate obesity-associated events in obesity-associated intestinal tumorigenesis and prevent spontaneous intestinal tumor development in the model. The model was developed by breeding db/m mice with Min/+ mice and then backcrossing of db/m mice with the offsprings born from both strains of mice. The db/db-Min/+ mice obtained will give us the important implications for further exploration of the possible underlying events that affect the positive association between CRC and chronic diseases, obesity and diabetes. Our main goal was to assess the involvement of obesity-associated events, such as hyperinsulinemia, in intestinal carcinogenesis *in vivo*.

2. Materilas and Methods

2.1. Animals

C57BL/KsJ-*db*+/+m (db/m) and C57BL/6J-*Apc*$^{Min/+}$ (Min/+) mice were purchased from the Japan SLC, Inc. (Shizuoka, Japan) and from the Jackson Laboratory (Bar Harbor, ME), respectively. They were bred and genotyped in our facility for the project. In brief, after the db/m females were mated with a db/m-Min/+ male, we finally obtained three mouse strains, the db/db-Min/+, the db/m-Min/+, and the m/m-Min/+ mice at the N9 backcross generation by backcrossing db/m mice with the offsprings born from the mating between the db/m mice and the Min/+ mice (Figure S1), The *Apc* gene was detected by polymerase chain reaction (PCR), while the db/m gene was recognized by the body shape and coat color of mice.

Mice used for the study were maintained in the well-controlled room with a high-efficiency particulate air (HEPA) filter, a 12 h lighting (7:00–19:00), 25 ± 2 °C room temperature, and 55 ± 15% humidity. Mice (3–6 mice/cage) were housed in polycarbonate cages measuring W225 × D338 × H140 mm (Japan CLEA, Inc., Tokyo, Japan) with the floor covered with a sheet of roll paper (Japan SLC, Hamamatsu City, Japan). MF (Oriental Yeast Co., Ltd., Tokyo, Japan) was used as a basal diet throughout the study. Groundwater that was chlorine-treated and subjected to ultraviolet disinfection was used as drinking water in a bottle. We fully complied with the "Guidelines Concerning Experimental Animals" issued by the Japanese Association for Laboratory Animal Science and exercised due consideration so as not to cause any ethical problem.

2.2. Experimental Procedure

After weaning at 4 weeks of age, a total of 94 mice aged 5 weeks were used in the study. They included 18 males and 13 females of db/db-Min/+ mice, 23 males and 19 females of db/m-Min/+ mice and 11 males and 10 females of m/m-Min/+ mice, at 15 weeks of age, the mice were weighed and measured their lengths for BMI calculation, and thereafter underwent blood withdrawal from abdominal aorta under anesthetic and intestinal evisceration for counting intestinal adenomas.

2.3. Pathological and Immunohistochemical Analyses

In addition to the macroscopic examination, the volume of colonic tumors was determined. The mean volumes (mm^3) of colonic tumors were calculated by the formula, (a) × (b) × (b)/2 where (a) and (b) were major axis and minor axes, respectively. After careful macroscopic observation, the small and

large intestines were fixed in 10% buffered formalin and paraffin-embedded tissue blocks of whole intestine were made. These tissues were subjected to hematoxylin and eosin (H & E) staining for histopathology. In addition, immunohistochemistry of β-catenin and IGF-1R were performed using the labeled streptavidin-biotin method (LSAB kit; DAKO, Glostrup, Denmark), as previously described. Anti-β-catenin antibody (1:1,000 final dilution) obtained from Transduction Laboratories (catalog no. 610154; San Jose, CA, USA) and anti-IGF-1R antibody (1:100 final dilution) purchased from Santa Cruz Biotechnology, Inc. (sc-7907; Santa Cruz, CA, USA) were applied as primary antibodies. Negative control sections were immunostained without the primary antibodies.

