Mesenchymal mode of migration participates in pulmonary metastasis of mouse osteosarcoma LM8

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Abstract The outcomes of osteosarcoma patients still remain poor because of intractable pulmonary metastasis. We previously established a highly metastatic osteosarcoma cell line, LM8 from Dunn mouse osteosarcoma by in vivo selection. We herein aimed to clarify the characteristic biological features related with high metastatic potential and new target molecules to suppress pulmonary metastasis of osteosarcoma, using this syngeneic spontaneous metastatic model. LM8 cells acquired fibroblastic morphology with striking fibropodia on the cell surface. Immunostaining showed faint stress fiber formation and peripherally localized integrin β1, and biochemical analyses showed the activated Cdc42 and autophosphorylation of focal adhesion kinase (FAK) in LM8 cells when compared to Dunn cells. LM8 cells had activated motility in single cell migration mode. LM8 migration was increased by a Rho-associated kinase (ROCK) inhibitor, Y-27632, while decreased by Cdc42 silencing using RNA interference system. We found that a clinically approved camptothecin analog, irinotecan suppressed the migration, Cdc42 activity, and autophosphorylation of FAK, and attenuated integrin β1 distribution selectively in LM8 cells. Daily oral administration of irinotecan significantly reduced the rate and size of pulmonary metastasis in syngeneic C3H mice. The fibroblastic morphology and activated cell migration with the dependency on Cdc42 but not Rho-ROCK signaling pathway argued that LM8 moved in mesenchymal mode of cell migration. This activated mesenchymal migration was a key component of the pulmonary metastasis of LM8 cells. The inhibition of mesenchymal migration by irinotecan, in addition to its cytotoxic effects, might be effective in preventing pulmonary metastasis of osteosarcoma.

Keywords Osteosarcoma · Metastasis · Mesenchymal migration · Cdc42 · Irinotecan

Abbreviations
ECM Extracellular matrix
3D Three dimensions
FAK Focal adhesion kinase
MMP Matrix metalloproteinase
ROCK Rho-associated kinase

Introduction
Osteosarcoma is the most common malignant bone tumor in childhood. The 5-year survival for osteosarcoma has
remained less than 70% [1–3]. Approximately 15–20% of patients with osteosarcoma present with visible macro-metastatic disease, and more than one-third of children presenting without metastasis will relapse after current therapy. The outcome for patients with metastatic disease is poor [4], and more than 95% of patients who died of metastatic disease had lung involvement at the time of death. Thus, the elucidation and inhibition of the mechanisms of pulmonary metastasis is strongly needed to improve the prognoses of osteosarcoma patients.

We previously established a highly metastatic osteosarcoma cell line, LM8 from Dunn mouse osteosarcoma [5] through eight cycles of in vivo selection consisting of tail vein injection, preparation of pulmonary metastasis, in vitro culture, and repeat tail vein injection. This syngeneic spontaneous metastasis model was outstanding compared to xenografting metastasis models in the following three points. Firstly, this syngeneic mouse model mimicked the clinical metastasis under a normal immune environment. Secondly, LM8 cells representatively showed pulmonary metastasis within 3–4 weeks after orthotopic transplantation, while Dunn formed little colonies in the lungs. Thirdly, we could investigate the biological features of metastasis through comparing LM8 and Dunn cells under the similar genetic background. We reported that LM8 cells revealed fibroblastic morphology with abundant filopodia and invasion property compared to Dunn cells [6].

Motility is crucial for cancer cells to spread and seed new tumors [7–10]. According to the reports by Friedl et al. [11, 12], tumor cell migration can be classified into collective and single cell migration, and single cell migration consists of two alternative mechanisms of migration, namely ameboid and mesenchymal migration. Number of evidences showed that tumor cells could shift the mode of migration according to its microenvironment. The inhibition of matrix metalloproteinase (MMP) induces the shift of migration mode from mesenchymal to ameboid, while a Rho-associated kinase (ROCK) inhibitor, Y-27632 induces the opposite shift. Epithelial-mesenchymal transitions, which is closely associated with epithelial tumor progression, could be interpreted the shift from collective to single cell migration in the context of cell migration [12, 13]. However, the relationship between the progression of osteosarcoma and the mode of migration has been hardly examined.

