

## Therapeutic and Analysis Model of Intrahepatic Metastasis Reflects Clinical Behavior of Hepatocellular Carcinoma

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This study was designed to establish an intrahepatic metastasis model to investigate the biology and therapy of hepatocellular carcinoma (HCC) in mice. A fragment of mouse HCC tumor CBO140C12 was orthotopically implanted into the mouse liver. The number of intrahepatic metastatic colonies and the volume of the implanted tumor increased in a time-dependent manner. At 28 days after fragment implantation, all mice showed intrahepatic metastasis. Intravenous administrations of cisplatin and doxorubicin at 7 and 21 days after the implantation significantly suppressed the growth of the primary tumor nodule, but tended to inhibit intrahepatic metastasis. However, a marked decrease of body weight was observed during the experiment. On the other hand, an inhibitor of matrix metalloproteinases (MMPs), ONO-4817, decreased the gelatinase activity of MMP-9 secreted by CBO140C12 cells, and significantly reduced the number of colonies of intrahepatic metastasis when administered orally. Our established model, which is focused on intrahepatic metastasis, is suitable for evaluating the therapeutic effect of HCC and for analyzing intrahepatic metastasis, because this model reflects the clinical features of HCC and all the steps of tumor metastasis.

Key words: Intrahepatic metastasis — Hepatocellular carcinoma — Metastasis model — Orthotopic implantation — MMP inhibitor

Despite recent advances in imaging techniques for the diagnosis of hepatocellular carcinoma (HCC), improvements in surgical procedures, and the introduction of new therapies such as transarterial embolization and percutaneous ethanol injection therapy, the prognosis of patients with HCC remains relatively poor.<sup>1)</sup> The reason for this is the high frequency of recurrence within the liver.<sup>2)</sup> Intrahepatic metastasis is one of the modalities of recurrence within the liver in HCC,<sup>3)</sup> and is known to occur more frequently than extrahepatic metastasis. It has been generally accepted that intrahepatic metastasis, as well as venous invasion and primary tumor size, are important risk factors for intrahepatic recurrence of HCC.<sup>2,4–6)</sup> Therefore, there is a need for biological and therapeutic studies of intrahepatic metastasis of HCC. To do this, animal models that can confirm clinical behavior are necessary to clarify the mechanism of intrahepatic metastasis, to evaluate the efficacy of new drugs and to search for new therapies.

Several studies have shown that orthotopic implantation of tumor cells into the relevant organ of mice can provide an *in vivo* model to study the biology and therapy of these cells.<sup>7,8)</sup> Indeed, models of orthotopic implantation of tumor cells have been developed for lung cancer,<sup>9)</sup> colon cancer,<sup>10)</sup> pancreatic cancer<sup>11)</sup> and also HCC.<sup>12,13)</sup> However,

orthotopic implantation of cell suspensions may induce changes in the nature and biological behavior of the original tumor.<sup>14)</sup> Considering these points, Fu *et al.*<sup>15)</sup> have constructed metastasis models of colon cancer produced by orthotopic implantation of histologically intact tumor tissue and they reported that the model could show the variety of clinical behaviors that occur in human subjects.

In the present study, we attempted to establish a model for intrahepatic metastasis that reflects the clinical features of HCC by orthotopic implantation of a fragment of mouse HCC tumor. In addition, to see whether this model was useful for evaluating the therapeutic effect and for analyzing the mechanism of intrahepatic metastasis, we examined the therapeutic efficacy of anti-cancer drugs and a matrix metalloproteinase (MMP) inhibitor ONO-4817<sup>16)</sup> in this model.

### MATERIALS AND METHODS

**Mice** Five-week-old, specific-pathogen-free female B6C3F1 mice were purchased from Japan SLC, Hamamatsu. The mice were maintained in the Laboratory for Animal Experiments, Institute of Natural Medicine, Toyama Medical and Pharmaceutical University, under laminar air-flow conditions with a 12-h light and dark cycle at a temperature of 22–25°C. All animals had free access to standard laboratory mouse food and water. This study was

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conducted in accordance with the standards outlined in the Guidelines for the Care and Use of Laboratory Animals of Toyama Medical and Pharmaceutical University.

