Anti-Angiotensin and Hypoglycemic Treatments Suppress Liver Metastasis of Colon Cancer Cells

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Key Words

Angiotensin • Renin • Hyperglycemia • Colon cancer • Liver metastasis

Abstract

The effect of diabetic conditions on liver metastasis was examined using CT26 mouse colon cancer cells. CT26 cells produced angiotensin (A)-I and A-II from angiotensinogen; the production was abrogated by inhibitors of renin and chymase. Renin expression and A-II production increased with an increase in the concentration of glucose in the medium. In a streptozotocin-induced BALB/c mouse diabetes model that was fed a high-calorie diet, the blood sugar level increased and was associated with an increasing size and number of CT26 liver metastases. In this diabetic mouse model, liver metastasis of CT26 cells was suppressed by anti-angiotensin treatment with a chymase inhibitor, a renin inhibitor, and an A-II receptor blocker. Moreover, concurrent hypoglycemic and anti-angiotensin treatments showed a synergistic inhibitory effect on CT26 cell liver metastasis. These results suggest that angiotensin activation ability associated with

diabetic conditions enhances liver metastasis of colon cancer. Therefore, treatment with anti-angiotensin and hypoglycemic agents might be relevant for baseline management of colon cancer patients with the diabetic condition for the prevention of liver metastasis. This scheme needs to be examined in a clinical setting.

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Introduction

Colorectal cancer (CRC) is the third leading cause of cancer death in Japan and the leading cause of cancer death in women. CRC mortality continues to increase as the Western dietary style is gaining popularity in Japan [1]. About 30% of CRC patients die from liver metastasis [2], and the 5-year survival rate of CRC patients with liver metastasis is less than 20% [1]. Thus, the diagnosis and treatment of liver metastasis is a pivotal problem in conquering CRC.

Type 2 diabetes is one of the most notorious outcomes of the Western dietary style. Diabetes is associated with

increased disease risk and CRC mortality [3, 4]. The blood level of glycated hemoglobin (HbA1c) is considered a marker of CRC risk [5]. The mechanism underlying the protumoral role of diabetes is explained by the significance of plasma insulin levels [3]. An increased blood concentration of insulin and insulin-like growth factor enhances CRC risk [6].

Angiotensin-II (A-II) is known to be a protumoral factor which leads to vasoconstriction and proliferation in neoplastic tissues [7]. A-II induces the activation of protein kinase C and the induction of angiotensin 2, vascular endothelial growth factor (VEGF), VEGF receptors, fibroblast growth factor, platelet-derived growth factor, transforming growth factor beta, epidermal growth factor, nitric oxide synthase, and metalloproteinase [7, 8]. These properties enhance colon cancer progression, particularly its metastasis.

In the present study, we aimed to elucidate the anti-metastatic effect of anti-angiotensin and hypoglycemic therapies in a hyperglycemic condition using a streptozotocin (STZ)-induced mouse diabetes model.

Materials and Methods

Cell Culture and Reagents

The CT26 mouse colon cancer cell line was kindly provided by Prof. Isaiah J. Fidler (MD Anderson Cancer Center, USA) [9]. Cells were maintained in Dulbecco's modified essential medium (Sigma Chemical Co., St. Louis, Mo., USA) containing 10% fetal bovine serum (Sigma) under conditions of 5% CO₂ in air at 37°C. The glucose concentration was 200 mg/dl in the regular medium. Angiotensinogen (ATG, 40 nM; Calbiochem, Darmstadt, Germany), D-glucose (Sigma), aliskiren (ALI; renin inhibitor, 1 µM; Santa Cruz Biotechnology, Inc., Santa Cruz, Calif., USA), chymostatin (CMS; chymase inhibitor, 50 µM; Peptide Research Institute), and captopril (CAP; ACE inhibitor, 1 mM; MP Biomedicals LLC, Solon, Ohio, USA) were purchased.

Immunoblot Analysis

Whole-cell lysates were prepared as described previously [10]. Anti-renin antibody (AnaSpec, Inc., San Jose, Calif., USA) was used as the primary antibody. An anti-tubulin antibody was used as a loading control (Oncogene Research Products, Cambridge, Mass., USA). The immune complex was visualized using an enhanced chemiluminescence Western blot detection system (Amersham, Aylesbury, UK). Whole-cell lysates were also used for ELISA.

Animal Model

BALB/c mice (male, 4 weeks old) purchased from Japan SLC, Inc. (Shizuoka, Japan) were used as a metastasis model. The mice were maintained according to the institutional guidelines approved by the Committee for Animal Experimentation of Nara Medical University. Single-cell suspensions of CRC cells (1×10^6)

in Hank's balanced saline solution were injected into the mouse spleen. The mice were sacrificed to determine the number and size of metastatic foci in the liver [11].

