A hyaluronan synthase suppressor, 4-methylumbelliferone, inhibits liver metastasis of melanoma cells

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Abstract 4-Methylumbelliferone (MU) inhibits the cell surface hyaluronan (HA) formation, and that such inhibition results in suppression of adhesion and locomotion of cultured melanoma cells. Here, we examine the effect of MU on melanoma cell metastasis in vivo. MU-treated melanoma cells showed both decreased cell surface HA formation and suppression of liver metastasis after injection into the mice. Oral administration of MU to mice decreased tissue HA content. These HA knock-down mice displayed suppressed liver metastasis. Thus, both cell surface HA of melanoma cells and recipient liver HA can promote liver metastasis, indicating that MU has potential as an anti-metastatic agent.

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1. Introduction

Metastasis, a clinical and histological feature of malignant tumor cells, is initiated by detachment of tumor cells from the primary lesion. These cells enter the vascular system and finally become established in remote tissues [1]. Metastasis worsens the clinical prognosis of the patient and, in spite of improvements in therapeutic methods such as surgery, radiotherapy, chemotherapy and immunotherapy, frequently results in death from cancer. Therefore, blockade of metastasis is an important strategy to increase the survival of patients with malignant tumors and the development of more effective anti-metastatic agents is required [2].

Hyaluronan (HA) is a non-sulfated glycosaminoglycan (GAG) of high molecular mass that consists of repeated β-1,4-GlcUA-β-1,3-GlcNAc disaccharide units. It is well established that HA participates in various phases of the metastasis of malignant tumors [3–5]. HA-rich matrices surrounding malignant tumor cells frequently lead to metastasis and hence to a worse clinical prognosis [6–8]. Indeed, the metastatic potency of malignant tumor cells is enhanced by transfection with the gene coding for HA synthase (HAS), an HA-synthesizing enzyme [6,9,10]. Conversely, inhibiting HAS expression in tumor cells with an antisense HAS construct reduces cell adhesion [11,12]. Thus, HA is a risk factor for metastasis of malignant tumors, suggesting that metastasis could be suppressed by inhibition of HA production.

We have reported that 4-methylumbelliferone (MU) inhibits HA synthesis in human dermal fibroblasts [13–15]. Together, these results suggest that MU, as a suppressor of HAS activity, may have an inhibitory effect on the metastasis of malignant tumor cells. Consequently, we recently examined the effect of MU on the adhesion and locomotion of malignant tumor cells, which are important properties during the early stages of metastasis. We demonstrated that HA enhances both the adhesion and the locomotion of B16F-10 melanoma cells, a highly malignant tumor cell line, and that these increases are inhibited by MU [16].

In the present study, we examined whether MU inhibits the metastasis of melanoma cells to mouse tissues in vivo. We here report that MU has an inhibitory effect on the liver metastasis of B16F-10 melanoma cells and therefore shows potential as an anti-metastatic agent.

2. Materials and methods

2.1. Materials

MU and umbelliferone (UM) were purchased from Sigma Chemical Co. (St. Louis, MO) and actinase E was obtained from Kaken Pharmaceutical Co. (Tokyo, Japan). HA and Streptomyces hyaluronidase were purchased from Seikagaku Co. (Tokyo, Japan). All other reagents were of analytical grade and obtained from commercial sources.

2.2. Tumor cells

B16F-10 melanoma cells (henceforth referred to as melanoma cells) were maintained in RPMI-1640 (Nissui, Tokyo, Japan) supplemented with 10% fetal calf serum (FCS), l-glutamine and antibiotics. Addition of MU to the medium was carried out as described previously [13].

2.3. Mice

Ten-week-old female C57BL/6 mice (19–22 g body weight) were used throughout the experiments. All mice were purchased from Japan Clea (Tokyo, Japan) and housed in conventional conditions with water and food ad libitum. MU was suspended in 1% arabic gum at a density
of 20%, and 300 µl of the suspension was administered orally to each mouse. Mice were placed under light diethyl ether anesthesia and blood samples were taken by cardiac puncture, then the total liver was removed. All animal experiments were performed according to the Guidelines for Animal Experimentation of Hirosaki University.

2.4. Assay of blood MU
The concentration of blood MU was assayed by using HPLC [17]. Briefly, the internal standard (30 µl of a 50 µg/ml solution of UM in methanol) was added to 100 µl of a whole blood sample. After mixing the blood sample with 1.0 ml of ethyl acetate, the mixture was centrifuged at 2000 × g for 10 min. The ethyl acetate layer was removed, evaporated to dryness under air and reconstituted in 500 µl of methanol. The methanol solution was centrifuged at 4000 × g for 10 min and 200 µl of injection sample was obtained. The HPLC system consisted of an L-6200 liquid chromatograph, L-7200 autosampler, F-1050 fluorescence spectrophotometer and D-7500 integrator (Hitachi Co., Tokyo, Japan). Reverse phase chromatography was carried out on a TSK-GEL ODS-120T column (0.46 cm × 25.0 cm; Tosoh Co., Tokyo, Japan) with a mobile phase of 40% methanol in water at a flow rate of 0.5 ml/min. The column temperature for this HPLC procedure was 35 °C and detection was performed at excitation and emission wavelengths of 350 and 450 nm, respectively.