**Cells** A mouse HCC cell line, CBO140C12, was kindly provided by Dr. K. Ogawa (Department of First Pathology, Asahikawa Medical College). These cells were maintained as monolayer cultures in Dulbecco's minimal essential medium/F-12 (D-MEM/F-12) (GIBCO BRL, Life Technologies, Inc., NY) supplemented with 10% fetal bovine serum (FBS), L-glutamine and 2 g/liter glucose.

**Chemicals** Cisplatin (CDDP, *cis*-diamminedichloroplatinum; "randa," 0.5 mg/ml) was purchased from Nippon Kayaku Co., Ltd., Tokyo. Doxorubicin hydrochloride (DOX, (2*S*,4*S*)-4-(3-amino-2,3,6-trideoxy- $\alpha$ -L-lyxo-hexopyranosyloxy)-2-glycero-1,2,3,4-tetrahydro-2,5,12-trihydroxy-7-methoxy-6,11-naphthacenedione monohydrochloride; "Adriacin," 10 mg) was purchased from Kyowa Hakko Industries, Ltd., Tokyo. ONO-4817 (an inhibitor of MMPs; (2*S*,4*S*)-*N*-hydroxy-5-ethoxymethyloxy-2-methyl-4-(4-phenoxybenzol)aminopentanamide) was kindly provided by ONO Pharmaceutical Co., Ltd. For *in vivo* experiments, ONO-4817 was suspended in 0.5% hydroxypropoxyl methylcellulose. For *in vitro* experiments, ONO-4817 was dissolved in dimethyl sulfoxide at a concentration of 20 mM for the stock solutions, and kept at  $-20^{\circ}\text{C}$  until use.

**Procedure for orthotopic implantation of mouse HCC tumor fragments** Log-phase cultures of CBO140C12 cells were harvested with 1 mM EDTA in phosphate-buffered saline (PBS), washed three times with serum-free D-MEM/F-12, and suspended in D-MEM/F-12 to a final concentration of  $1 \times 10^7$  cells/ml. The cell suspensions of CBO140C12 (200  $\mu\text{l}$ /mouse) were injected subcutaneously into the upper abdomen of B6C3F1 mice. After 3 weeks, solitary tumors were obtained at each injected site. The necrotic and non-cancerous tissues were removed and then the tumor was divided into fragments of approximately 1 mm<sup>3</sup>. A small incision in the left upper abdomen was made and the left lobe of the liver was carefully exposed under ether anesthesia. A fragment of CBO140C12 tumor was implanted using an implanting needle (Natsume Co., Ltd., Tokyo) into the left liver lobe of B6C3F1 mice. To stop any bleeding, a cotton swab was pressed on the site of the puncture for approximately 30 s after the implanting needle was withdrawn. The liver was then returned to the peritoneal cavity and the skin incision was closed with a surgical skin clip.

**Macroscopic findings and histopathological study** Mice were sacrificed at various periods (on day 7, 14, 21 and 28) after fragment implantation. In the survival time experiment, the mice were examined frequently for health status in accordance with the United Kingdom Co-ordinating Committee on Cancer Research (UKCCCR) Guidelines<sup>17</sup>; any moribund mice were sacrificed and any deaths recorded. The number of visible colonies (exclud-

ing the tumor nodule at the implanted site) in the liver and the long and short diameters of the tumor mass at the implanted site were measured manually. The tumor volume was calculated by use of the following formula: tumor volume (mm<sup>3</sup>) =  $1/2 \times (\text{long diameter}) \times (\text{short diameter})^2$ . After the evaluation, the samples were fixed in 4% paraformaldehyde and stained with hematoxylin and eosin for histopathological analysis.

***In vivo* treatment with CDDP, DOX and ONO-4817** CDDP and DOX were administered intravenously (*i.v.*) at the maximum tolerated dose (MTD) of 10 mg/kg<sup>18</sup> and 12 mg/kg<sup>18</sup> at 7 and 21 days after the orthotopic implantation of the tumor fragment, respectively. ONO-4817 was administered orally three times on day 0 and then twice a day for 27 days, starting on day 1 after the implantation. Mice were sacrificed on day 28 after fragment implantation, and then the number of visible colonies in the liver and the long and short diameters of the tumor mass at the implanted site were measured manually.