Mice were fed a normal diet (CE-2; total calories 343.1, NFE 50%, crude fat 4.8%, mainly consisting of soybean oil; Clea Japan, Inc., Tokyo, Japan) or a high-calorie diet (HCD; QuickFat, total calories 405.5, NFE 46.5%, crude fat 13.6%, mainly consisting of beef tallow; Clea Japan) with or without 10% glucose consumption (Otsuka Pharmaceutical Co., Tokushima, Japan). Mice were treated with STZ (200 mg/kg of body weight, i.p., once; Wako Pure Chemical Industries, Ltd., Osaka, Japan) for a diabetes model, gliclazide (GCZ; 3.7 mg/kg of body weight, p.o., in the evening, once a day; Wako), porcine insulin (INS; 4 units/kg of body weight, s.c. in the evening, once a day; MP Biomedicals), ALI (25 mg/kg of body weight, s.c., in the evening, once a day), losartan (LOS; 2 mg/kg of body weight, intragastric administration, in the evening, once a day; Toronto Research Chemicals, Inc., Toronto, Ont., Canada), or CMS (10 mg/kg body weight, i.p., in the evening, once a day).

Mouse blood sugar and A-II in serum or cultured medium were measured using a Mediasafe Reader (Terumo Co., Tokyo, Japan) and A-II ELISA kits (Phoenix Pharmaceuticals, Inc., Belmont, Calif., USA), respectively. Renin and chymase protein levels in whole-cell lysates were examined using ELISA kits (Uscn Life Science, Inc., Wuhan, China; and DRG International Inc. East Mountainside, N.J., USA, respectively). ELISA was performed according to the provider's instructions.

Statistical Analysis

Statistical analyses of experimental data were performed using the Mann-Whitney U test and the Kruskal-Wallis test with Dunn's multiple comparisons test (nonparametric ANOVA). The survival rates of mice were examined using the Kaplan-Meier method. A two-sided $p < 0.05$ was considered statistically significant.

Results

Angiotensin Activation by CT26 Cells

We examined the angiotensin activation ability of CT26 cells (fig. 1a). CT26 cells produced A-I and A-II from ATG. CT26 cells were treated with inhibitors of angiotensin activation-related enzymes. Treatment with chymase inhibitor abrogated A-II production and increased A-I levels. Renin inhibitor abrogated the production of A-I and A-II. In contrast, ACE inhibitor did not affect the production of A-I or A-II. These results suggest that CT26 cells activate angiotensin via renin and chymase. The A-II level depended on the glucose concentration of the culture medium (fig. 1b). When CT26 cells were cultured with different concentrations of glucose, the renin protein levels were increased in a dose-dependent manner. In contrast, chymase production was not altered by the glucose concentration (fig. 1c).

Fig. 1. Angiotensin activation in CT26 cells. **a** The A-II concentration in the culture medium was examined by ELISA. CT26 cells were treated with ATG (40 nM) and CMS (50 μ M) or CAP (1 mM) or ALI (1 μ M) for 12 h. **b** A-II concentration in the culture me-

dium. CT26 cells were cultured in a medium containing various concentrations of glucose. **c** Renin and chymase protein levels in the CT26 cells were examined by ELISA. Error bars represent the SD.

Effect of Hyperglycemia on Liver Metastasis of CT26 Cells

To examine the effect of hyperglycemia on liver metastasis of CT26 cells, nude mice fed an HCD and/or given STZ injection were used (fig. 2). The body weights of the mice in each group did not significantly differ; however, the blood sugar level was higher in the HCD, STZ, and combined HCD/STZ groups (groups B, C, and D) than in the control group (group A) ($p < 0.0001$) (fig. 2c, d). Renin expression levels were associated with blood sugar levels (fig. 2b). The size and number of metastatic foci were higher in the STZ-treated groups (groups B and D) than in the control group (group A) (fig. 2e, f). These results suggest that a high blood sugar level enhances liver metastasis by increasing renin expression.

Inhibitory Effect of Anti-Angiotensin Agents on Liver Metastasis

To evaluate the anti-metastatic effect of anti-angiotensin agents (fig. 3), a liver metastasis model using mice fed an HCD and treated with STZ injection was modified by the administration of chymase-I (group F), renin-I (group G), and A-II type 1 receptor blocker (ARB; group H) (fig. 3a). Fewer metastatic foci were found in the groups treated with anti-angiotensin agents (groups F, G, and H) compared to the untreated group (group E) ($p < 0.001$) (fig. 3c, d). Moreover, the overall survival in the treated groups was improved compared to that in the

untreated group (fig. 3b). Thus, anti-angiotensin treatment is effective in suppressing colon cancer liver metastasis.

Inhibitory Effect of Hypoglycemic Agents on Liver Metastasis

Finally, we examined the effect of concurrent treatment with anti-angiotensin and hypoglycemic agents on the liver metastasis of CT26 cells (fig. 4). Insulin and GCZ were administered with or without renin-I in the liver metastasis model using mice fed an HCD and treated with STZ injection (fig. 4a). Treatment with insulin and GCZ resulted in lower blood sugar levels compared to those in the untreated mice (fig. 4b). The mice treated with hypoglycemic agents (groups J and K) showed a decrease in the number of metastatic foci and improved survival compared to the untreated group (group I) (fig. 4d, e). Concurrent treatment with anti-angiotensin and hypoglycemic agents (groups L and M) resulted in a lower serum A-II concentration, a smaller number of metastatic foci, and longer survival compared to the untreated mice (group I) or the mice treated with hypoglycemic agents alone (groups J and K) (fig. 4c–f). The mice treated with the combination showed suppression of liver metastasis and improved survival which was also distinguishable from that of the control mice (group N) (fig. 4d, e).

