Antimetastatic Effect of an Orally Active Heparin Derivative on Experimentally Induced Metastasis

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Abstract Purpose: Orally active anticancer drugs have great advantages for the treatment of cancer. Compelling data suggest that heparin exhibits critical antimetastatic effects via interference with P-selectin–mediated cell-cell binding. However, heparin should be given parenterally because it is not orally absorbed. Here, we evaluated the inhibitory effect of orally absorbable heparin derivative (LHD) on experimentally induced metastasis.

Experimental Design: We developed LHD, which is a chemical conjugate of low molecular weight heparin and deoxycholic acid, and measured the plasma concentration of LHD after oral administration. To evaluate the antimetastatic effect of LHD, we carried out experimental lung metastasis assays in vivo using murine melanoma or human lung carcinoma cells and interruption assay between murine melanoma cells and activated platelets and human umbilical vascular endothelial cells in vitro.

Results: In mice, the plasma concentration was ~7 µg/mL at 20 minutes after oral administration of LHD (10 mg/kg), indicating that bleeding was not induced at this dose. Interestingly, we found that LHD dramatically attenuated metastasis experimentally induced by murine melanoma or human lung carcinoma cells and that its antimetastatic activity was attributed to the interruption of the interactions between melanoma cells and activated platelets and between melanoma cells and human umbilical vascular endothelial cells by blocking selectin-mediated interactions. Furthermore, it prevented tumor growth in secondary organs.

Conclusions: On the basis of these findings, the present study shows the possibility of LHD as a suitable first-line anticancer drug that can be used for preventing metastasis and recurrence because it has therapeutic potential as an antimetastatic drug, has lower side effects, and can be orally absorbed.

Metastasis continues to be the major cause of morbidity and mortality in malignancies. Hence, initiation and maintenance of tumor growth in secondary organs may serve as an attractive target for therapeutic intervention (1). The process of tumor metastasis is highly complicated, involving dependent and independent coagulation mechanisms. Tumor cell–induced platelet aggregation and activation plays an important role in tumor cell protection and in the successful adherence to and invasion through the endothelial wall. Various independent studies have indicated that spontaneous and experimental metastasis is significantly inhibited by the anticoagulant heparin in different experimental models (2). In addition to its functions as an antithrombotic agent, the many other biological effects of heparin that have recently been elucidated may account for their therapeutic efficacy in tumor metastasis (2, 3). Compelling data also suggest that the critical antimetastatic effects by heparin are mediated primarily via interference with P-selectin–mediated binding (4–6).

Heparin is a naturally occurring glycosaminoglycan and a potent inhibitor of blood coagulation, which is primarily achieved via the formation of an inhibitory complex with antithrombin III (7, 8). In current clinical practice, low molecular weight heparin (LMWH) is used for the initial therapy of venous thromboembolism, which is a common complication in cancer patients (9). Recently, it is known that heparin can affect cancer progression via a number of physiologic processes like angiogenesis and tumor metastasis (2–6). Interestingly, the diverse clinical efficacies of heparin have been shown without severe toxicity. However, heparin-based therapeutic approaches are limited because heparin is not absorbed in the intestine due to its high molecular weight,
negatively charged structure, and hydrophilic properties, and for these reasons, heparin should be given parenterally (10, 11).

It is interesting to develop an orally active heparin because the long-term use of continuous parenteral heparin is not practical. On the other hand, cancer progression can be prevented by chronic administration of orally active heparin. For oral absorption of heparin, various approach types, such as liposomes, enteric coatings, and enhancers, have been studied (12–15). In the previous reports, we introduced an orally active LMWH-deoxycyclic acid conjugate (LHD) produced by the covalent bonding between the amine group of N-deoxycycl-

lethyleneamine (DOCA-NH₂) and the carboxylic acids of heparin via amide linkage (Fig. 1; refs. 16–20). Interestingly, we found that this new heparin derivative could be orally absorbable in the rodent and monkey. The oral absorption of heparin derivative was attributed to the conjugated DOCA molecule constituent, which could promote intestinal absorption by enhancing the hydrophobic properties of heparin and by increasing the interaction between heparin and the intestinal membrane. Therefore, we can expect that cancer progression can be prevented by chronic administration of orally active heparin derivative.