2.5. Assay of GAG in mouse liver
Liver samples (0.95–1.1 g wet weight) were washed gently with cold isotonic saline, sliced with scissors and homogenized in 2 volumes of saline at 0 °C. The homogenate was centrifuged and the pellet was delipidated as described previously [18], then dried with diethyl ether and weighed to obtain the dry weight. The dry liver was incubated with actinase E (0.1 mg/mg of dry weight) and GAG in the liver was analyzed by HPLC as described previously [16].

2.6. Assay of liver metastasis
On the day of inoculation, melanoma cells were harvested from the cultures and washed twice in culture medium. Cells suspended at a density of 1 × 10⁶ cells in 0.05 ml of PBS were injected into the lateral tail vein. To investigate the effect of cell surface HA, cells pre-incubated with 0.5 mM MU for 48 h were inoculated. To determine the role of liver HA, mice were administered MU for 2 weeks and inoculated with melanoma cells untreated with MU. Controls were injected with cells untreated with MU and administered 1% arabic gum. At 14 days after the injection of melanoma cells, the liver was removed and surface colonies were counted under the dissecting microscope.

3. Results and discussion
3.1. Effect of MU on HA synthesis by mouse liver
In order to investigate the effect of MU on HA synthesis in mouse tissues, MU suspended in 1% arabic gum was administered orally at a dose of 3 mg/g body weight, and its kinetics and dynamics were studied. The concentration of MU in whole blood was analyzed by HPLC as described in Section 2. As shown in Fig. 1A, the concentration of MU was highest at 3 h after administration and then decreased quickly. MU could not be detected at 24 h.

Next, we also investigated the effect of MU on the HA content of various tissues, including liver, skin, lung and brain. As shown in Fig. 1B, the HA content of liver tissue decreased after administration of MU (Fig. 1B), whereas no significant decrease was observed in other tissues (data not shown). The liver content of HA decreased in a time-dependent manner, showing a significant decrease at 6, 9 and 12 h after administration, but was restored at 24 h. These observations suggest that liver HA can be suppressed continually by administration of MU every 12 h. Therefore, mice were given MU every 12 h to maintain HA knock-down conditions. Under these conditions, the liver content of HA was suppressed continually by 31.8% compared with that in mice not given MU (Fig. 1C). We also investigated whether MU inhibits the synthesis of other GAGs such as chondroitin sulfate, dermatan sulfate, and heparin sulfate in mice liver. The significant inhibition of MU on the synthesis of these GAGs was not observed (data...
dissecting microscope (A). Each bar is the mean for 10 animals per group +S.D. Grouped data were analyzed for significance in comparison with the control group by using the Mann-Whitney test; $^*P < 0.05$. Metastatic colonies on the liver can be observed as black spheroids (B).

not shown). These results suggest that MU selectively inhibited HA synthesis in mice liver.

3.2. Effect of HA on metastasis

To examine the effect of MU-mediated inhibition of cell surface HA formation on liver metastasis of melanoma cells in vivo, cells were treated with 0.5 mM of MU for 48 h and subsequently injected into the tail vein of mice. The HA content of injected melanoma cells was decreased by MU treatment (by 32%, 5.2 pmol/10^4 cells) (data not shown), which were in agreement with the data published by Kudo et al. [16]. After 14 days, mice were killed and metastatic nodules were counted. As shown in Fig. 2, many metastatic nodules, appearing as black spheroids, were observed on the liver surface of mice injected with control melanoma cells without MU treatment. On the other hand, the number of metastatic nodules was markedly decreased (by 64%) in mice injected with melanoma cells treated with MU. Similar results were observed on the surface of lung, kidney, spleen, and mesentery (data not shown). Collectively, these results suggest that the formation of cell surface HA is necessary for metastasis of melanoma cells.

As mentioned above, oral administration of MU selectively inhibits the HA synthesis in liver tissue (Fig. 1B), whereas no significant inhibitory effect was observed in other tissues. Therefore, we examined the effect of recipient HA synthesized by liver cells on the metastasis of melanoma cells. Melanoma cells untreated with MU were injected into the tail vein of mice, and subsequently MU was administered twice daily for 14 days to maintain HA knock-down conditions in the liver. As shown in Fig. 2, oral administration of MU reduced the number of metastatic nodules in liver tissue by 32%. Thus, recipient HA synthesized by the liver is also crucial for liver metastasis of melanoma cells, suggesting that it might be a scaffold for, rather than a barrier against, metastasizing melanoma cells.

