The role of angiotensin II in liver metastasis formation from colorectal cancer

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Objective: Liver metastasis from colorectal cancer (CRC) is a significant clinical problem. The renin-angiotensin system (RAS) is involved in growth and progression of tumor invasion and metastasis. The objective of this study was to evaluate the role of angiotensin II (ANG II) in the formation of liver metastasis in CRC.

Methods: A liver metastasis model was made by injecting CMT93 mouse colon cancer cells into male C57Bl/6 mice via the portal vein inoculated into the spleen.

Results: The liver metastatic area was significantly suppressed in mice treated with angiotensin covert enzyme inhibitor (ACEI) or angiotensin II type 1 receptor (AT1) blocker (ARB) compared to vehicle-treated mice. The expression of F4/80 and transforming growth factor-β (TGF-β) were suppressed in ACEI- and ARB-treated mice. Immunofluorescence analysis revealed that F4/80+ Kupffer cells (KCs) co-stained with TGF-β in metastatic area in the vehicle-treated mice were increased as compared with ACEI- and ARB-treated mice. In vitro studies revealed that the clonal murine KC line (KUP5) enhanced proliferation under ANG II treatment compared to the control. Moreover, the expression of CD31 and type I collagen were up-regulated in the vehicle-treated mice but not in the ACEI- or ARB-treated mice.

Conclusion: These results suggested that ANG II induces liver metastasis of CRC through TGF-β production from accumulated KCs.

Key words: angiotensin II, liver metastasis formation, Kupffer cells, TGF-β

Introduction

Colorectal cancer (CRC) is one of the most common cancers related to mortality.1 The liver is the most common site of metastases of tumor sites that drain initially via the portal circulation.2 Treatment of colorectal liver metastases requires a multidisciplinary approach that may involve such approaches as chemotherapy, surgery, interventional radiology, and portal vein embolization.3 Though only 5−10% of patients with colorectal liver metastases were resectable to date, with the advances in diagnostic methods and new therapies, resectability rates have increased up to 25%.4 Angiotensin II (ANG II) is a multifunctional bioactive peptide, and ANG II causes vasoconstriction and subsequently increases blood pressure.5 ANG II exerts its actions through ANG II type 1 receptor (AT1) and ANG II type 2 receptor (AT2). ANG II also stimulates neovascularization, which is a requirement for tumor growth and stimulates cell proliferation.6

Angiotensin-converting enzyme inhibitors (ACEIs) and AT1 blockers (ARBs) (or AT1 antagonists) are two classes of commonly used antihypertensive agents that mediate their effect by the renin-angiotensin system (RAS). Several reports showed that treatment of patients with ACEIs and ARBs decreases the risk of CRC or overall cancer.7-10 Recently, using AT1-antagonist and AT1-deficient mice, we have shown that the blocking of AT1 signaling inhibits growth of tumor cells and lung metastasis formation.11,12
RAS is also known for having a significant role in liver morphology and function. Increased activity of the ACE/ANG II/AT1 axis is related to the induction of fibrosis. Previous reports showed that ANG II regulates liver fibrosis through AT1 signaling. ANG II induces fibrogenic action via AT1 located in Kupffer cells (KCs), resulting in development of liver fibrosis such as cirrhosis. Those findings lead us to formulate the hypothesis that ANG II induces the microenvironment of liver metastasis formation via AT1 by accumulating or proliferating KCs. Elucidate the mechanism of liver metastasis formation through ANG II/AT1 axis provides a new tool for prevention of recurrence of CRC.

**Materials and Methods**

For the liver metastasis model, mice were anaesthetized by intraperitoneal injection with pentobarbital sodium (50 mg/kg) for all the experiments, monitored by the disappearance of the pedal withdrawal reflex. The left flank hair was shaved, and the revealed skin was rubbed with ethanol pads. A 0.5 cm incision in the left flank was made adjacent to the spleen, as previously described. The CMT93 (ATCC® CCL-223™) cells were maintained in the culture medium DMEM (Sigma, Tokyo) and RPMI (Roswell Park Memorial Institute) 1640 medium (Sigma), containing 10% fetal bovine serum. These cancer cells (2.0 × 10^6) in 200 μl of phosphate-buffered saline (PBS) were slowly injected into the spleen of each rat using a 27 G needle. Five minutes after the injection, the spleen was removed and the incision was closed with surgical clips. An ACEI, lisinopril (100 mg/kg/d; Shionogi Pharmaceutical, Osaka), and an ARB (or AT1 antagonist), candesartan (100 mg/kg/d; Takeda Chemical Industries, Osaka), were orally administered daily. At 2 weeks after the CMT93 cells injection, the mice were euthanized with an intraperitoneal overdose of pentobarbital sodium (100 mg/kg). The liver was resected and weighed, and the metastatic area was measured.