2.4. Blood Chemistry

At 15 weeks of age, blood samples (0.5–1.0 mL/mouse) were collected for determination of total cholesterol, triglyceride and glucose by a simple measurement device (DRICHEM Fujifilm Medical Co., Ltd., Tokyo, Japan). The concentration of blood insulin was measured by the LBIS insulin measuring kit for mice (Shibayagi Co., Ltd., Gunma, Japan).

2.5. RNA Extraction and Quantitative Real-Time RT-PCR Analysis

A quantitative real-time RT-PCR analysis was carried. Total RNA was isolated from the scraped colonic mucosa of the male mice (n = 6 from each strain) using the RNAqueous-4PCR kit (Ambion Applied Biosystems, Austin, TX, USA). The cDNA was synthesized from 0.2 μg total RNA using the SuperScript III First-Strand Synthesis System (Invitrogen, Carlsbad, CA, USA). The primers used for the amplification of IGF-1, IGF-2, and IGF-1R specific genes were as follows: IGF-1 forward, 5-CTGGACCAGAGACCCTTTGC-3 and reverse, 5-GGACGGGGACTTCTGAGTCTT-3; IGF-2 forward, 5-GTGCTGCATCGCTGCTTAC-3 and reverse, 5-ACGTCCCTCTCGGACTTGG-3; and IGF-1R forward, 5-GTGGGGGCTCGTGTTTCTC-3 and reverse, 5-GATCACCGTGCAGTTTTCCA-3. Real-time PCR was done in a LightCycler (Roche Diagnostics Co., Indianapolis, IN, USA) with SYBR Premix Ex Taq (TaKaRa Bio, Shiga, Japan). The expression levels of the IGF-1, IGF-2, and IGF-1R genes were normalized to the β-actin gene expression level.

2.6. Statistical Analysis

Measurements are expressed as the mean value ± standard deviation (Mean ± SD), and differences if present were compared by one-way analysis of ANOVA and Tukey-Kramer's multiple comparison's test. The incidences of intestinal tumors were compared by Fisher's exact probability test. The results were considered statistically significant if the p values were <0.05.

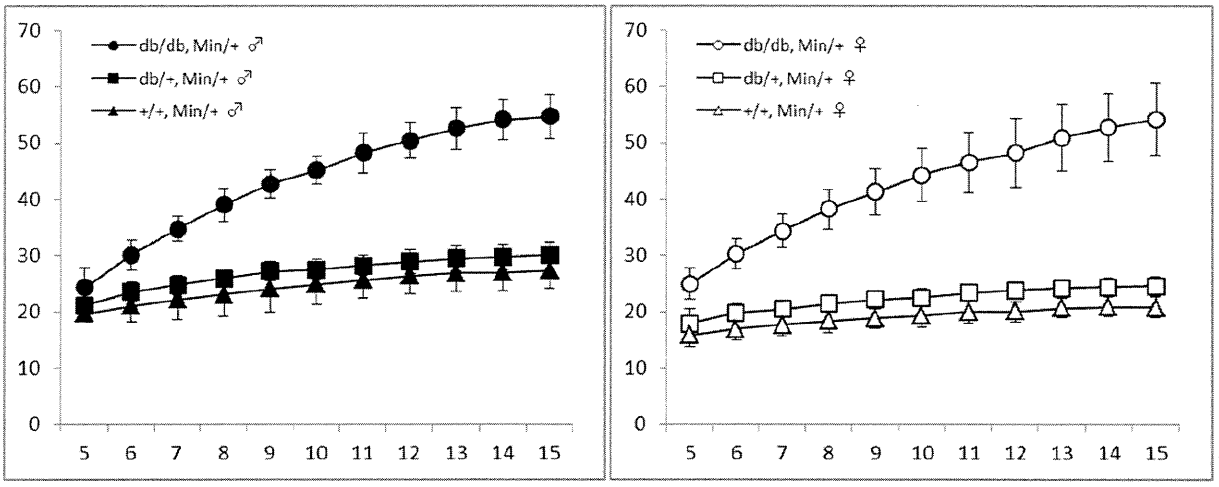
3. Results

3.1. General Observations

As shown in Figure 1, the mean body weights of the db/db-Min/+ mice, either males (Figure 1(A)) or females (Figure 1(B)), were significantly heavier even at the age of 5 weeks than that of the db/m-Min/+ and m/m-Min/+ mice. The differences continued up to 15 weeks of age. The mean body