In the present study, we focused on the differences in morphologies and modes of migration between LM8 and Dunn cells, and found that LM8 showed the activated motility with mesenchymal movement maintained by the outside-in signals linking extracellular matrices (ECMs), Cdc42 and actin cytoskeleton, which reflected the highly metastatic potential. We then re-evaluated several clinically approved anti-cancer drugs, and found that irinotecan most effectively reduced LM8 mesenchymal migration. Finally, we confirmed that irinotecan inhibited pulmonary metastasis of mouse osteosarcoma in spontaneous metastasis models.

Materials and methods

Reagents

Irinotecan was purchased from Daiichi-Sankyo (Tokyo, Japan) as a 2-ml vial solution (40 mg). SN38 was purchased from Tocris Cookson (Ellisville, MO). The following anticancer drugs were screened for the suppressive effects on migration: Etoposide and cyclophosphamide (Sigma, Saint-Louis, MO), doxorubicin (Kyowa Hakko Kirin, Tokyo, Japan), methotrexate (Wyeth, Tokyo, Japan), and cisplatin (Maruko Pharmaceutical, Kasugai, Japan). Anti-Rac1 polyclonal antibody and anti-integrin β1 monoclonal antibody for function blocking were purchased from BD Pharmingen (San Jose, CA). Anti-FAK polyclonal antibody and anti-RhoA polyclonal antibody were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-phosphotyrosine397-FAK polyclonal antibodies were purchased from Biosource (Camarillo, CA). Anti-Cdc42 polyclonal antibody was purchased from Cell Signaling (Beverly, MA). Anti-actin monoclonal antibody and anti-integrin β1 polyclonal antibody were purchased from Millipore Bioscience Research (Temecula, CA). Rhodamine-phalloidin was purchased from Molecular Probes (Eugene, OR). Y-27632 was supplied by Mitsubishi Pharma Co. (Tokyo, Japan). Dishes with collagen I were purchased from Sumilon (Tokyo, Japan).

Cells and animals

The highly metastatic osteosarcoma cell line, LM8 was derived from Dunn osteosarcoma through eight repeated cycles of the procedure of Poste and Fidler [14]. Cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% FBS. All cells were cultured at 37°C in a fully humidified incubator under 5% CO₂. Specific pathogen-free 5- to 6-week-old C3H/He mice were used in this study (SLC, Sizuoka, Japan). All animal experiments were approved by the institutional animal experiments review committee (protocol no. 80902), and all animals were euthanized with diethyl ether at the end of experiments.

Number of filopodia

Microspikes longer than 20 μm were defined as filopodia. More than 150 cells from three separate experiments were analyzed by two independent individuals.
Pull down assay and Cdc42 activation assay

Levels of activated Rho-GTPases (GTP-RhoA, GTP-Rac1 and GTP-Cdc42) were measured by pull down assay [15, 16]. Briefly, cells were washed with cold HEPES-buffered saline, scraped into lysis buffer. Cell lysates were clarified by centrifugation at 13000×g at 4°C for 10 min. Lysates containing an equal volume of protein were incubated with glutathione-Sepharose beads (Amersham Pharmacia Biotech, Buckinghamshire, UK) coupled with bacterially expressed GST-ankyrin fusion protein for GTP-RhoA, or bacterially expressed GST-p21 activated kinase (PAK) fusion protein for GTP-Rac1 and GTP-Cdc42, for 2 h at 4°C. Activated Rho-GTPase bound to beads and total Rho-GTPase in cell extracts was detected by immunoblotting. Rho-GTPase activities are indicated by the amount of bead-bound Rho-GTPase normalized against the amount of Rho-GTPase in whole cell lysates. Cdc42 activation was also measured by G-LISA Cdc42 Activation Assay Biochem Kit (Cytokeleton, CO) according to the manufacturer's protocol.