**Immunohistochemical analyses**

Liver tissue was immediately fixed with 10% neutral buffered paraformaldehyde. After fixation, the tissue was dehydrated with a graded ethanol series and then embedded in paraffin. Each section (4 μ) of the paraffin-embedded tissue was mounted on glass slide, and either stained with hematoxylin-eosin or processed for immunohistochemistry. For the latter, sections were activated using Histo VT One (Nakarai Tesque, Yokohama) and then incubated overnight at 4℃ with one of the following primary antibodies; (a) anti-mouse F4/80 antibody (1:200, rat monoclonal, Santa Cruz Biotechnology, sc-52664, CA, USA), (b) anti-mouse TGF-β antibody (1:200, rabbit polyclonal, Abcam, ab92486), (c) anti-mouse CD31 antibody (1:200, rabbit polyclonal, Abcam, ab28364), and (d) anti-mouse type I collagen antibody (1:100, rabbit polyclonal, Abcam, ab21286). For primary antibodies of (a) and (b), after washing in PBS, the sections were incubated for 2 hours at room temperature with Alexa Fluor 488-conjugated donkey anti-rabbit IgG (Molecular Probes; Eugene, OR, USA) and Alexa Fluor 594-conjugated donkey anti-rat IgG (Molecular Probes). For primary antibodies of (c) and (d), after being immersed in 3% solution of hydrogen peroxide (H₂O₂) for 3 minutes, the sections were incubated for 2 hours at room temperature with Alexa Fluor 488-conjugated donkey anti-rabbit IgG (Molecular Probes; Eugene, OR, USA) and Alexa Fluor 594-conjugated donkey anti-rat IgG (Molecular Probes). For primary antibodies of (c) and (d), after being immersed in 3% solution of hydrogen peroxide (H₂O₂) for 30 minutes, the sections were incubated for 30 minutes at room temperature with N-Histofine Simple Stain Mouse MAX PO (Nichirei Bioscience, Tokyo) and were immersed in 0.02% 3,3'-diaminobenzene and 0.3% nickel ammonium sulfate in 50 mM Tris-HCl buffer (pH 7.4) containing 0.005% H₂O₂ for 3 minutes. Images were captured with a light microscope and a fluorescence microscope (Biozero BZ-9000 Series; Keyence, Osaka).

**Real-time polymerase chain reaction (PCR) analysis**

Transcripts encoding F4/80, TGF-β CD31, collagen type I alpha 1 (Col1a1), and GAPDH were quantified by real-time polymerase chain reaction (PCR) analysis. Total RNA was extracted from liver tissues with TRIzol reagent (Gibco-BRL, Life Technologies, Rockville, MD, USA), and single-stranded cDNA was generated from 1 μg of total RNA via reverse transcription with ReverTra Ace (Toyobo, Osaka). Quantitative PCR was performed with SYBR Premix Ex Taq (Takara Bio, Shiga). The real-time PCR primers were designed using Primer 3 software (http://primer3.sourceforge.net/) based on data from GenBank (https://www.ncbi.nlm.nih.gov/Genbank/). The following primers were used for real-time PCR:

5'-TCTTTTCTCCCTGGCTTCTC-3' (sense) and 5'-CACCACCTTCAGGTCTTCAC-3' (antisense) for F4/80;
5'-AACATCCCTGGCGTTACCTT-3' (sense) and 5'-TGTATTCCGTCCTTTGGGTTTC-3' (antisense) for TGF-β;
5'-ACTTCTGAACTCCAACAGCGA-3' (sense) and 5'-CCATTTCTGTTGGGGTCTTTAT-3' (antisense) for CD31;
5'-AGGCATAAAAGGTCATCGTG-3' (sense) and 5'-GACGCTTGGATCCCCTGTTG-3' (antisense) for Col1a1;
5'-CCAATGAGTAGGCTGGAGAG-3' (sense) and 5'-AAAGTGAGAGGTTGAGGATT-3' (antisense) for GAPDH. Data were normalized to the level of GAPDH in each sample.
Cell cultures
Murine KC line (KUP5) was seeded on 12-well chamber glass slides (#354118, Corning) at the density of $1 \times 10^5$ cells/well with the growth medium. ANG II ($10 \mu M$) was added into serum-free media for 3 hours.

Proliferation assays
A total of $2.5 \times 10^5$ cells/wells KUP5 was suspended in 96 wells. ANG II ($0.1 \mu M$) was added into the serum-free media for 24 hours. Cell proliferation was monitored and analyzed using IncuCyte ZOOM™ (St. Andrews, KY, USA) cell proliferation assays, and the percentage of the occupied areas of the cells was calculated.

Statistical analyses
Data are expressed as means ± SD. All statistical analyses were performed using the Graph-Pad version 5.01 (La Jolla, CA, USA). Statistical comparisons between the two groups were made by Student’s t-test. Comparisons of more than two groups were analyzed using one-way ANOVA. A P value of <0.05 was considered to indicate statistical significance.

Results
ACEI and ARB suppressed liver metastasis formation. We examined the effect of ACEI and ARB (or AT1

![Figure 1. Induction of liver metastasis formation by ANG II](image)

A. Typical appearance of liver metastasis formation macroscopically. The metastatic area is outlined in yellow and marked with a T.
B. Typical appearance of H&E staining of liver metastasis formation. Metastatic area is outlined in black and marked with a T. Scale bar = 100 μm.
C. The metastatic area (cm²) was smaller in ACEI- and ARB-treated mice at 2 weeks after inoculating tumor cells. Data are expressed as the means ± SD of 6 mice per group. *P < 0.05 vs. vehicle-treated mice by Student’s t-test.
antagonist) on liver metastasis formation following intrasplenic injection of CMT93 cells into mice. Both macroscopically (Figure 1A) and histologically (Figure 1B), the appearance of liver metastasis formation was suppressed in the ACEI- and ARB-treated mice. Furthermore, compared to the vehicle-treated mice, the metastatic area was significantly suppressed in the ACEI- and ARB-treated mice (Vehicle: 2.11 ± 0.199, ACEI: 0.51 ± 0.14, ARB: 0.54 ± 0.12, n = 6, P < 0.05) (Figure 1C). These results indicated that ANG II induced liver metastasis formation via AT1 signaling.

**ACEI and ARB inhibited the accumulation of KCs in the metastatic area.**

Infiltration of macrophages in the liver stimulates tumor growth, migration, and invasion. KCs, tissue-fixed macrophages located in the sinusoids of the liver, have an essential role in the liver immune system and inflammation. F4/80-positive KCs in the sinusoids and blood-derived immature macrophages induce liver metastasis by forming fibrosis. Based on these previous reports, we first, determined the expression of mRNA levels of F4/80 in the liver. Compared to the vehicle-treated mice, the expression of F4/80 was significantly

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**Figure 2.** ANGII/AT1 axis enhanced TGF-β expression in F4/80-positive KCs.

A. The gene expression of F4/80 in the liver 2 weeks after inoculating tumor cells were suppressed in mice treated with ACEI or ARB compared to only with the vehicle. Data are expressed as the means ± SD of 4 mice per group. *P < 0.05 vs. vehicle-treated mice by Student's t-test

B. The gene expression of TGF-β in the liver 2 weeks after inoculating tumor cells suppressed in mice treated with ACEI or ARB compared to vehicle. Data are expressed as the means ± SD of 4 mice per group. *P < 0.05 vs. vehicle-treated mice by Student's t-test

C. Double immunofluorescence staining against TGF-β (green) and F4/80 (red) in the liver metastatic area at 2 weeks after inoculating tumor cells. The metastatic area is outlined in white and marked with a T. Scale bar = 100 μm
suppressed in the ACEI- and ARB-treated mice (Figure 2A). TGF-β is known as a regulator for tumor metastasis formation. The expression of TGF-β in the liver was significantly suppressed in the ACEI- and ARB-treated mice (Figure 2B). To determine, whether or not TGF-β is expressed on F4/80-positive KCs, immunohistochemical analyses for TGF-β and F4/80 were performed. Compared to the vehicle-treated mice, the numbers of TGF-β/F4/80-positive KCs were diminished in the ACEI- and ARB-treated mice (Figure 2C). These results suggested the ACE/ANG II/AT1 axis induced the accumulation of TGF-β/F4/80 positive KCs in liver metastases.