weights of males and females of db/m-Min/+ mice were significantly higher than that of the m/m-Min/+ mice in either sex at 5 weeks of age, and this trend continued until 15 weeks of age. Table 1 summarizes the body length, body weight, and body mass index (BMI) at 15 weeks of age. The BMIs of the db/db-Min/+ mice (0.62 ± 0.04 in males and 0.63 ± 0.08 in females, $p < 0.001$ for each comparison) were significantly greater than those of the db/m-Min/+ (0.35 ± 0.03 in males and 0.30 ± 0.03 in females) and m/m-Min/+ mice (0.34 ± 0.05 in males and 0.27 ± 0.03 in females).

Figure 1. Body weight gains during the study: (**A**) males and (**B**) females.



(**A**) (**B**)

Table 1. Body weight, body length and BMI of mice at 15 weeks of age.

Gender	Genotype		No. of mice examined	Body weight (g)	Body length (cm)	BMI (g/cm^2)
	db/db type	Apc$^{Min/+}$ type				
Males	db/db	Min/+	18	54.7 ± 3.9 [a,b]	9.38 ± 0.30 [c]	0.62 ± 0.04 [b]
	db/m	Min/+	23	30.2 ± 2.3 [d]	9.23 ± 0.38	0.35 ± 0.03 [e]
	m/m	Min/+	11	27.3 ± 3.2 [f]	9.00 ± 0.30	0.34 ± 0.05 [f]
Females	db/db	Min/+	13	54.2 ± 6.4 [g]	9.30 ± 0.24 [h]	0.63 ± 0.08 [g]
	db/m	Min/+	19	24.5 ± 1.8	9.13 ± 0.33	0.30 ± 0.03
	m/m	Min/+	10	20.8 ± 1.8	8.75 ± 0.23	0.27 ± 0.03

[a] Mean ± SD; [b] Significantly different from the db/m-Min/+ and m/m-Min/+ males ($p < 0.001$); [c] Significantly different from the m/m-Min/+ males ($p < 0.05$); [d] Significantly different from the db/m-Min/+ females ($p < 0.001$); [e] Significantly different from the db/m-Min/+ females ($p < 0.01$); [f] Significantly different from the m/m-Min/+ females ($p < 0.01$); [g] Significantly different from the db/m-Min/+ and m/m-Min/+ females ($p < 0.001$); [h] Significantly different from the m/m-Min/+ females ($p < 0.01$).

3.2. Serum Levels of Glucose, Total Cholesterol, Triglyceride and Insulin in Experimental Mice

The blood concentrations of glucose, total cholesterol, triglyceride, and insulin in the db/db-Min/+, db/m-Min/+, and m/m-Min/+ at 15 weeks of age are listed in Table 2. The measures of the db/db-Min/+ mice were higher than that of the db/m-Min/+ or m/m-Min/+ mice ($0.001 < p < 0.05$). However, the values of the db/m-Min/+ and m/m-Min/+ mice of either sex were comparable.

Table 2. Serum levels of glucose, total cholesterol, triglyceride, and insulin of mice at 15 weeks of age.