Immunoblotting

Immunoblotting was performed as described previously [17]. Band intensity was analyzed using ImageJ (NIH Software, Bethesda, MD).

Cell migration assay

For vertical migration, modified Boyden chamber migration assay was performed as described previously [18]. In brief, cell migration assay was performed for 12 h using 24-well Bio-Coat cell migration chambers (BD Biosciences, Bedford, MA). The lower surface of the membrane was coated with 30 μg/ml of fibronectin for haptotactic migration. Horizontal migration was measured by EZ-TAXIScan (GE Healthcare Biosciences, Tokyo, Japan), according to the manufacturer's protocol. Briefly, cells were applied to a cover glass coated with collagen I. After incubation for cell spreading on cover glass, chemotactic migration was conducted using 10% FBS as a chemoattractant. Chemotactic migration was allowed to proceed for 5 h, and images were captured every 3 min. To analyze cell counts passing through the indicated area, accessory analysis software (TaxisScan Analyzer2) [19] was used.

Cell proliferation assay

Cell proliferation was measured using WST-8 Cell Counting Kit (Dojindo, Kumamoto, Japan) according to the manufacturer's protocol.

Transfection of Cdc42 siRNA and pcDNA3-FLAG-tagged wild type Cdc42

In the knock-down and rescue experiment, LM8 cells were transfected with the Cdc42 siRNA targeting to 3' UTR of mouse Cdc42 (target sequence: 5'-ATGGATTGAGTTCC TAATTTA-3'), non-targeting siRNA (Qiagen, Valencia, CA) and pcDNA3-FLAG-tagged wild type Cdc42 according to the protocol for Lipofectamine 2000. LM8 cells were analyzed for migration 36–48 h after the transfection, and for Cdc42 protein expression and activation 48 h after the transfection.

Immunofluorescence and phase contrast microscopy

Immunofluorescence analysis was performed as described previously [18]. Fluorescence and phase contrast images obtained with an AxioCam photomicroscope (Carl Zeiss Vision GmbH, Hallbergmoos, Germany) and IX70 (Olympus) microscope using a 40× objective (NA 0.6), respectively. Images were analyzed and processed for presentation using brightness and contrast adjustment with ImageJ.

Measurement of the concentration of irinotecan and SN38 in mouse serum and lungs

The concentration of irinotecan and SN38 was measured as described previously [20].

Mouse model

Irinotecan was orally administered at 10 ml/kg mouse weight with sterile physiological saline immediately before use. In order to generate the primary tumor resection model of osteosarcoma, LM8 cells (1 × 10⁷ per 200 μl PBS) were injected into the subcutaneous tissue of the backs of syngeneic C3H mice. The primary tumor was resected under anesthesia after 2 weeks. C3H mice were estimated to die from pulmonary metastasis 5–6 week after primary tumor resections. Thus, for ethical reasons, the histological evaluation and count of pulmonary metastasis foci were performed 3 weeks after primary tumor resections. In order to prepare the tail vein injection model, LM8 cells (1 × 10⁶ per 100 μl PBS) were injected into tail vein of syngeneic C3H mice. C3H mice were estimated to die from pulmonary metastasis 4–5 week after tail vein injection. Thus, for ethical reasons, metastatic foci in both lungs were counted 3 weeks after tail vein injection.
Statistical analysis

Results are expressed as means ± SD. The statistical differences were determined by two-sided Student's t-test in biological assays, and two-sided Mann–Whitney's U test in animal experiments. Values of less than 0.05 were considered to be statistically significant.