Fig. 2. Effect of STZ-induced diabetic conditions on liver metastasis of CT26 mouse colon cancer cells. **a** Protocol of a liver metastasis model using the STZ-induced diabetic mouse. Mice were fed a control diet or an HCD. Each group consisted of 6 mice. **b** Renin protein expression was examined by immunoblotting in groups A, B, C, and D. Tubulin was examined as a loading control. **c–f** Body weight, blood sugar, and size and number of liver metastatic foci in each group. Error bars represent the SD. isp = intrasplenic inoculation; ip = intraperitoneal inoculation; w = week.

Fig. 3. Effect of anti-angiotensin agents on liver metastasis of CT26 mouse colon cancer cells. **a** Protocol of a liver metastasis model using STZ-induced diabetic mice. Mice were treated with chymase inhibitor (chymase-I, CMS), ALI, and losartan (LOS). Each group consisted of 6 mice. **b** The survival of mice in each group was calculated and compared using the Kaplan-Meier method. Statistical significance was compared between group E and all other groups. **c, d** Size and number of liver metastatic foci in each group. Error bars represent the SD. * Compared with group E. isp = intrasplenic inoculation; ip = intraperitoneal inoculation; w = week.

Fig. 4. Effect of concurrent treatment with an anti-angiotensin agent and a hypoglycemic agent on liver metastasis of CT26 mouse colon cancer cells. **a** Protocol of a liver metastasis model using STZ-induced diabetic mice. Mice were treated with a combination of ALI and INS or GCZ. There were 8 mice in each group. **b–e** Blood sugar, serum A-II, and size and number of liver metastatic foci in each group. Error bars represent the SD. **f** The survival of mice in each group was calculated and compared using the Kaplan-Meier method. Statistical significance was compared between group I and all other groups. *, ** Compared with group I. # Compared with group J and K. isp = Intraperitoneal inoculation; w = week.

Discussion

CT26 CRC cells possess an angiotensin activation mechanism provided by expression of renin and chymase. In fact, CT26 cells express renin in association with the glucose concentration in a dose-dependent manner. The angiotensin activation ability of CT26 cells was confirmed by A-II production which was inhibited by both a renin inhibitor and a chymase inhibitor. Thus, the renin-chymase system is thought to be employed to activate ATG in CT26 cells. Renin expression was induced by the glucose in the culture medium. A high glucose level increases renin expression and the subsequent A-II production in the cardiac fibroblasts [12]. We also found that

renin expression was increased by the hyperglycemic status in the STZ-induced diabetic mice. Moreover, hyperglycemia also enhanced liver metastasis in the STZ-induced diabetic mice. These findings suggest that hyperglycemia in diabetic animals enhances liver metastasis via angiotensin activation.

Based on these results, we examined the effects of anti-angiotensin treatment and/or hypoglycemic treatment on liver metastasis of CT26 cells. Inhibitors of the renin-angiotensin system are widely used to treat hypertension. In the present study, some anti-angiotensin agents, inhibitors of renin and chymase, and ARB suppressed liver metastasis of CT26 cells. Moreover, hypoglycemic treatment by GCZ and insulin showed improvement of liver

metastasis. Combined treatment with anti-angiotensin and hypoglycemic agents showed a synergistic inhibitory effect on liver metastasis. ACE inhibitors and/or ARB have been reported to improve the disease prognosis or reduce progression in pancreatic and urogenital cancer [13, 14]. Our results suggest that anti-angiotensin system therapy should also be tested for prevention of liver metastasis in colon cancer.

In the present study, we used an STZ-induced mouse diabetes model. Since STZ damages pancreatic beta cells, this is not really the most appropriate model to study type II diabetes [15]. The STZ dosage used in this study results in partial damage to the beta cells which maintain insulin production at levels lower than those in normal mice (data not shown). We fed the mice an HCD which results in a metabolism resembling that observed in type II diabetes [16]. Thus, the STZ-induced diabetes model combined with an HCD is a suitable simulation of type II diabetes [15]. To confirm the effect of diabetic conditions on liver metastasis, further experiments using spontane-

ous or genetically engineered diabetes model rodents are required.

Considering that hyperglycemia is associated with liver metastasis of colon cancer via renin upregulation, diabetic status is thought to be a risk factor for liver metastasis. Control of blood sugar could, therefore, be important in preventing liver metastasis in colon cancer patients. The effect of anti-angiotensin treatment and blood sugar control for baseline management of colon cancer patients with the diabetic condition needs to be examined in the clinical setting for the prevention of liver metastasis.

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