**Gelatin zymography** To prepare the tumor-conditioned medium, CBO140C12 cells were grown to subconfluence in 6-well tissue culture plates in a 1:1 mixture of D-MEM/F-12 containing 10% FBS. After several washes with serum-free D-MEM/F-12, the medium was replaced with D-MEM/F-12 containing 0.1% BSA and the culture was continued for a further 24 h. The culture supernatants were collected and centrifuged to remove debris. The conditioned media were used to detect gelatinase activity. Gelatin zymograms were performed according to the method reported by Heussen and Dowdle, with some modifications.<sup>19</sup> Briefly, samples were applied to 7.5% polyacrylamide gel containing 0.1% sodium dodecyl sulfate (SDS) and 0.1% gelatin at 4°C. After electrophoresis, gels were washed twice with a rinsing buffer containing 50 mM Tris-HCl, 2.5% Triton X-100, 5 mM CaCl<sub>2</sub>, 1  $\mu\text{M}$  ZnCl<sub>2</sub> and 0.05% NaN<sub>3</sub> at room temperature for 1 h to remove SDS. Gels were incubated with the incubation buffer (50 mM Tris-HCl, 5 mM CaCl<sub>2</sub>, 1  $\mu\text{M}$  ZnCl<sub>2</sub> and 0.05% NaN<sub>3</sub>) in the presence or absence of ONO-4817 for 24 h at 37°C, and stained with 0.1% Coomassie blue, 10% acetic acid and 10% isopropanol, destained in 10% acetic acid and 10% isopropanol, and dried. Enzyme-digested lesions were identified as white bands against a blue background.

**Statistical analysis** The statistical significance of differences between the groups was determined by applying Student's two-tailed *t* test.

## RESULTS

**Intrahepatic metastasis by orthotopic implantation of a fragment of CBO140C12 tumor** To investigate growth at the inoculated site and intrahepatic metastasis, a fragment of murine HCC (CBO140C12) was orthotopically implanted into the liver under ether anesthesia. The opera-

tive mortality was less than 5% and the mean survival time of the mice successfully implanted with a CBO-140C12 tumor fragment was  $39.2 \pm 6.6$  (range 32–56) days. The tumor colonies other than the implanted tumor in the liver were visible to the naked eye on days 14, 21 and 28 after orthotopic implantation (Fig. 1). Most of the intrahepatic metastatic colonies were observed in the implanted left lobe. In some cases, a few tumor colonies were detected in other lobes of the liver. Histopathological determination was performed using the resected liver on day 28 after implantation. The metastatic colonies in the liver were distant from the tumor nodule at the implanted site and developed in areas of the portal vein (Fig. 2A). Furthermore, vascular tumor thrombosis was also observed in other sections (Fig. 2B). The incidences of intrahepatic metastasis were 0, 14.3, 85.7 and 100% on days 7, 14, 21 and 28 after the implantation, respectively (Table I). As shown in Fig. 3, the number of metastatic colonies in the liver increased in a time-dependent manner, and was 2,  $5.2 \pm 1.7$  and  $11.0 \pm 6.0$  on day 14, 21 and 28 after the implantation, respectively. The tumor volumes at the implanted sites were  $2.1 \pm 0.7$ ,  $14.5 \pm 7.6$ ,  $78.1 \pm 19.3$  and  $157.4 \pm 27.2$  mm<sup>3</sup> on day 7, 14, 21 and 28 after the implantation, respectively (Fig. 3B). On day 28 after implantation, 2 out of 7 mice showed lung metastasis.

