Isoleucine, an Essential Amino Acid, Prevents Liver Metastases of Colon Cancer by Antiangiogenesis

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Abstract
In spite of recent advances in the treatment of colon cancer, multiple liver metastases of colon cancer are still difficult to treat. Some chemotherapeutic regimens have been reported to be efficient, but there is a high risk of side effects associated with these. Here, we show that isoleucine, an essential amino acid, prevents liver metastases in a mouse colon cancer metastatic model. Because isoleucine is a strong inducer of ß-defensin, we first hypothesized that it prevented liver metastases via the accumulation of dendritic cells or memory T cells through up-regulation of ß-defensins. However, neither ß-defensin nor immunologic responses were induced by isoleucine in both mouse livers and spleens. Furthermore, isoleucine prevented liver metastasis in nude mice, which lack T cells and natural killer T cells. Finally, we discovered a novel mechanism of isoleucine: down-regulation of angiogenesis via inhibition of vascular endothelial growth factor, partially through the mammalian target of the rapamycin pathway, independent of hypoxia-inducible factor 1-α. Importantly, isoleucine is safe for administration to humans because it does not affect cell viability. Isoleucine could be a novel prophylactic drug for the prevention of liver metastases of colon cancer. [Cancer Res 2007;67(7):3263–8]

Introduction
Colorectal cancer is one of the leading causes of deaths from cancer worldwide. In Japan, the incidence of colorectal cancer has increased in association with diets becoming more "Westernized." In spite of recent advances in the treatment of colon cancer, the prognosis of patients with multiple liver metastases of colon cancers is still poor, and systemic chemotherapy prolongs survival only by a few months (1). Moreover, there is a high risk of severe side effects (2, 3), although methods for reducing the side effects of systemic chemotherapy [e.g., infusion chemotherapy through a reservoir pump (4) or chronotherapy (5)] can be used.

Antimicrobial peptides, such as defensins, play an important role in the innate immunity of all life forms, including plants or animals. Several types of antimicrobial peptides are induced at epithelial surfaces in response to inflammation. These antimicrobial peptides exhibit a broad spectrum of antifungal, antibacterial (6), and antiviral activities (7). Impairment of the function of defensin leads to susceptibility to infection in the airways of cystic fibrosis (8), and susceptibility to salmonella infection in mouse intestinal tracts (9). In addition to their direct antimicrobial activities, ß-defensins are strong chemotactic factors for memory T cells and dendritic cells, suggesting that they also play an important role in acquired immunity (10–12). ß-defensins are also inducible by inflammatory cytokines, such as tumor necrosis factor-α and interleukin-1ß (13, 14). Recently, Fehlbaum et al. (15) reported that isoleucine and its analogues are highly specific inducers of ß-defensins. We thus originally hypothesized that isoleucine may contribute to tumor immunity through both innate and acquired immunity by induction of ß-defensins.

Angiogenesis is a key process in tumor growth (16–18), and vascular endothelial growth factor (VEGF), which stimulates angiogenesis in a paracrine fashion, seems to be essential for the progression of various solid tumors. VEGF production is initiated by phosphorylation of eukaryotic initiation factor (eIF) 4E-binding protein 1 (4E-BP1) by the mammalian target of the rapamycin (mTOR) following disruption of their binding to eIF-4E, enabling eIF-4E to translate most mRNAs, including VEGF, through the m7GpppN cap (19–21). Rapamycin, an immunosuppressive drug, interacts with mTOR, following dephosphorylation of 4E-BP1 and impairment of VEGF production (22). Recent studies have shown that leucine promotes albumin production partially through the mTOR pathway, which stimulates VEGF through activation of eIF-4E (23, 24). However, the association between isoleucine and angiogenesis remains unclear.

Here, we describe remarkable results indicating that isoleucine actually prevents tumor growth in a mouse colon cancer liver metastatic model. The mechanism by which isoleucine exerts its antitumor effect was not, as we expected, via an immunologic pathway through the up-regulation of ß-defensins. Rather, isoleucine acts through a novel mechanism whereby it inhibits VEGF at the translation initiation level, partially through the mTOR pathway. Importantly, isoleucine did not affect cell viability.