In the present study, we evaluated the therapeutic efficacy of LHD as a new oral ant metastatic agent by attesting its antitumorigenic behavior in vitro and in vivo.

Materials and Methods

Synthesis of LHD. LHD was prepared as previously described (16–20). In brief, it was synthesized by conjugating the carboxylic group of DOCA with the carboxylic groups of LMWH (fraxiparin, average MW of 4.5 kDa; GlaxoSmithKline). The initial stage of its preparation involved activating the carboxylic group of DOCA, as follows. DOCA (196 mg) was mixed with dicyclohexylcarbodiimide (Sigma Chemical Co.; 165 mg) and hydroxysuccinimide (Sigma; 92 mg) in 15 mL of dimethylformamide (Merck) at a molar feed ratio of 1:1.6:1.6, respectively. Dicyclohexylcarbodiimide and hydroxysuccinimide levels were slightly higher than that of DOCA to ensure complete DOCA activation. This mixture was reacted for 5 h at room temperature in vacuum, precipitated by adding 1 mL of distilled water and then removed by filtration. The solution obtained was then added to 15 mL of 1:1.6:1.6 mixture prepared by gentle mixing was incubated for 20 min at 37°C. B16F10 melanoma cells were incubated in DNA/Lipofectamine mixture in 5% CO₂ for 4 h at 37°C. Culture media were then changed to normal RPMI 1640 to allow cells to recover for 2 d. The transfection efficiency achieved was ~30% to 40% in B16F10 melanoma cells. To separate transfected cells from nontransfected cells, selection medium containing G418 (1 mg/mL; Life Technologies) was added for 2 d, and this medium was then changed weekly. Transfected melanoma cells that expressed the neomycin resistance gene survived in the selection media, whereas nontransfected melanoma cells died at higher G418 concentrations. Selected RFP-transfected B16F10 melanoma cells were cultured in RPMI 1640 containing 25 mmol/L HEPES (Life Technologies) and G418 (0.5 mg/mL); passages 2 to 15 were expanded as pools of stably transfected cells.

Experimental lung metastasis assays in vivo using murine melanoma cells. B16F10 melanoma cells (3 × 10⁵/100 μL HBSS) were injected into the lateral tail veins of 8-wk-old male C57BL/6J mice (21–24). Briefly, LHD (1 or 10 mg/kg/d) or LMWH (10 mg/kg/d) was orally administered into the mice 20 min before tumor cell injection. In addition, LHD (1 mg/kg/d) was also i.v. injected into the mice to be used as an effective control of LHD group. In addition, as a positive control, the dissolved water solution without LHD (0 mg/kg/d) was orally administered into the mice. Twenty minutes after oral or i.v. administration, B16F10 melanoma cells were i.v. inoculated into the tail vein. Starting the next day, different doses of LHD or LMWH were administered into the mice everyday for 14 d after daily fasting for 4 h. Two weeks after administration, lung tissues were harvested from the mice and fixed in 4% paraformaldehyde. Numbers of tumor nodules (colonization of B16F10 melanoma cells) on lung surfaces were counted by two observers in a blinded fashion. After that, fixed lungs were embedded in paraffin, cut into 5-μm sections, and stained with H&E. Tumor nodules within the lungs were observed and also counted in randomly selected fields (×100, 10 fields) under a light microscope.

On the other hand, to visualize the distribution of tumor nodules in the whole lung tissue, RFP-transfected B16F10 melanoma cells (3 × 10⁵/100 μL HBSS) were separately carried out. On day 14, RFP fluorescence intensity of whole lung tissue was measured by using a Kodak Image Station 4000 MM (Digital Imaging Systems) equipped with a special C-mount lens and a TRI TC emission filter. The mean
intensities of RFP fluorescence were normalized by calculating the fluorescence intensities of normal lung tissues (background, 0%) and lung tissues with no treatment of LHD (positive control, 100%).