Results

LM8 cells showed more fibroblastic morphology with striking filopodia and activated Cdc42

LM8 cells showed characteristic morphology with striking filopodia on plastic culture plates (data not shown). The difference in filopodia formation was more apparent with collagen I (Fig. 1a, left, arrowheads) and fibronectin (data not shown). LM8 showed significantly higher number of filopodia per cell (Fig. 1a, right), but the difference in the average length of filopodia was not significant (data not shown). The same tendency was observed in three dimensional (3D) collagen culture, in which LM8 cells showed more numerous and longer filopodia with dynamic turnover, and proliferated more sparsely than Dunn cells (Supplementary Figure 1a, Supplementary Movies 1a, b). The immunostaining of F-actin and vinculin showed that LM8 cells had fewer actin stress fibers than Dunn cells (Fig. 1b, top). Immunostaining also showed differences in the distribution of integrin β1. The peripheral localization of integrin β1 was more apparent in LM8 cells (Fig. 1b, bottom, arrowheads), although FACS analysis showed similar expression levels of integrin β1 on the cell surface [18]. We next investigated the activation of three representative Rho family GTPases, RhoA, Rac1 and Cdc42. The levels of biochemically active Cdc42 (GTP-Cdc42) were significantly higher in LM8 cells than in Dunn cells on the pull down assay, which is consistent with the characteristic filopodia formation of LM8 cells. By contrast, levels of Rac1 and RhoA activity did not differ (Fig. 1c). The autophosphorylation level of FAK was also significantly higher in LM8 cells, compared with Dunn cells (Fig. 1d, left). Double immunostaining revealed that the autophosphorylated FAK was co-localized with vinculin at focal adhesion sites in LM8 cells, whereas it was hardly seen at focal adhesion sites in Dunn cells (Fig. 1d, right).

LM8 cells had activated motility in single cell migration mode

LM8 migration was twice as that of Dunn cells on the modified Boyden chamber migration assay measuring vertical migration (Fig. 2a, left). LM8 cells also showed twice the chemotactic migration of Dunn cells on EZ-TAXI Scan measuring horizontal migration [19] (Fig. 2a, right). When mixed with Dunn cells, LM8 cells tended to move in single cell migration mode with filopodia formation on the co-culture wound-healing assay (Supplementary Figure 1b, arrowheads).

The activated motility of LM8 cells correlated with activated Cdc42

We previously reported that Y-27632 suppressed hepatoma cell migration and peritoneal invasion [21]. Thus, we firstly investigated the effect of Y-27632 on LM8 and Dunn cell migration. Unexpectedly, Y-27632 increased LM8 migration at the dose of 10 µM, whereas decreased Dunn migration in a dose dependent fashion (Fig. 2b, left). Immunostaining showed that Y-27632 suppressed focal adhesion formations and stress fibers more prominently in LM8 cells compared to Dunn cells (Fig. 2b, right). The increase of LM8 migration by Y-27632 appeared with the activation of Cdc42 (Fig. 2c, left). Function blocking antibody against integrin β1 decreased Cdc42 activity in LM8 cells on collagen I, which indicated that the outside-in signals from ECM participated in Cdc42 activation in LM8 cells (Fig. 2c, right). We had no convenient molecules to selectively suppress Cdc42 activity. Thus, we conducted the knock-down of Cdc42 using RNA interference system. In the knock-down and rescue experiment, the protein expression level of total Cdc42 was reduced by Cdc42 siRNA transfection in LM8 cells and recovered by co-transfection of FLAG-tagged wild type Cdc42 in addition to Cdc42 siRNA (Fig. 2d, top). The Cdc42 activation level decreased to 50% by the knock-down of Cdc42 compared to control, and recovered by the re-expression of Cdc42 (Fig. 2d, middle). Accordingly, the migration rate of LM8 cells synchronized with the level of Cdc42 activation (Fig. 2d, bottom). We also confirmed that LM8 migration was reduced by another Cdc42 siRNA, and that Dunn migration was not changed by Cdc42 suppression (data not shown). Since the level of Cdc42 activation and rate of migration induced by Cdc42 knock-down were similar to those of Dunn cells, these results indicated that the different Cdc42 activation levels between LM8 and Dunn cells could represent their different migration rates.