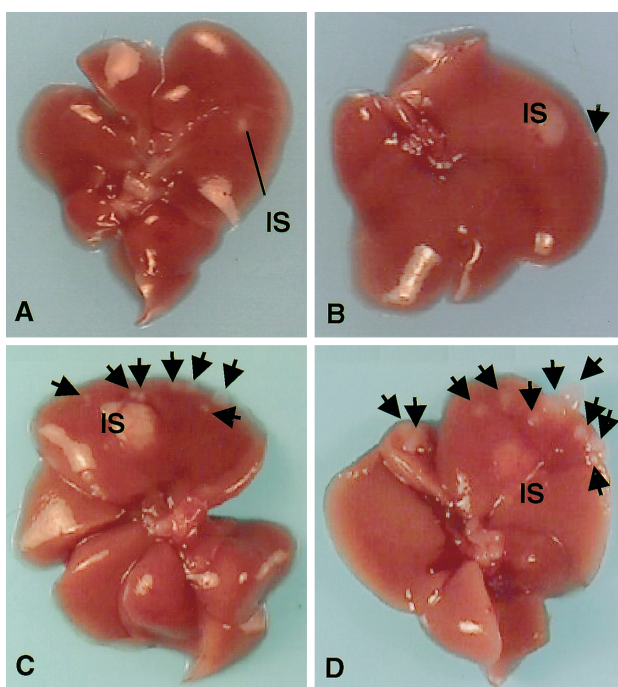


Fig. 1. Macroscopic findings of resected liver on days 7 (A), 14 (B), 21 (C) and 28 (D) after orthotopic implantation of a fragment of CBO140C12 tumor. IS, a tumor nodule at the implanted site; arrow, intrahepatic metastatic colony.

However, the tumor colonies in the lung were very small and numbered only one or two per mouse (data not shown). **Effect of CDDP and DOX on growth at the implanted site and intrahepatic metastasis after orthotopic implantation of a CBO140C12 tumor fragment** To evaluate the therapeutic efficacy of anti-cancer drugs in this model, mice were administered i.v. CDDP or DOX at the MTD on days 7 and 21 after the orthotopic implantation of a tumor fragment. Administration of CDDP or DOX tended to reduce the number of tumor colonies in the liver as compared with the untreated controls, but the effects were not significant (Fig. 4A). The numbers of metastatic colonies in the livers of the control, CDDP-

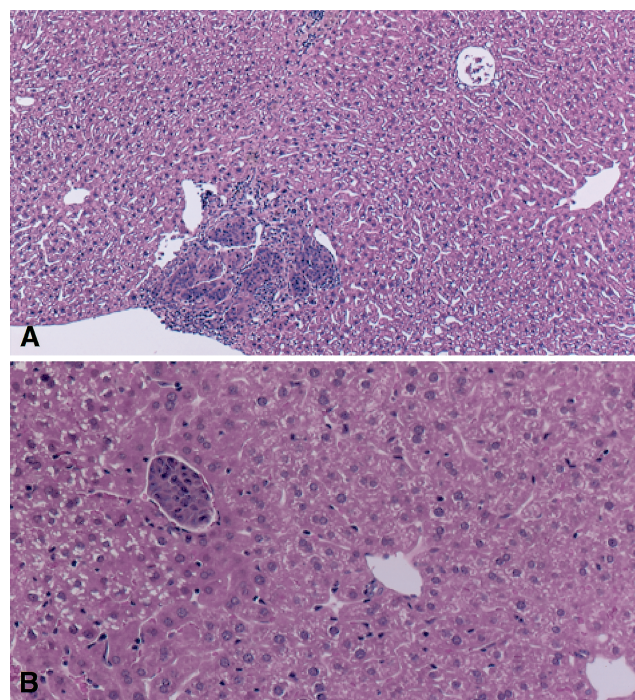


Fig. 2. Pathological findings of the small tumor colonies in the liver 28 days after orthotopic implantation of a fragment of CBO140C12 tumor. Tumor colonies developed at the portal vein area (A,  $\times 40$ ), and vascular tumor thrombosis was seen (B,  $\times 100$ ).