**ANG II enhanced the expression of TGF-β in KCs.**
To determine whether or not KCs induce TGF-β expression, in vitro studies were done using the murine KC line, KUP5 cells, under ANG II treatment. We confirmed that KUP5 cells express AT1 (data not shown). The gene expression of TGF-β in KUP5 cells was enhanced by ANG II treatment compared to the control (Figure 3A).

**ANG II induced the proliferation of KCs.**
That ANG II has an effect on the proliferation of cells including tumor cells, endothelial cells, and hepatic stellate cells is well documented. We tried to determine whether or not ANG II induces KC proliferation. The proliferation of KUP5 was significantly enhanced under treatment of ANG II compared to the control at 24 hours (control: 21.67 ± 0.69%, ANG II: 23.21 ± 0.29%, P < 0.05) (Figure 3B). These results indicated that ANG II has an effect on the proliferation of KCs.

**ANG II promoted angiogenesis in liver metastases.**
Because tumor metastasis formation depends on angiogenesis, we tried to determine whether or not ANG II-induced liver metastasis was dependent on angiogenesis. The mRNA level expression of CD31, specific marker for endothelial cells, in the liver was significantly suppressed in the ACEI- and ARB-treated mice compared to the vehicle-treated mice (Figure 4A). Furthermore, immunohistochemical analysis of CD31 showed that CD31-positive cells in the metastasis areas were significantly fewer than those in the ACEI- and ARB-treated mice compared to the vehicle-treated mice (Figure 4B).

**ANG II induced the type I collagen expression in the metastatic area.**
It has been shown that RAS induces liver fibrosis via the ANG II/AT1 axis. Fibroblastic tissue is important for metastasis formation. Fibrosis is characterized by the presence of an excess of fibrous connective tissue in an organ particular by deposition of type I collagen. To investigate whether or not the deposition of type I collagen is related to metastasis formation, real-time PCR analyses and immunohistochemical analyses were performed.

The expression of collagen in the liver was significantly suppressed in the ACEI- and ARB-treated mice compared to that in the vehicle-treated mice (Figure 5A). Furthermore, immunohistochemical analyses of type I collagen showed that type I collagen-positive cells were located in the metastatic areas in the vehicle-treated mice but were significantly diminished in the ACEI- and ARB-treated mice (Figure 5B). These results suggested that type I collagen was enhanced by the ANG II/AT1 axis.

**Discussion**
The objective of the present study was to elucidate the
Figure 4. ANG II/AT1 axis induced metastasis formation by angiogenesis.

A. The gene expression of CD31 in the liver 2 weeks after inoculating tumor cells. Data are expressed as the means ± SD of 4 mice per group.

B. Immunohistochemical staining of CD31 in liver metastases 2 weeks after inoculating tumor cells. Yellow arrows indicate CD31-positive cells. The metastatic area is outlined in black and marked with a T. Scale bar = 100 μm

Figure 5. ANG II/AT axis induced deposition of type I collagen in metastasis area.

A. The gene expression of type I collagen in the liver 2 weeks after inoculating tumor cells. Data are expressed as the means ± SD of 4 mice per group. *P < 0.05 vs. vehicle-treated mice by Student's t-test

B. Immunohistochemical staining of type I collagen in the liver metastatic area 2 weeks after inoculating tumor cells. Metastatic area is outlined in black and marked with a T. Scale bar = 100 μm
role of ANG II/AT1 signaling in liver metastasis formation from CRC. This study showed that liver metastasis formation was suppressed by treatment with ACEI and ARB (or AT1 antagonist). Furthermore, we showed liver metastasis formation was induced by not only the accumulation of F4/80+ KCs expressing TGF-β but also by the enhancement of angiogenesis and type I collagen deposition. These results indicated that liver metastasis formation depend on the ANG II/AT1 axis.