**Experimental lung metastasis assays in vivo using human lung carcinoma cells.** A549 cells (human lung carcinoma; 5×10⁶/100 μL HBSS) were injected into the lateral tail veins of 8-wk-old male BALB/c nu/nu nude mice. Briefly, LHD (0 or 10 mg/kg/d) was orally administered into the mice 20 min before tumor cell injection. Twenty minutes after oral administration, A549 lung carcinoma cells were i.v. inoculated into the tail vein. Starting the next day, LHD was orally administered into mice everyday for 14 d after daily fasting for 4 h. Two weeks after oral administration, lung tissues were harvested from the mice. The numbers of tumor nodules on lung surfaces were counted by two observers in a blinded fashion. After that, fixed lungs were embedded in paraffin, cut into 5-μm sections, and stained with H&E. Tumor nodules within the lungs were observed and also counted in randomly selected fields (×100, 10 fields) under a light microscope.

**Interruption of interaction between B16F10 melanoma cells and activated platelets or activated HUVECs by LHD.** Adhesion assays of B16F10 melanoma cells to activated platelets were carried out as previously described with minor modification (25). Briefly, isolated platelets from platelet-rich plasma were added to six-well plates (2×10⁶ per well) and incubated for 1 h at 37°C. Platelets were then activated with mouse thrombin (1 unit/well; Sigma) for 30 min. B16F10 cells (1×10⁵ per well) were further added to each well in the presence of LMWH or LHD and then were allowed to settle for 30 min. After the nonadherent cells had been washed out with HBSS, the numbers of tumor cells attaching to platelets were counted by photographing randomly selected fields (×200, seven fields). On the other hand, adhesion assays of B16F10 cells to activated HUVECs were done as follows. HUVECs were added to six-well plates (1×10⁵ per well) in EGM-2 medium (Cambrex) and were allowed to grow to confluence. The cells were then activated with recombinant tumor necrosis factor α (25 ng/mL; R&D Systems) for 4 h at 37°C (26), and then B16F10 cells (1×10⁵ per well) were added to the HUVECs in the presence of LMWH or LHD and were allowed to settle for 30 min. Nonadherent cells were then removed by washing thrice with HBSS, and the number of tumor cells adherent to HUVECs was counted in photographs of randomly selected fields (×100, seven fields).

**Interruption of interaction between B16F10 melanoma cells and immobilized P-selectin protein by LHD.** The interruption by LHD of the interaction between B16F10 melanoma cell and immobilized mouse P-selectin was confirmed (27). Microtiter plates (Nunc A/S) were coated with 400 ng of soluble protein A (Sigma) and 100 μL of 50 mmol/L sodium carbonate/sodium bicarbonate buffer (pH 9.5).
absorbed LHD was maintained in the range of 0 to 0.5 IU/mL in the plasma, and at this concentration level, no side effects nor bleeding was induced. In addition, toxicity was not detected up to 2 IU/mL of plasma concentration of LHD in monkeys (data not shown). The maximum plasma concentration of LHD at 1 and 10 mg/kg was three to six times higher than the recommended minimum effective concentration (0.1 IU/mL) for the prevention of venous thromboembolism (30, 31).

**LHD inhibited experimental lung metastasis of murine melanoma in vivo.** To evaluate that the orally absorbed LHD could inhibit lung metastasis induced experimentally by B16F10 murine melanoma, LHD (at 0, 1 or 10 mg/kg) was orally administered 20 minutes before injection of B16F10 melanoma via tail vein. Subsequently, LHD was orally administered once a day for 14 days. Tumor nodules on the surface of lung tissue and in the lung interior at day 14 were counted (Fig. 2A and B). The numbers of tumor nodules on the surface of untreated tissue or lung tissues treated with 1 and...
10 mg/kg LHD were 139.1 ± 22.9, 66.0 ± 12.6, and 20.0 ± 7.9, respectively, whereas the numbers of tumor nodules in the lung interior were 25.3 ± 0.7, 7.7 ± 0.3, and 4.3 ± 0.7, respectively. A similar tendency was noted on observation of tumor nodules in histologic sections. Tumor nodules usually formed around the vessels or in the periphery of the tissue (Fig. 2C and D, arrow). When LHD was i.v. injected into the mice everyday for 14 days, the number of tumor nodules on the lung surface or in the lung interior was 26.5 ± 4.4 and 4.5 ± 0.9, respectively (Fig. 2A and B). When the mice were orally treated with LMWH (10 mg/kg/d) for 14 days, the number of tumor nodules on the lung surface or in the lung interior was 122.9 ± 21.3 and 19.8 ± 1.3, respectively (Fig. 2A and B). In the meantime, no animals in all of the experimental cases died during the entire experimental period.