Table I. Incidence of Intrahepatic Metastasis after Orthotopic Implantation of a CBO140C12 Tumor Fragment

Days after implantation	7	14	21	28
Intrahepatic metastasis	0/7 <sup>a)</sup> (0) <sup>b)</sup>	1/7 (14.3)	6/7 (85.7)	7/7 (100)

a) Number of mice with intrahepatic metastasis/number of mice tested.

b) Number in parentheses represents % incidence of intrahepatic metastasis.

treated and DOX-treated groups were  $9.9 \pm 4.1$ ,  $6.8 \pm 5.3$  and  $5.8 \pm 6.0$ , respectively. The volume of the implanted tumor was significantly decreased by the administration of CDDP ( $P < 0.001$ ) or DOX ( $P < 0.01$ ). We also examined the body weight of controls, CDDP-treated and DOX-treated mice on various days after the implantation. The

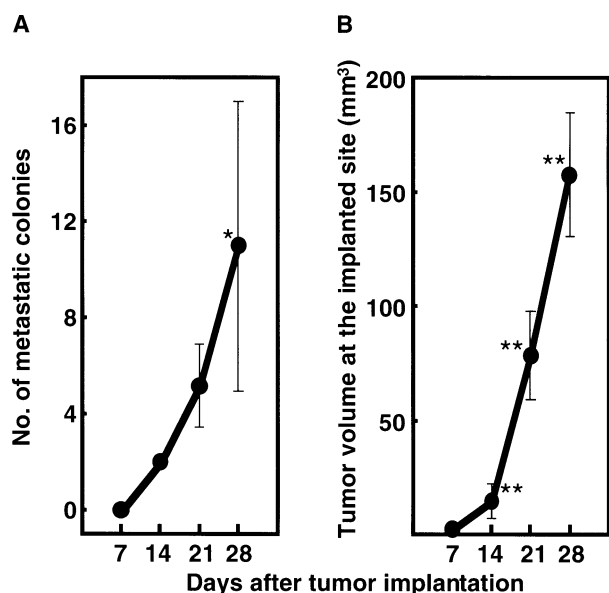


Fig. 3. Changes in the number of metastatic tumor colonies in the liver (A) and tumor volume at the implanted site (B) on various days after orthotopic implantation of a fragment of CBO140C12 tumor. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ .

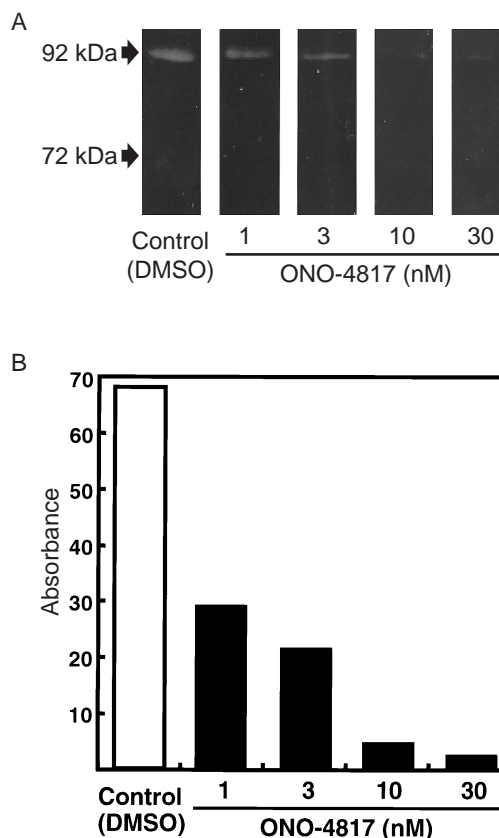


Fig. 5. Direct effect of ONO-4817 on the gelatinolytic activity of MMPs secreted by CBO140C12 cells (A). The gelatinolytic activity was quantified by the Master Scan Analysis System (B). Control, dimethylsulfoxide.