Gender	Genotype		No. of mice examined	Glucose (mg/dL)	Total cholesterol (mg/dL)	Triglyceride (mg/dL)	Insulin (ng/dl)
	db/db type	$Apc^{Min/+}$ type					
Males	db/db	Min/+	18	250.6 ± 35.6 [a,b]	161.8 ± 28.7 [b]	193.1 ± 14.8 [c]	3.3 ± 1.4 [b]
	db/m	Min/+	23	185.7 ± 27.6 [d]	109.4 ± 13.1	162.6 ± 27.6	0.4 ± 0.2
	m/m	Min/+	11	187.0 ± 21.8 [e]	96.1 ± 13.0	165.2 ± 21.5	0.3 ± 0.1
Females	db/db	Min/+	13	226.2 ± 28.7 [f]	145.1 ± 18.5 [g]	215.0 ± 38.0 [h,i]	3.5 ± 1.1 [f]
	db/m	Min/+	19	181.6 ± 24.5	119.1 ± 12.5	182.3 ± 29.8	0.3 ± 0.1
	m/m	Min/+	10	195.0 ± 20.4	115.3 ± 10.3	170.9 ± 18.2	0.3 ± 0.1

[a] Mean ± SD; [b] Significantly different from the db/m-Min/+ and m/m-Min/+ males ($p < 0.001$); [c] Significantly different from the db/m-Min/+ males ($p < 0.01$); [d] Significantly different from the db/m-Min/+ females ($p < 0.01$); [e] Significantly different from the m/m-Min/+ females ($p < 0.01$); [f] Significantly different from the db/m-Min/+ and m/m-Min/+ females ($p < 0.001$); [g] Significantly different from the m/m-Min/+ females ($p < 0.01$); [h] Significantly different from the db/m-Min/+ females ($p < 0.05$); [i] Significantly different from the m/m-Min/+ females ($p < 0.01$).

3.3. Tumors in the Intestinal Tract

Intestinal nodular tumors were observed in three strains of mice of each sex. As shown in Table 3, the total numbers of tumors, histopathologically tubular adenomas (Figure 2(A)), in small and large intestine of the db/db-Min/+ mice (60.5 ± 14.6 in males and 57.8 ± 12.7 in females, $p < 0.001$ for each comparison) were significant larger than the db/m-Min/+ mice (32.1 ± 7.5 in males and 32.6 ± 5.9 in females) and m/m-Min/+ mice (30.5 ± 7.7 in males and 30.9 ± 6.3 in females). The values of the db/m-Min/+ mice and m/m-Min/+ mice were comparable. The mean numbers of small intestinal tumors of the db/db-Min/+ mice of both sexes at the age of 15 weeks were also significantly greater than that of the db/m-Min/+ and m/m-Min/+ mice ($p < 0.001$). The incidence and multiplicity of colonic adenomas of the male and female db/m-Min/+ mice were greater than those of the db/m-Min/+ and m/m-Min/+ mice, and significant differences in the incidence were observed between the db/db-Min/+ and db/m-Min/+ ($p < 0.005$) or m/m-Min/+ males ($p < 0.005$), but not between the females. Histopathology of intestinal tumors developed in three strains of mice did not significantly differ. There were no significant differences in proliferation activities and apoptotic index in the tumor cells among three strains of mice (data not shown).

The mean volumes (mm^3) of colon tumors were 11.95 ± 11.70 (n = 31) in the db/db-Min/+ mice, 13.63 ± 8.78 (n = 12) in the db/m-Min/+ mice, and 15.52 ± 13.67 (n = 10) in the m/m-Min/+ mice, and the values did not significantly differ among three strains of both sexes.

Table 3. Multiplicity (no. of tumors/mouse) and incidence of intestinal tumors (adenomas) of mice at 15 weeks of age.

Gender	Genotype		No. of mice examined	Total number of adenomas/mouse (incidence)	Small intestine (incidence)	Colon (incidence)
	db/db type	$Apc^{Min/+}$ type				
Males	db/db	Min/+	18	60.5 ± 14.6 [a,b] (18/18, 100%)	58.8 ± 13.7 [b] (18/18, 100%)	1.7 ± 2.6 (12/18, 66.7% [c])
	db/m	Min/+	23	32.1 ± 7.5 (23/23, 100%)	31.6 ± 6.7 (23/23, 100%)	0.6 ± 1.2 (5/23, 21.8%)
	m/m	Min/+	11	30.5 ± 7.7 (11/11, 100%)	29.5 ± 6.1 (11/11, 100%)	0.9 ± 2.1 (3/11, 27.3%)
Females	db/db	Min/+	13	57.8 ± 12.7 [d] (13/13, 100%)	58.6 ± 12.3 [d] (13/13, 100%)	1.2 ± 1.5 (7/13, 53.8%)
	db/m	Min/+	19	32.6 ± 5.9 (19/19, 100%)	32.4 ± 5.9 (19/19, 100%)	0.2 ± 0.4 (4/19, 21.1%)
	m/m	Min/+	10	30.9 ± 6.3 (10/10, 100%)	30.7 ± 6.3 (10/10, 100%)	0.2 ± 0.4 (2/10, 20.0%)