To visualize the distribution of tumor nodules in the whole lung tissue, RFP-transfected B16F10 melanoma cells were inoculated via lateral tail vein 20 minutes after daily oral administration of LHD. At day 14, RFP fluorescence intensity was measured by using a Kodak Image Station 4000 MM equipped with a special C-mount lens and a TRITC emission filter (Fig. 3). RFP fluorescence was significantly reduced by orally administered LHD and the relative intensities of RFP at 1 and 10 mg/kg/d LHD were 44.7 ± 8.4% and 15.8 ± 6.6%, respectively. Therefore, orally absorbed LHD significantly reduced tumor nodules, indicating that the metastasis event in the tumor progression could be attenuated by the orally active LHD.

**LHD inhibited experimental lung metastasis of human lung carcinoma in vivo.** To investigate that the orally absorbed LHD could inhibit lung metastasis induced experimentally by A549 human lung carcinoma, LHD (at 0 or 10 mg/kg) was orally administered once a day for 14 days. Tumor nodules on the surface of lung tissue and in the lung interior at day 14 were counted (Fig. 4A and B). The numbers of tumor nodules on the surface of lung tissue without or with 10 mg/kg LHD were
Although the IC\textsubscript{50} values of LMWH and LHD were significantly different, the inhibitory effect of LHD was similar to that of LMWH. The IC\textsubscript{50} values of LMWH and LHD were 11.5 and 72.1 \textmu g/mL, respectively. Collectively, these results represent evidence that P-selectin–mediated melanoma cell-platelet or P-selectin–mediated melanoma cell-endothelial cell adhesion can be blocked by LHD, which suggests that orally absorbed LHD can inhibit selectin-mediated cell-cell interactions \textit{in vitro}.

\textbf{Inhibition of tumor growth by orally active LHD.} To provide further evidence on the inhibitory effect of orally active LHD on tumor growth, which is the end stage of metastasis, the antitumoral effect of LHD was investigated in an \textit{in vivo} tumor graft model using B16F10 melanoma cells. B16F10 melanoma cells were s.c. implanted into the flanks of C57BL/6 mice, and subsequent tumor growth was monitored during the oral administration of 1 or 10 mg/kg/d LHD for 14 days (Fig. 6). Compared with the control group, the orally administered LHD significantly delayed tumor growth. At day 14, the tumor volumes in control mice and in mice treated with 1 or 10 mg/kg/d LHD were 3,324.7 \pm 245.3, 1,998.1 \pm 350.4, and 1,332.5 \pm 172.9 mm\textsuperscript{3}, respectively. Therefore, these results showed that the orally administered LHD could attenuate the process of tumor metastasis, and thus tumor growth, in secondary organs.

\section*{Discussion}

The metastatic spread of solid tumors is directly or indirectly responsible for most cancer-related deaths, yet no specific antitumoral strategy that inhibits tumor settlement in distant organs exists. There are three types of cell-cell interactions mediated by L- or P-selectin that are relevant for hematogenous metastasis. Cancer cell-platelet interactions are mediated by P-selectin and provide protection for circulating tumor cells via a platelet cloak. Cancer cell-endothelium interactions are also mediated by P-selectin and play a role in the arrest of circulating tumor cells. Finally, cancer cell-leukocyte interactions are mediated by L-selectin. The present study shows that orally absorbed LHD could inhibit experimentally induced metastasis, further revealing, in part, P-selectin–dependent attenuation of lung metastasis.