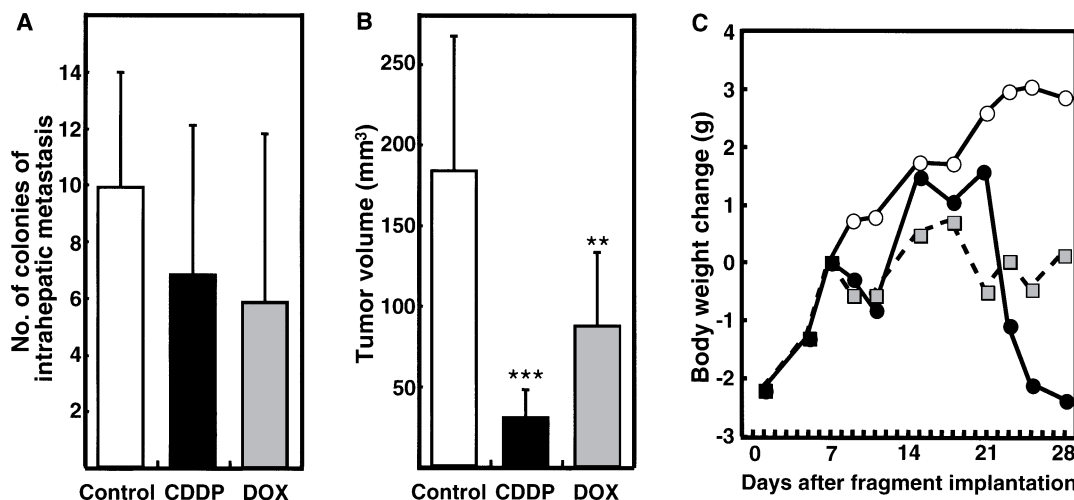


Fig. 4. Effect of CDDP and DOX on our model. A, The number of metastatic colonies in the liver; B, Tumor volume at the implanted site; C, Body weight change of mice. ○, control; ●, CDDP-treated; □, DOX-treated. \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$ .

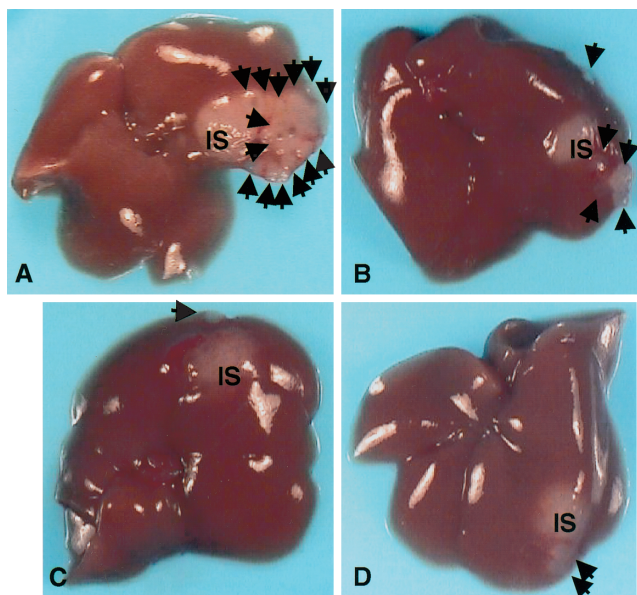


Fig. 6. Macroscopic findings of resected liver on day 28 after implantation. A, control; B, C or D, 50, 100 or 200 mg/kg ONO-4817, respectively. IS, a tumor nodule at the implanted site; arrow, intrahepatic metastatic colony.

body weight change was calculated by use of the following formula: Body weight change (g) = (Body weight on various days after the implantation) – (body weight on day 7 after the implantation, i.e. at the first injection of drugs). As shown in Fig. 4C, a marked decrease in body weight was observed after the administration of CDDP or DOX, as compared with untreated controls. Notably, a significant

loss of body weight was seen after the second administration of CDDP.

**Effect of ONO-4817, an inhibitor of MMPs, on the growth of the implanted tumor and intrahepatic metastasis after orthotopic implantation of a murine HCC fragment** We first investigated the effect of ONO-4817 in CBO140C12 tumors on the activity of MMPs, considered key molecules in the intrahepatic metastasis of HCC.<sup>20)</sup> As shown in Fig. 5, the gelatinase activity of MMP-9 secreted from CBO140C12 cells in a zymogram was inhibited by the addition of ONO-4817 in a concentration-dependent manner. Consecutive oral administrations of ONO-4817 for 4 weeks resulted in a significant inhibition of intrahepatic metastasis, as compared with the control group (Fig. 6). When ONO-4817 was administered at doses of 0, 50, 100 and 200 mg/kg, the numbers of metastatic colonies in the livers on day 28 after the implantation were  $9.5 \pm 6.1$ ,  $5.9 \pm 4.4$ ,  $3.3 \pm 2.4$  ( $P < 0.05$ ) and  $3.6 \pm 3.2$  ( $P < 0.05$ ), respectively (Fig. 7A). Tumor volumes at the implanted sites also decreased on administration of ONO-4817 (Fig. 7B). During this examination, there were no discernible differences in body weight change between the treated and non-treated groups (Fig. 7C).