Materials and Methods

Animals and cell lines. Male BALB/c mice (6–8 weeks old) and male BALB/c nude mice (nu/nu, 6–8 weeks old) were obtained from SLC (Shizuoka, Japan) and maintained with ad libitum access to food and water. Colon 26 cells (BALB/c syngenic colon cancer cells) were maintained in DMEM (Sigma, Tokyo, Japan) containing 10% FCS. Yae-1 cells were maintained in 10% FCS containing RPMI 1640 (Invitrogen Corp., Carlsbad, CA).

Liver metastatic model. Isoleucine, leucine (3 mg/d dissolved in 0.2 mL PBS), or PBS alone was given daily to BALB/c nude mice or nude mice using a feeding tube, starting from 5 days before the tumor implantation until sacrifice. On the operation day, 1 x 10⁶ colon 26 cells in 0.2 mL PBS were implanted into the spleens of anesthetized and laparotomized mice. Three weeks after the operation, the mice were sacrificed and examined for liver
metastases. Blood samples were taken by retro-orbital puncture and serum albumin concentrations were measured using a standard clinical analyzer. The liver of each mouse was weighed. Livers, spleens, and tracheas were removed and frozen until they were used for reverse transcription-PCR (RT-PCR) and Western blotting studies.

Immunologic experiments. Seven days after administration of isoleucine or leucine (3 mg/d, n = 5 each), mice were sacrificed and intraperitoneal lymphocytes were prepared as reported previously (25). The intraperitoneal lymphocytes and splenic lymphocytes were stained with anti-CD3 and anti-panNK (anti-DX5; BD Biosciences, San Jose, CA). The intraperitoneal lymphocytes and splenic lymphocytes were subjected to a standard natural killer (NK) cytotoxic assay. Briefly, various numbers of intraperitoneal lymphocytes and splenic lymphocytes were cultured with 3,000 51Cr-coated Yac-1 cells for 5 h. The amount of 51Cr in the supernatant was measured. The CTL concentration was measured using a standard 5-h chromium releasing assay. Briefly, splenocytes (nontumor site) from the isoleucine-treated mice (3 mg/ml; n = 5) 3 weeks after implantation of colon 26 cells or naive mice were incubated with irradiated (10,000 rad) colon 26 cells in complete culture medium with 10% T-stim (Becton Dickinson, Billerica, MA) for 7 days following coculture with chromium-coated colon 26 cells for 5 h. The amount of chromium released into the medium was measured. Specific killing was calculated using the following equation: (experimental release – spontaneous release) / (maximum release – spontaneous release) × 100.

In vitro experiments. Two days after seeding of colon 26 cells at a cell density of 1 × 104 cells/cm2 to a 24-well plate, the medium was replaced with isoleucine or leucine dissolved in 2.5% FCS containing DMEM, and the cells were cultured for another 48 h. The supernatant was used to measure the VEGF concentration by ELISA (mouse VEGF ELISA kit; R&D Systems, Minneapolis, MN). These cells were used for 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as well for checking for any effects of the amino acids on cell viability. RT-PCR, cells were harvested 24 h after replacement of the medium.

RT-PCR. RT-PCR was used to measure the concentration of mouse β-defensin (mBD)-3 and VEGF in the mouse tissues or in the colon 26 cells. Poly(A)+RNA was isolated using RNA Zol (Biotecx Laboratories, Houston, TX) from mouse tissues (liver, spleen, and trachea) or cells and a 1 µg aliquot was reverse transcribed into cDNA (Taqman; Applied Biosystems, Foster City, CA). The following primers were used for PCR: BD-3, 5′-CTCTTTGCATTTCTCCTGTTGCTGCTG-3′ (forward) and 5′-CATC TTCATGGAGAGCGCAATTCTTG-3′ (reverse); VEGF, 5′-GGCTGCCTGCGGATGTC-3′ (forward) and 5′-TACCCGCCTTGGCTTGTA-3′ (reverse). RT-PCR using primers specific for mouse glyceraldehyde-3-phosphate dehydrogenase was used as a positive control. Trachea tissue was used as a positive control for mBD-3 mRNA.