The orally absorbed LHD could effectively prevent the experimentally induced metastasis of melanoma. The numbers of tumor nodules of B16F10 murine melanoma and A549 human lung carcinoma on the surfaces and in the interiors of lungs were significantly attenuated by orally administered LHD compared with the control. The formation of tumor nodules, which is colonization in the lung tissue, is directly related to intravascular coagulation of circulating tumor cells in the bloodstream because circulating tumor cells are thromboplastic. By depriving circulating tumor cells of their platelet cloak by the orally absorbed LHD, circulating tumor cells become more readily cleared by the immune system. In the present study, we show that LHD could inhibit the interaction between B16F10 melanoma cells and activated platelets. Furthermore, we show that adhesion of the tumor cells to the activated vascular endothelium was also blocked by the orally absorbed LHD, suggesting that LHD could attenuate extravasation of tumor cells attached on the wall of vasculature. The suppression of the interactions was due to the competitive inhibition by LHD of the selectin-mediated interactions. To clearly confirm the inhibition of the selectin-mediated interaction by LHD, we carried out an \textit{in vitro} adherence experiment of B16F10 melanoma cells to the immobilized P-selectin molecules.
Furthermore, in our previous study, we showed the inhibition of early retention of tumor cells and clot formation in lung in vivo (22). Five minutes after tumor cell injection, mice were sacrificed, their lungs were harvested, and lung tissues were histologically analyzed. When compared with saline-treated group, the heparin derivative could inhibit clot formation (coagulation of tumor cells and platelets) in lung microvessels, suggesting that the heparin derivative could inhibit tumor cell residence in lung capillaries by blocking the interaction between tumor cells and platelets in the blood stream.

On the other hand, the metastasis steps may also be interrupted by LHD at subsequent steps, namely the establishment and subsequent growth (i.e., colonization) of the metastasis at the new site. Here, we show that tumor growth was significantly delayed by orally absorbed LHD after s.c. implantation of B16F10 melanoma cells. In the previous study, we found that the orally absorbed LHD could inhibit tumor growth via inhibition of angiogenesis in the tumor tissues, which was confirmed from the immunohistologic analysis with anti-CD34 antibody to the tumor tissue (20). In addition, the LHD could inhibit the in vitro tubular formation of HUVECs, the formation of microvessels around chicken chorioallantoic membrane, and the formation of microvessels into Matrigel plugs in the flank of mice. Collectively, these findings suggested that the orally absorbed LHD could attenuate metastasis via inhibition of selectin-mediated interaction and tumor growth in secondary organs by inhibiting angiogenesis. Furthermore, in the previous study, we showed the antimetastatic effect of the heparin-DOCA derivative on the incidence of lung metastasis induced by SCC7 (head and neck squamous cell carcinoma; ref. 22). Therefore, the heparin derivative could have the antimetastatic effect on the lung metastasis induced by B16F10, SCC7, and A549, which means that the antimetastatic effect of the heparin derivative does not depend on the specific type of tumor cells (i.e., B16F10, SCC7, and A549 at least).

Conventional cancer therapies with their severe toxicities often have frustrated patients and have prompted cancer research to focus increasingly on therapies that target molecular abnormalities specific to tumorigenesis. Currently, it has been proposed that antimetastatic cancer therapy designed to target...
selectin molecules might be more broadly effective and safer than either targeted or traditional therapies aimed at cancer cells due to traditional impediments to successful cancer therapy such as drug resistance and inadequate drug delivery. Various P-selectin antagonists have been discovered at present, such as monoclonal antibodies, sLe\(\alpha\), sLe\(\beta\), and its mimetics, as well as recombinant PSGL-1 (32). However, because these selectin inhibitors have various drawbacks such as narrow cross-reactivity, weak affinity, relatively low selectivity among the selectins, short circulating half-life, great expense to generate, potential antigenecity, and a very limited track record as i.v. therapeutic agents, the development of these compounds into effective drugs for clinical use has been greatly limited.

Accumulating evidence indicates that many of the in vivo antimetastatic effects of heparin reflect its action on P-selectin–mediated binding (6, 33). Several cell-surface glycoconjugates bearing selectin-binding sites have previously been detected in melanoma cells. Lewis antigens (e.g., sialyl Lewis X and sialyl Lewis A) and heparin sulfate–like proteoglycans have been identified as potential P-selectin ligand determinants (32). The interaction of tumor cell glycoconjugates with P-selectin expressed by platelets and endothelial cells may therefore facilitate extravasation and survival of circulating melanoma cells (26, 33). On these interactions, the P-selectin blocking effect of heparin is most likely attributed to charged carboxylates and sulfate esters. The 6-O-sulfate group of the glucosamine units is critical for heparin inhibition of the selectins (25, 32). Although the effect of heparin on cancer progression remains an issue for debate, various studies have suggested that heparin is an attractive candidate in anticancer therapy, especially for the fact that heparin is an efficient inhibitor of P- and L-selectin. In spite of these attributes of heparin, heparin-based therapeutic approaches are limited because its high molecular weight and negatively charged hydrophilic structure require heparin to be given parenterally only, and not orally.