## DISCUSSION

Tumor metastasis is a complex cascade of events and can be subdivided into sequential steps.<sup>21–24)</sup> Since many molecules such as cadherins, MMPs, integrins and vascular endothelial growth factor (VEGF) are positively and/or negatively involved in the development of tumor metastasis, they are potential targets for therapy to control tumor

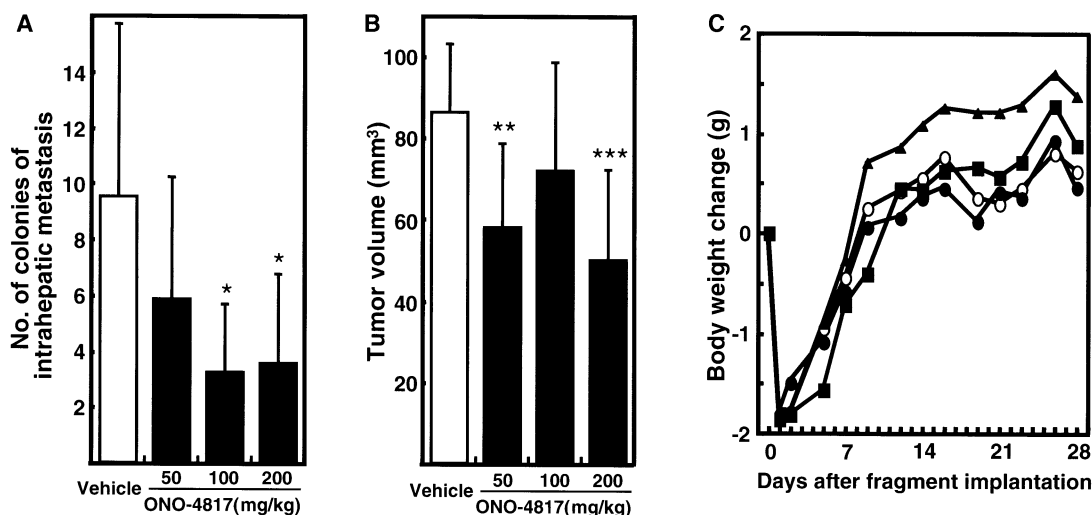


Fig. 7. Effect of oral administration of ONO-4817 on our model. A, The number of metastatic colonies; B, Tumor volume at the implanted site; C, Body weight change of mice. ○, control; ●, ■ or ▲, 50, 100 or 200 mg/kg ONO-4817, respectively. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$ .

metastasis. If the expression of some of these molecules were controlled, tumor metastasis might be inhibited. Therefore, tumor metastasis models which feature all the processes of tumor metastasis along with its clinical behavior are required to analyze the mechanisms involved and to search for new therapies.

Several papers have described models of intrahepatic metastasis produced by implantation of a tumor cell suspension into mouse spleen or liver.<sup>12, 13, 25, 26)</sup> It is thought that these models, however, do not include all steps of cancer metastasis, such as release from the primary tumor, invasion of surrounding tissues or extravasation. Recently, Sun *et al.*<sup>27)</sup> have reported a metastatic model of human HCC, not a model of intrahepatic metastasis. Although this model was established in nude mice by fixing histologically intact tissues within the liver, lung metastasis was seen in all cases, as well as intrahepatic metastasis. Therefore, a model for intrahepatic metastasis of HCC that reflects the clinical behavior has been required to analyze the mechanism of intrahepatic metastasis and to evaluate the efficacy of new therapies against intrahepatic metastasis.