Our previous studies have shown that MU-mediated inhibition of formation of cell surface HA by melanoma cells results in suppression of their adhesion and locomotion [16]. On the contrary, HA, which is released from the cell surface and finally forms an HA-rich matrix, increased the adhesion and locomotion of melanoma cells [16], suggesting that HA is crucial for promoting the metastasis of melanoma cells in vitro.

In this study, we examined the effect of MU-mediated inhibition of HA synthesis by melanoma cells on their ability to form liver metastases in vivo. Furthermore, we investigated the effect of modulating the liver content of HA in the recipient mice on the formation of liver metastases by melanoma cells.

Firstly, we demonstrated that HA synthesis in mouse liver is inhibited by oral administration of MU. The inhibitory effect of MU was observed only in the liver, and no clear inhibition was detected in other tissues. Several studies have reported that liver appears to be the main organ for MU metabolism [19,20]. Therefore, accumulation of a large amount of MU may specifically reduce HA synthesis by liver cells. MU selectively inhibited HA synthesis, whereas other GAGs such as chondroitin sulfate, dermatan sulfate, and heparin sulfate were not influenced by administration of MU. Recently, Kakizaki et al. [15] demonstrated that MU inhibits the HA synthesis involving the glucuronidation of MU by endogenous UDP-glucuronosyltransferase (UGT) resulting in a depletion of UDP-GlcUA. They considered two possible molecular mechanisms related to the selective inhibition of the synthesis by MU [15]. They supposed that MU specifically targets HAS due to its cellular localization, which is different from other glycosyltransferases, exostosin family and chondroitin synthases involved in the synthesis of heparan sulfate and chondroitin sulfate, respectively. Indeed, all the GAGs, with the single exception of HA, are synthesized at the intracellular Golgi network, whereas HA is synthesized on the inner side of the plasma membrane by a membrane-associated HAS. Another explanation for the selective inhibition would be the preferential effects of MU on the chain elongation of a large molecular mass of HA.

Secondly, the inhibitory effect of MU on cell surface HA formation led to suppression of liver metastasis by melanoma cells. Cell surface HA synthesized by HAS is secreted into, and finally forms, the extracellular matrix [21] and therefore is at the front line during intercellular interactions. It is well established that HA-rich matrices surrounding malignant tumor cells indicate a worse prognosis because of higher
metastatic potency [7,8]. Indeed, some types of malignant tumor cells, such as mesothelioma and Wilms' tumor, produce large amounts of HA, and HA overproduction by tumor cells enhances their metastatic potential [6]. Moreover, introduction of the HAS gene into malignant tumors also enhances their metastatic potential [6,9,10], whereas inhibition of HAS expression in tumor cells by introduction of antisense HAS diminishes it [11,12]. Taken together with these findings and our previous observation that MU inhibits the adhesion and locomotion of cultured melanoma cells [16], the present study strongly suggests that inhibition of cell surface HA synthesis may inhibit the metastasis of melanoma cells, and that HA-rich matrices surrounding melanoma cells provide a suitable environment for metastasis.

The role of recipient HA in the remote tissues into which malignant tumor cells invade is unclear, except that our previous studies demonstrated that the cell adhesion and locomotion of cultured melanoma cells were enhanced by exogenous HA [16]. Therefore, thirdly, we investigated the effect of recipient HA on liver metastasis of melanoma cells by using HA knock-down mice generated by oral administration of MU. We demonstrated that recipient liver HA promotes the liver metastasis of melanoma cells, suggesting that recipient HA works as a scaffold to accelerate metastasis.

Recently, Camenisch et al. [22] produced genetically knock-out mice lacking the HAS-2 gene and reported that these animals underwent fetal death due to disordered development of the cardiovascular system. This observation underlines the difficulty in obtaining animals that have suppressed HA levels in tissues, and suggests that MU may be a useful tool for the examination of the functions of HA. Although several studies have shown that the metastatic potency of tumor cells is enhanced by transfection with the gene for HAS [6,23], we have adopted the opposite approach by using the inhibitor MU to demonstrate that both the formation of cell surface HA and the synthesis of host liver HA are necessary for liver metastasis by melanoma cells. Thus, this study is the first demonstration of MU-mediated inhibition of liver metastasis by melanoma cells in vivo.

In summary, by using HA knock-down mice treated with MU, we have demonstrated that the metastasis of melanoma cells is enhanced both by cell surface HA on the tumor cells and by recipient HA in the liver. Our results strongly indicate that MU has potential as a novel anti-metastatic agent to inhibit the metastasis of melanoma cells.

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