RAS is a complex network for enzymes and peptides that regulates blood pressure. Experimentally, ANG II enhances tumor cell proliferation and angiogenesis via AT1. In vitro studies have shown that ANG II induces the expression of vascular endothelial growth factor in several solid tumors, including those of the neck and head, ovary, prostate, pituitary gland, and kidney. We also have reported that the AT1 blockade inhibits angiogenesis in murine Lewis lung tumours and lung metastasis formation in melanoma cells. Shimomoto et al. showed that suppressed ANG II production in the liver inhibited liver metastasis formation. Consistent with those, the present study showed that liver metastasis formation was suppressed in the ACEI- and ARB-treated mice, indicating that ANG II induces liver metastasis formation via AT1.

KCs are known as specialized macrophages in the liver. KCs produce a wide variety of molecules, such as growth control mediators, inflammatory agents, proteolytic and hydrolytic enzymes, oxygen and nitrogen species, and lipid metabolites. F4/80 antigen is a mature mouse cell surface glycoprotein expressed at high levels on various macrophages including KCs, splenic red pulp macrophages, microglia, gut lamina propria, and Langerhans cells in the skin. KCs are present around tumors, and TGF-β was detected in the stroma cells of tumors. Activated the KC secret TGF-β that is required for liver fibrosis, and the blunting of TGF-β signaling reduces fibrogenesis.

In carcinogenesis, TGF-β signaling is redirected to epithelial-to-mesenchymal transition. TGF-β may favor cancer progression and metastasis. In the present study, the gene expression of F4/80 and TGF-β in the metastatic liver was attenuated in the ACEI- and ARB-treated mice. Furthermore, double immunostaining showed that F4/80+ KCs co-expressed with TGF-β and those double-positive cells were suppressed in the ACEI- and ARB-treated mice. These results indicated that the accumulation of F4/80+ KCs expressing TGF-β in the metastatic areas depended on the ANG II/AT1 axis.

That KCs express ANG II receptors, AT1, and AT2 has previously been shown. To clarify whether or not ANG II induces the expression of TGF-β and proliferation of KCs, we used KUP5, the murine KC line. In preliminary studies, we confirmed that KUP5 cells predominantly expressed AT1 and AT2. In the present study, treatment with ANG II enhanced expression of TGF-β in KUP5 cells and proliferation of KUP5 cells. These results suggested that ANG II induces expression of TGF-β in KCs and KC proliferation via AT1.

TGF-β plays an important role as a mediator of interactions between stromal cells and tumor cells. TGF-β regulates the tumor microenvironment that includes fibroblasts, immune cells, and cells that comprise the blood vessels. Indeed, TGF-β has an effect on angiogenesis, a phenomenon grows new vessels from the existing vessels. The present study showed that expression of CD31 in metastatic areas was significantly suppressed in the ACEI- and ARB-treated mice. TGF-β is known as a key player in fibrogenesis via promoting activation and proliferation of resident fibroblasts, which leads to excessive synthesis of type I collagen. It was already reported that the AT1 receptor in KCs plays a role in regulating fibrogenic factors including TGF-β and fibronectin, thus presumably activating the hepatic stellate cells. ANG II exerts its effects directly by stimulating TGF-β1 production and by triggering fibroblast proliferation and differentiation into collagen-secreting myofibroblasts.

The present study showed that the expression of type I collagen was significantly suppressed in the ACEI- and ARB-treated mice. These results supported that liver metastasis was due to expression of type I collagen, which was dependent on AT1 signaling. Taken together, the ANG II/AT1 axis induces liver metastasis by fibrogenesis. But the precise mechanism by which ANG II interacts with KCs and induces fibrosis remains unclear. Therefore, further experiments are warranted using those subtype-deficient mice.

In conclusion, the present study showed that AT1 signaling plays an essential role in liver metastasis by proliferated KCs secreting TGF-β which is involved in epithelial-to-mesenchymal transition. Therefore, AT1 antagonist may be a useful therapy to prevent tumor recurrence and liver metastasis from CRC.

References