In the present study, we focused our attention on inducing intrahepatic metastasis by orthotopic implantation of a small fragment of murine HCC. In our model, the incidence of intrahepatic metastasis was 100%, whereas that of lung metastasis was 28.6% on day 28 after the implantation. The metastatic colonies were visible in the area surrounding the implanted tumor as satellite nodules (see Fig. 1). In addition, vascular tumor thrombosis and development of tumor in the portal vein area were observed on histopathological examination. These findings suggest that the lesion leading to metastatic colonies in this model using a tumor fragment can be defined as intrahepatic metastasis,<sup>28, 29)</sup> and our model may reflect the clinical features of HCC. Also, the intrahepatic metastasis in our model depends upon the metastatic ability of the CBO140C12 tumor, not upon the implant procedure, because the implantation of a fragment of other murine HCC caused growth at the implanted site, but did not yield any intrahepatic metastasis.<sup>20)</sup> We are now investigating the metastatic behavior of other types of cell lines not related to HCC by means of the same implantation procedure. This may be useful for clarifying the mechanisms of intrahepatic metastasis of HCC.

To evaluate whether our model of intrahepatic metastasis is useful for determining the therapeutic potential of anticancer drugs, we also investigated the effect of anticancer drugs, CDDP and DOX, which have a well known anti-tumor mechanism, on primary tumor growth and intrahepatic metastasis in the model. Administration of the MTD of CDDP and DOX on days 7 and 21 after orthotopic implantation of a fragment of CBO140C12 tumor resulted in a significant inhibition of primary tumor

growth, but only tended to inhibit intrahepatic metastasis (Fig. 4). Since CDDP and DOX exhibit antineoplastic activity by virtue of binding to DNA and disrupting template functions,<sup>30, 31)</sup> the inhibition of intrahepatic metastasis by these drugs may be primarily due to the suppression of tumor growth at implanted and metastatic sites, and not to direct inhibition of intrahepatic spread. Also, administration of the MTD of CDDP and DOX caused a loss of body weight in the treated mice, as compared with untreated controls. Although further study will be needed to investigate the significantly different effects of drugs on intrahepatic metastasis and implanted tumor growth, the relationship of these data with clinical observation is interesting. In addition, the body weight change in the mice during administration seems to be a good parameter for evaluating the side effects of the drug administration. This model will be useful when many mice are to be used to evaluate the efficacy of new drugs, because the procedure for implantation in this model is straightforward and the whole process can be performed without bleeding within 2 min per mouse. Furthermore, this model has several benefits for therapeutic examination, such as syngeneic and immunocompetent features and low operative mortality.

We also examined the effect of an MMP inhibitor (ONO-4817) in the model to see whether this model was useful for analysis of the molecular mechanism of intrahepatic metastasis. Previous studies have examined the efficacy of synthetic MMP inhibitors on tumor growth and metastasis using animal models.<sup>32, 33)</sup> Wang *et al.*<sup>34)</sup> revealed that the model of orthotopic implantation of histologically intact tissues could be adapted to examine the therapeutic efficacy of MMP inhibitors on the process of malignant progression as a whole compared with implantation of cell suspensions. ONO-4817 is a derivative of hydroxamic acid and is being developed as an orally active MMP inhibitor. It was shown to inhibit the activities of MMP-2, 3, 8, 9, 12, and 13, but not the activity of MMP-1 or 7.<sup>16)</sup> A previous clinicopathological study has found that the activity of MMP-9 is closely involved in capsular infiltration of HCC.<sup>35)</sup> Recently, we have shown that the expression of MMP-9 in CBO140C12 cells was partly associated with intrahepatic metastasis.<sup>20)</sup> As shown in Figs. 5 and 7, oral administration of ONO-4817 decreased the number of intrahepatic metastatic colonies partly through the down-regulation of MMP-9 activity in CBO140C12 cells. These results strongly suggest not only that tumor-related enzymes, including MMP-9, are involved in the formation of intrahepatic metastasis, but also that our model will be useful for analyzing the molecular mechanism of intrahepatic metastasis.

We have here established a model for the formation of a solitary primary nodule and intrahepatic metastasis by orthotopic implantation of a fragment of CBO140C12 liver tumor. Considering the clinical features of HCC, this

model may provide a basis for evaluating the efficacy of anti-cancer drugs against tumor growth and intrahepatic metastasis and their adverse effects, and for analyzing the mechanism of intrahepatic metastasis in HCC.

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