It is interesting to develop an orally active heparin because long-term use of continuous parenteral heparin is not practical.

Fig. 5. Interruption of interaction between B16F10 murine melanoma cells and activated platelets, activated HUVECs, or immobilized P-selectin protein by LMWH (○) or orally active LHD (△). A, inhibition of adhesion of B16F10 melanoma cells (1 × 10^5 per well) to platelets (2 × 10^8 per well) activated with thrombin (1 units/well) in the presence of different concentrations of LMWH or LHD. Points, mean (n = 7); bars, SE. B, inhibition of adhesion of B16F10 melanoma cells (1 × 10^5 per well) to HUVECs (1 × 10^5 per well) activated with tumor necrosis factor α (25 mg/mL) in the presence of different concentrations of LMWH or LHD. Points, mean (n = 7); bars, SE. C, inhibition of adhesion of calcein AM–labeled B16F10 melanoma cells (5 × 10^4 per well) to P-selectin protein (400 ng/well) immobilized on the bottom of the microplate in the presence of different concentrations of LMWH or LHD. After lysis of bound B16F10 melanoma cells, fluorescence intensities were measured and normalized. Points, mean (n = 5); bars, SE.

Fig. 6. Tumor growth profiles of B16F10 murine melanoma cells (1 × 10^6 per mouse) s.c. implanted in the flank of C57/BL/6J mice during daily oral administration of the dissolved water alone (0 mg/kg/d; ○), 1 mg/kg/d (●), or 10 mg/kg/d (▲) of LHD for 2 wk. Tumor size was measured in two dimensions daily using a caliper, and volumes were calculated. Points, mean (n = 8); bars, SE. *, P < 0.01, no treatment versus 1 or 10 mg/kg/d LHD.
The main advantage of orally active heparin is that cancer progression such as metastasis and tumor growth can be prevented and/or delayed by chronic administration of orally active heparin. This chronic administration of orally active heparin can be applicable in the clinic due to the nontoxicity of heparin. During the course of our study, we developed an amphiphilic heparin derivative by coupling hydrophobic DOCA molecules to hydrophobic LMWH, with the goal of enhancing its intestinal absorption. The chemically modified heparin derivative LHD had either the increased lipophilicity or the interaction between the conjugated DOCA molecules and bile acid transporters in the intestine, thereby enabling oral absorption. In the previous study, we experimentally showed the direct interaction between LHD and ileal brush border membrane surface by using the surface plasmon resonance technique and the increased lipophilicity and partition coefficient (K<sub>C<sub>19</sub>W</sub>) in the octanol/water solution of LHD (18, 19). In addition, LHD was actually absorbed efficiently in monkeys and mice at clinical acceptable doses. Furthermore, (18, 19). In addition, LHD was actually absorbed efficiently in monkeys and mice at clinical acceptable doses. Consequently, the rapid appearance of LHD in plasma may contribute to its possibility of causing bleeding, which restricts the application of heparin in the prevention of metastasis. Peak levels of LMWH in plasma above the upper limit of the recommended therapeutic range (0.6-1.0 IU/mL) may be associated with an increased risk for bleeding (34). In the current study, because the peak levels of orally absorbed LHD in plasma at 1 and 10 mg/kg/d were below the recommended minimum effective concentration for the prevention of venous thromboembolism, these doses of LHD, showing potent antimetastatic activities, did not increase the risk for bleeding. Therefore, we expect that LHD could exhibit antimetastatic activities and prevent venous thromboembolism without any bleeding problems.

In summary, our findings showed that orally active LHD could have antimetastatic effect via inhibition of cell-cell interaction between B16F10 melanoma cells and platelets or HUVECs by interrupting selectin-mediated interactions. Furthermore, orally active LHD could also attenuate tumor growth in secondary organs. Although the present study shows the possibility of LHD as a first-line anticancer drug, we think that LHD could be more suitable for use in preventing metastasis and recurrence because LHD has the therapeutic potential of an antiangiogenic and antimetastatic drug, has lower side effects, and can be orally absorbed.

References

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