Western blotting. The cell extracts were resolved by SDS-PAGE and transferred to nitrocellulose membranes. After blocking, the membranes were incubated overnight at 4°C with antibodies against hypoxia-inducible factor 1-α (HIF1-α; Santa Cruz Biotechnology, Santa Cruz, CA) or phosphospecific antibodies against 4E-BP1 (Thr37/46; Cell Signaling Technology, San Diego, CA) and signal transducers and activators of transcription 3 (Stat3; Santa Cruz Biotechnology). Antibodies against α-tubulin were used as an internal control. The membranes were washed and then incubated for 30 min with horseradish peroxidase–conjugated secondary antibodies. The membranes were washed again, and the proteins were detected using electrochemiluminescence techniques (Pierce Chemicals, Rockford, IL).

Immunohistochemical staining for CD31. Immunohistochemistry was done using a peroxidase-streptavidin method on frozen livers, using antibodies against mouse CD31 (BD PharMingen, San Diego, CA). The antibodies were detected with 3,3′-diaminobenzidine. The sections were counterstained with 10% hematoxylin (Wako Pure Chemical, Osaka, Japan).

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

Effect of isoleucine on liver metastasis of colon cancer. We first hypothesized that because isoleucine induces β-defensins, it could contribute to tumor regression through innate or acquired immunity. Thus, we attempted to determine whether isoleucine prevents tumor growth in a mouse liver metastatic model of colon cancer. Remarkably, none of the isoleucine-treated mice (3 mg/d) had any liver metastases (n = 5). In contrast, no tumor growth inhibition was observed in the leucine-treated mice (n = 5), relative to mice treated with PBS (data not shown). These effects were observed with a rather low concentration of isoleucine (0.6 mg/d), and the effect on the number of liver metastases was dose dependent (Fig. 1A and B). The effects of isoleucine against liver metastases were supported by observations with respect to both liver weight (Fig. 1C) and serum albumin levels (Fig. 1D). Interestingly, tumor cells in the spleen were found to be growing even in the isoleucine-treated mice (3 mg/d), which did not have any liver metastases. Furthermore, the sizes of the tumors in the spleen in the isoleucine-treated mice were similar to the sizes of those in the leucine-treated mice (data not shown).

Isoleucine does not induce mBD-3 in mice. Because isoleucine is a strong inducer of β-defensins (15), we initially hypothesized that β-defensins would be induced in the liver by isoleucine treatment. Of the many β-defensins, we focused on mBD-3 because human β-defensin-2, which is the homologue of mouse BD-3, causes the accumulation of dendritic cells or memory T cells around tumors and contributes to tumor immunity (10). Additionally, mBD-2 and mBD-3 are chemotactics for immature dendritic cells (12) and mBD-3 is inducible in the liver (26). Therefore, using RT-PCR, we examined mBD-3 mRNA expression in the livers and the spleens of mice treated with isoleucine or leucine. However, no expression of mBD-3 was observed in either liver or spleen tissue, even in mice, in which liver metastases were prevented by isoleucine treatment, whereas mBD-3 mRNA was clearly detected in trachea tissue (positive control; Fig. 2). Thus, it seems that the expression of mBD-3 does not contribute to the prevention of liver metastases by isoleucine treatment in our model.

Isoleucine does not induce innate or acquired immunity in liver or spleen. To investigate the immunologic processes participating in the prevention of liver metastases of colon cancer by isoleucine, NK cytotoxicity was examined. NK cytotoxicity was not induced in either the intraperitoneal lymphocytes (3.8 ± 2.1%; E:T, 50:1) or the splenocytes (6.9 ± 5.1%; E:T, 50:1) of the isoleucine-treated mice, implying that neither NK nor NKT cells have an important role in the prevention of liver metastases (naive mice; intraperitoneal lymphocytes, 5.2 ± 0.3%; splenocytes, 8.3 ± 1.8%, respectively). Furthermore, no significant differences in the populations of intraperitoneal lymphocytes or splenocytes were observed between the isoleucine-treated mice and naive mice (data not shown). A CTL assay using splenocytes from the isoleucine-treated mice without any liver metastases showed that no specific killing of colon 26 cells was occurring (isoleucine, 5.9 ± 4.9%; leucine, 7.0 ± 2.1%, respectively; E:T, 50:1). Furthermore, isoleucine (3 mg/d) completely prevented liver metastases even in nude mice (n = 5), implying that neither T cells nor NKT cells have an important role in the prevention of liver metastases by isoleucine (Fig. 1). Taken together, these results show that neither innate nor acquired immunity plays a major role in the prevention of metastatic liver tumors by isoleucine.
Effect of isoleucine on VEGF-mediated angiogenesis in vitro.