Irinotecan most effectively reduced LM8 migration among anti-cancer drugs

LM8 cells showed the activated motility with above mentioned biological features (Table 1), which might be a promising target for the treatment of pulmonary metastasis of osteosarcoma. We thus re-evaluated six anticancer
Fig. 1 LM8 cells showed different morphology with Cdc42 activity compared to Dunn cells. a LM8 showed striking filopodia (arrowheads) and fibroblastic cell shape on collagen I (left). Bar 20 μm. Number of filopodia per cell was quantified (right). Dunn cells showed more stress fibers bridging focal adhesions (top). Integrin β1 was peripherally localized in LM8 cells (bottom, arrowheads), while diffusely present in Dunn cells. Bar 20 μm. The activity of Cdc42 was greater in LM8 cells than in Dunn cells, while RhoA and Rac1 activities were similar in both cell lines. The upper panel shows representative blots. The lower panel shows quantitative analysis of relative GTP-Cdc42/total Cdc42, GTP-Rac1/total Rac1 and GTP-RhoA/total RhoA ratios calculated by densitometry (Cdc42: n = 3, Rac1 and RhoA: n = 2). Bar SD. * P < 0.05 versus control. d The autophosphorylation of FAK was significantly up-regulated in LM8 cells compared to Dunn cells (left). The upper panel shows a representative blot. The lower panel shows quantitative analysis of relative Tyr397-phosphorylated FAK/total FAK ratio calculated by densitometry (n = 3). Bar SD. * P < 0.05 versus control. Immunostaining revealed that Tyr397-phosphorylated FAK (red) was co-localized with vinculin (green) at focal adhesion in LM8 cells, whereas it was hardly seen at focal adhesion in Dunn cells (right). Bar 20 μm.

Drugs, focusing on their suppressive effects on LM8 motility on the modified Boyden chamber migration assay at doses having little inhibitory effect on proliferation during migration assays. Each drug concentration was determined by cell proliferation assay which indicated the ineffective concentration on LM8 proliferation (Supplementary Figure 2). Among the six anti-cancer drugs clinically used for sarcoma treatment (etoposide, doxorubicin, cyclophosphamide, methotrexate, cisplatin and irinotecan), a camptothecin analog, irinotecan most effectively reduced
Fig. 2 LM8 cells showed active motility dependent on Cdc42 activity, not Rho-ROCK signaling pathway. a LM8 migration was twice as that of Dunn cells on the modified Boyden chamber migration assay (left) (n = 3). Bar SD. **P < 0.01 versus control. The same tendency was confirmed on the EZ-TAXIscan migration assay. The number of cells passing through the fixed region surrounded by red lines was counted at the indicated times on the EZ-TAXIscan migration assay (right). Bar 100 μm. b The migration of LM8 cells increased with Y-27632 (10 μM), while that of Dunn cells showed a dose-dependent decrease (left) (n = 3). Bar SD. *P < 0.05, **P < 0.01 versus control. The suppression of focal adhesion formations and stress fibers was more prominent in LM8 cells compared to Dunn cells (right). c The activity of Cdc42 in LM8 cells was up-regulated by Y-27632 (10 μM) (left), while down-regulated by the function blocking antibody of integrin β1 (right). The lower panel shows quantitative analysis of relative GTP-Cdc42/total Cdc42 ratios calculated by densitometry (n = 3). Bar SD. *P < 0.05 versus control. d The protein expression (top) and activity (middle) of Cdc42, and the migration (bottom) of LM8 cells were reduced by Cdc42 siRNA transfection and was recovered by additional transfection of FLAG-tagged wild type Cdc42. *P < 0.05, **P < 0.01 versus control.