[a] Mean ± SD; [b] Significantly different from the db/m-Min/+ and m/m-Min/+ males ($p < 0.001$); [c] Significantly different from the db/m-Min/+ and m/m-Min/+ males ($p < 0.005$); [d] Significantly different from the db/m-Min/+ and m/m-Min/+ females ($p < 0.001$).

Figure 2. Histopathology of a colonic tumor developed in db/db-Min/+ mice. The tumor is histopathologically diagnosed as a tubular adenoma. Immunohistochemistry of β-catenin and IGF-1R in the tumor shows positive reaction of β-catenin in their cell membrane and cytoplasm and IGF-1R in their cytoplasm. Bars = 50 μm. (**A**) H & E stain; (**B**) β-catenin immunohistochemistry; and (**C**) IGF-1R immunohistochemistry.

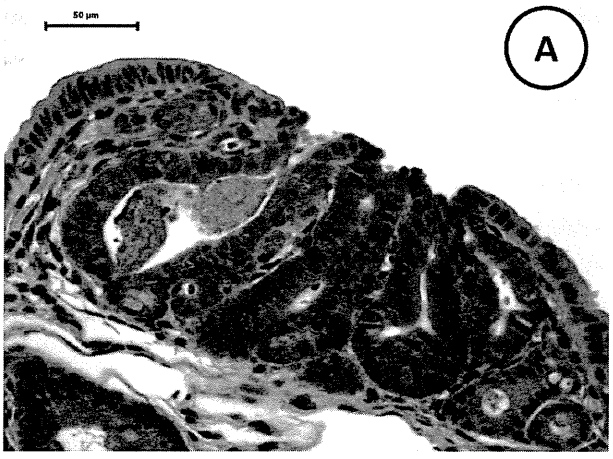
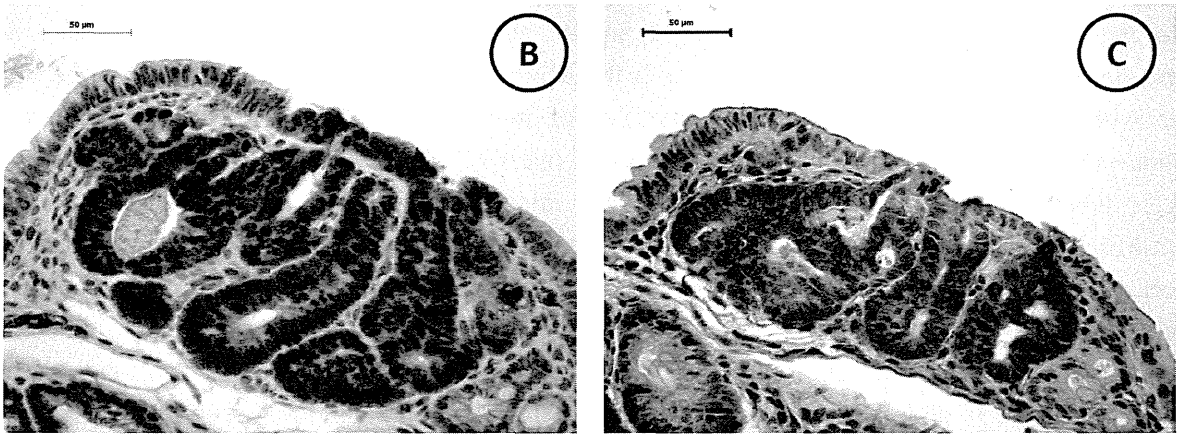