We next examined whether isoleucine affects angiogenesis, which is an important process in tumor growth. In in vitro experiments, isoleucine inhibited VEGF production by colon 26 cells in a dose-dependent manner (Fig. 3A), whereas leucine produced no effect on VEGF production relative to the medium alone. Interestingly, a MTT assay showed that neither isoleucine nor leucine affected cell viability, even with a rather high concentration of isoleucine or leucine (1 mg/mL; Fig. 3B). The expression level of VEGF mRNA in isoleucine-treated colon 26 cells was markedly lower than that in cells treated with leucine or medium alone (Fig. 3C and D).

Effect of isoleucine on angiogenesis in a mouse liver metastatic model. To determine whether the antiangiogenic effect of isoleucine could be seen in mice as well as in cell culture, VEGF mRNA levels were determined by RT-PCR using liver or spleen samples collected from mice treated with isoleucine, leucine, or PBS. In nontumor lesions of the liver, the expression level of VEGF mRNA in the isoleucine-treated mice was markedly impaired in comparison with the levels in the leucine-treated mice. Furthermore, the VEGF mRNA level in the leucine-treated mice was up-regulated relative to the expression levels in the PBS-treated mice (Fig. 4A and B). The VEGF mRNA level in the cancerous lesions was further up-regulated. The expression levels of VEGF mRNA in spleens treated with either isoleucine or leucine (without colon 26 challenge) tended to be higher than those in the livers of naive mice. Western blotting of nontumor liver tissues revealed that the expression of phosphorylated 4E-BP1 and Stat3 were parallel to the VEGF mRNA level. HIF1-α, however, was up-regulated in the leucine-treated mice relative to the leucine-treated mice, and quite low expression levels were observed in the cancerous lesions. The expression level of HIF1-α was inversely correlated with the expression level of phosphorylated 4E-BP1 and Stat3. In addition, immunohistochemical staining of endothelial cells with anti-CD31 showed that the number of endothelial cells in the tumor was markedly reduced in the isoleucine-treated mice (0.6 mg/d; Fig. 5B) relative to the number in the leucine-treated mice (Fig. 5A). These results show that isoleucine exerts antiangiogenic effects in a mouse liver metastatic model as well as in in vitro cell cultures.

Discussion

We show here that isoleucine prevents tumor growth in a mouse liver metastatic model of colon cancer. Contrary to our
predictions, this occurred because isoleucine inhibited VEGF, thus producing antiangiogenic effects, not because of tumor immunity via induction of h-defensin. Furthermore, isoleucine inhibited VEGF production partially through a mTOR pathway, not through a HIF1-α–dependent pathway. Importantly, isoleucine did not show any signs of cytotoxicity in our in vitro experiments.

We initially hypothesized that isoleucine would prevent liver metastatic tumor growth via innate and acquired immunity, given that it is a strong inducer of h-defensins, which promote the accumulation of memory T cells and dendritic cells through CCR6 (10). We found that isoleucine prevented liver metastasis in a dose-dependent manner, but no mBD-3 expression and no immunologic cytotoxicity was observed, even in the isoleucine-treated mice, in which metastatic liver tumors were completely prevented. Furthermore, we found that isoleucine completely prevented liver metastases in nude mice, which lack T cells and NKT cells. These observations imply that immunologic responses do not contribute to the mechanism of prevention of liver metastases by isoleucine.

Next, we determined whether isoleucine has effects on angiogenesis because angiogenesis is generally an essential factor for the growth of various tumors (16–18). Our in vitro experiments showed that isoleucine inhibited VEGF production in a mouse colon cancer cell line at transcriptional levels. It is important that we did not use amino acid–deficient medium before cultivating of colon 26 cells with isoleucine or leucine, which should be similar condition that patients take additional isoleucine to daily food intake. Interestingly, isoleucine did not affect cell viability, implying that the inhibitory effect of isoleucine on VEGF production is not due to antiproliferative or direct effects on the VEGF-producing cells. These pieces of evidence were confirmed by an in vivo study, in which liver VEGF mRNA levels were found to be markedly reduced in the isoleucine-treated mice. The liver VEGF mRNA levels of the leucine-treated mice were up-regulated relative to the levels in the mice given PBS alone, and the VEGF mRNA levels were further up-regulated in the cancerous lesions of the leucine-treated mice.

Figure 3. Effects of isoleucine and leucine on VEGF and VEGF mRNA production and cell viability in colon 26 cells. A, ELISA for VEGF in the culture medium showed that isoleucine inhibits VEGF production in a dose-dependent manner. B, MTT assays showed that neither isoleucine nor leucine exhibited any cytotoxicity. C and D, 2 d after seeding of colon 26 cells, the culture medium was replaced with isoleucine, leucine, or medium alone in 2.5% DMEM and cells were harvested after 24 h. Representative gels of RT-PCR for VEGF mRNA expression.

Figure 4. A and B, effects of isoleucine and leucine on VEGF mRNA levels in vivo. After they were pretreated with isoleucine or leucine (3 mg/d) for 5 d, colon 26 cells (1 x 10^6) were implanted into the spleens of BALB/c mice. Three weeks later, mice were sacrificed and their livers were removed for further studies. Representative gel from RT-PCR of VEGF mRNA (n = 5 each group) in mice liver. Lane 1, liver of isoleucine-treated mice (nontumor tissue); lane 2, liver of leucine-treated mice (nontumor tissue); lane 3, liver metastatic lesion from leucine-treated mouse; lane 4, liver from PBS-treated mice (without colon 26 challenge); lane 5, spleen from isoleucine-treated mice (without colon 26 challenge); lane 6, spleen from leucine-treated mice (without colon 26 challenge). *, P < 0.05; **, P < 0.01. C, representative Western blot of HIF1-α and phosphorylated 4E-BP1 and Stat3 proteins in mouse liver. Lane 1, isoleucine-treated mice (nontumor liver); lane 2, leucine-treated mice (nontumor liver); lane 3, cancer lesion from leucine-treated mouse; lane 4, liver from PBS-treated mice (without colon 26 challenge).
the present study, the expression level of HIF1-α was inversely correlated with the expression level of phosphorylated 4E-BP1 and Stat3. These findings suggest that isoleucine down-regulates the transcriptional factor of VEGF through the mTOR pathway and that HIF1-α was up-regulated in a negative feedback process. Although the reason HIF1-α was inversely up-regulated in our experiment remains unclear, we have shown that isoleucine down-regulates VEGF expression via a HIF1-α-independent pathway. Many investigators have concluded that the phosphatidylinositol 3-kinase/PTEN/Akt pathway can increase VEGF expression via HIF1-α (28–30). However, Pore et al. (31) indicated that Akt can transactivate the VEGF promoter and lead to increased VEGF expression-independent of HIF1-α. Their findings seem to support our findings.

Leucine enhances albumin synthesis in rat hepatocytes, whereas isoleucine does not (23). In the present study, the serum albumin concentration of the leucine-treated mice was lower than that of the isoleucine-treated mice. This discrepancy may arise from the fact that the leucine-treated mice had multiple metastases in the liver, which may have impaired albumin synthesis, whereas the isoleucine-treated mice did not show any liver metastases and looked healthy. Thus, albumin synthesis has been unaltered, although isoleucine itself is not able to enhance protein synthesis. Clinically, treatment with branched-chain amino acids (BCAA) can actually improve serum albumin concentrations in patients with liver cirrhosis. However, the incidence of hepatocellular carcinoma seems to be similar between patients with and without BCAA treatment. In cirrhotic patients treated with BCAA, leucine may enhance angiogenesis as well as serum albumin concentrations partially through the mTOR pathway. At the same time, isoleucine may down-regulate angiogenicity by impairment of VEGF production. These opposing effects may counterbalance angiogenicity in cirrhotic patients being treated with BCAA.

In conclusion, we found that isoleucine prevents tumor growth in a mouse liver metastatic model. Furthermore, we determined that isoleucine prevents tumor growth via a novel mechanism, by impairment of VEGF production, partially through mTOR pathway-independent HIF1-α. Importantly, isoleucine is only an essential amino acid, and no significant cytotoxicities against cells were observed. Treatment with isoleucine could be a safe and effective prophylactic treatment for use in patients with colon cancer, and clinical trials should be considered.

Acknowledgments
Received 10/16/2006; revised 12/31/2006; accepted 1/30/2007.

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We thank M. Itoh and T. Nobori for helpful discussions and T. Inoue for technical assistance.
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