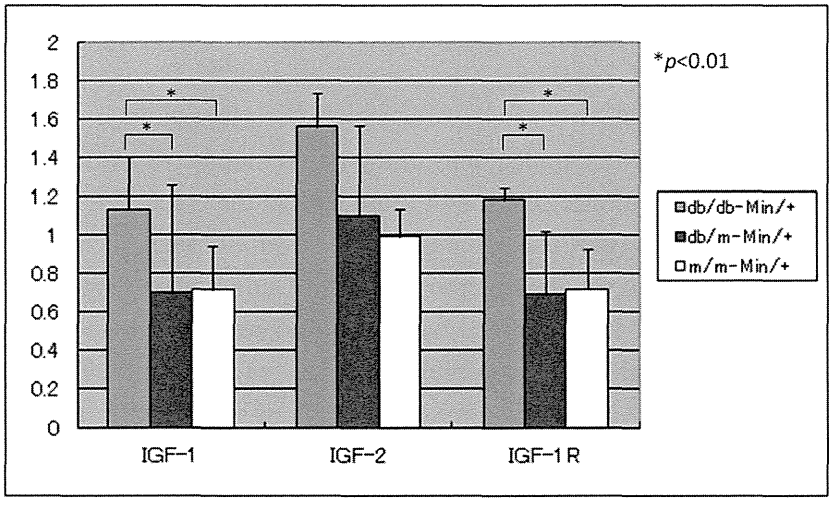
Figure 2. *Cont.*



3.4. Expression Levels of IGF-1, IGF-2, and IGF-1R mRNAs

The expression levels of IGF-1, IGF-2, and IGF-1R mRNAs in the colonic mucosa of the db/db-Min/+, db/m-Min/+ and m/m-Min/+ mice. As illustrated in Figure 3, the db/db-Min/+ mice showed significant increased mRNA levels of IGF-1, IRF-1R, and IGF-2 (1.4-fold to 2.6-fold increase), when compared to the db/m-Min/+ or m/m-Min/+ mice.

Figure 3. mRNA expression of IGF-1, IGF-2, and IGF-1R in the colonic mucosa of three strains of male mice (n = 6 from each strain).



3.5. Immunohistochemistry of β-Catenin and IGF-IR in the Colonic Tumors

Immunohistochemical expression of β-catenin was accumulated in the nucleus and cytoplasm of the colonic adenoma cells (Figure 2(B)) developed in three strains of mice. IGF-IR was immunohistochemically expressed in the cytoplasm and cell membrane of the adenoma cells (Figure 2(C)) and the sustainability did not significantly differ among adenomas developed in three strains of mice. Also, adenomas developed in small intestine shows similar expression of β-catenin and IGF-1R (data not shown).

4. Discussion

In the current study, we generated three mouse strains, the db/db-Min/+, db/m-Min/+, and m/m-Min/+ mice, by breeding db/m mice with the Min/+ mice, and then backcrossing of the db/m mice with the offsprings born from both strains of mice. All three strains developed intestinal adenomas and the number of tumors of either small intestine or colon in the db/db-Min/+ mice was greater than that of the db/m-Min/+ and m/m-Min/+ mice. The db/db-Min/+ mice were heavier than db/m-Min/+ and m/m-Min/+ mice. As expected, our findings were in accordance with our previous report showing that C57BL/KsJ-db/db obese mice were highly susceptible to AOM-induced colon carcinogenesis. In addition, the db/db-Min/+ mice had hyperinsulinemia, diabetes, and hyperlipidemia. Unexpectedly, development of colonic tumors including adenocarcinoma was not remarkable, but the number was slightly greater than that (0.5–1.1 colon tumors/mouse) described in previous reports [22,23]. The findings suggest that induction of a number of colonic tumors including adenocarcinoma needs other either intrinsic or external stimuli [24]. mRNA levels of IGF-1, IRF-1R, and IGF-2 in the colonic mucosa of the db/db-Min/+ mice were greater than the db/m-Min/+ or m/m-Min/+ mice. Immunohistochemically, the expression of β-catenin was accumulated in the nucleus and cytoplasm of small and large intestinal adenoma cells, and IGF-1R in the cytoplasm. These findings suggest that up-regulation of the expression of IGFs and the receptor might play a role in intestinal tumorigenesis [25], and that hyperinsulinemia could affect receptor-mediated signaling [10,11,26–28] in the db/db-Min/+ mice.

There is accumulating evidence suggesting that hyperinsulinemia and hyperlipidemia are involved in colon carcinogenesis in obese and diabetic rodents [29,30]. Several epidemiological studies indicate that diabetic patients with hyperinsulinemia have increased risk for CRC [27,31–35]. An experimental animal study also showed that continuous injections of insulin promote AOM-induced colon carcinogenesis in rats [36]. Hence, it seems likely that hyperinsulinemia in the db/db-Min/+ mice enhanced the development of intestinal adenomas in the current study. Regarding the mode of action, IGF-1 pathway plays a role in insulin-related tumor promotion in the colon. IGF-1 binds to the IGF-1R, activates a signal cascade, and triggers cell proliferation in several tissues, including colon [10,27,31]. Because of the homology with the insulin receptor, insulin at supra-physiological levels also binds to and activates the IGF-1R [37]. Furthermore, hyperinsulinemia was shown to indirectly increase bioavailability of IGF-1 by regulating the expression levels of IGF-binding proteins [38,39]. Our findings that IGF-1R expression was immunohistochemically up-regulated in the intestinal adenomas were in accordance with other studies showing that the IGF-1R is overexpressed in human CRC. Accordingly, it is possible that hyperinsulinemia in the db/db-Min/+ mice activates the signaling cascades involving the IGF-1R, resulting in a proliferative response.

In this study, we noticed hypelipidemia in the db/db-Min/+ mice, which is also a risk factor of human CRC development [40–43]. Experimentally, hyperlipidemia was found in intestinal carcinogenesis models with obese [14] and Min/+ mice [44]. Improvement of hyperlipidemia by certain drugs or natural compounds resulted in reduction and prevention of intestinal carcinogenesis in these models [10,13,14,22,23,28,44–47]. Thus, hyperlipidemia also contributes to a high incidence of spontaneous intestinal tumors in the db/db-Min/+ mice.

In the current study, we observed that the congenic db/db-Min/+ mice increase intestinal tumors compared to either transgenic counterpart. However, the volume of intestinal tumors did not significantly differ among the db/db-Min/+, db/m-Min/+ and m/m-Min/+ mice. Since differences in the indices of proliferation and apoptosis in the tumors were not observed among the three strains of mice (data not shown), there may be other factors that affect the growth of intestinal tumors. Recently Endo *et al.* [48] reported that the growth of colorectal tumors is dramatically inhibited in the *db/db* mice. Therefore, the leptin signaling might be important for colorectal tumor growth.

In conclusion, our findings described here indicate that the db/db-Min/+ mice with hyperinsulinemia and hyperlipidemia developed many intestinal adenoma, and the number was much greater than the db/m-Min/+ and m/m-Min/+ mice. The results observed were considered to occur through hyperinsulinemia and modulation of insulin-IGF axis. Since several biological events other than hyperinsulinemia/hyperlipidemia are also reported to be involved in the association between obesity/excess body weight, insulin resistance, and CRC development, such as chronic inflammation (inflammatory cytokines), glucose toxicity, advanced glycation end products product metabolism, and adipocytokies, further studies will be necessary to reveal the specific determinants that are responsible for the correlation between obesity and/or diabetes and colon carcinogenesis. Our db/db-Min/+ mice could be used for such studies.

Conflict of Interest

The authors declare no conflict of interest.

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