LM8 migration (Fig. 3a). Irinotecan reduced LM8 migration in a dose-dependent manner, and at lower doses than suppressed LM8 proliferation (IC_{50} of proliferation: 25.5 μM and IC_{50} of proliferation: 136.6 μM) (Fig. 3b, left). Since irinotecan was supposed to be metabolized to SN38 in vivo, we investigated the dose-dependent inhibitory effect of SN38 on the migration and proliferation of LM8 cells. SN38 also reduced LM8 migration at lower doses than
Table 1 The comparison between highly metastatic cell line, LM8 and parental Dunn cells

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<th>LM8</th>
<th>Dunn</th>
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<td>Metastatic ability</td>
<td>Subcutaneous transplantation</td>
<td>Massive pulmonary metastasis in 4 weeks</td>
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<td>Morphology</td>
<td>Filopodia formation</td>
<td>2.2 filopodia/cell</td>
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<td></td>
<td>Stress fiber formation</td>
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<td>Focal adhesion</td>
<td>Integrin β1</td>
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<td>FAK Y397</td>
<td>High phosphorylation</td>
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<td>Motility</td>
<td>Vertical migration</td>
<td>20% of applied cells (12 h)</td>
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<td>Haptotaxis</td>
<td>20% → 35%</td>
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<td>PDGF (30 ng/ml)</td>
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<td>LPA (10 μg/ml)</td>
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<td>mRNA and protein expression</td>
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<td>VEGF [6]</td>
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<td>MMP [6]</td>
<td>GTP-Cdc42</td>
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<td>Rho family GTPases</td>
<td>Y-27632 (10 μM)</td>
<td>20% → 32% (Haptotaxis)</td>
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<td>Cdc42 siRNA</td>
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proliferation (IC₅₀ of migration: 7.7 nM and IC₅₀ of proliferation: 333.3 nM) (Fig. 3b, right).

Irinotecan reversibly and selectively suppressed the biological features of LM8 cells

As shown in Supplementary Figure 3a and Supplementary Movie 2, irinotecan suppressed the filopodia formation of LM8 cells. Irinotecan reduced filopodia formation at 2 h after exposure, and the reduced filopodia recovered within 20 h after drug washout. The timing of inhibition and recovery of Cdc42 activity and migration rate induced by administration and washout of irinotecan were correlated with filopodia formation (Supplementary Figure 3b). Irinotecan suppressed the migration and Cdc42 activity (Fig. 4a), attenuated filopodia formation and integrin β1 distribution to the perinuclear region (Fig. 4b) selectively in LM8 cells, but did not affect those in Dunn cells. We finally compared the suppressive effect on FAK autophosphorylation by irinotecan between LM8 and Dunn cells. As expected, irinotecan reduced the levels of FAK autophosphorylation selectively in LM8 cells (Fig. 4c).

SN38 and topotecan also reduced LM8 migration

An active irinotecan metabolite, SN38 (5 nM) and another clinically approved camptothecin analog, Topotecan (100 nM) also reduced LM8 migration at a dose that showed little inhibitory effects on cell proliferation during migration assays (Supplementary Figure 4a). SN38 significantly reduced Cdc42 activity (Supplementary Figure 4b), and induced filopodia shrinkage (arrowhead, Supplementary Figure 4c) in a similar manner to irinotecan.

Irinotecan reduced migration and Cdc42 activity in other human osteosarcoma cell lines

Irinotecan (15 μM) suppressed migration in other human osteosarcoma cell lines: Saos-2, U2OS and HOS (Supplementary Figure 5a), but showed little inhibitory effects on cell proliferation during migration assay (data not shown). Irinotecan significantly reduced Cdc42 activity in Saos-2 cells (Supplementary Figure 5b).

Irinotecan significantly inhibited pulmonary metastasis in vivo

Several reports presented the effectiveness of daily oral administration of irinotecan [22–24], because protracted exposure of irinotecan at low concentration increases its effect. In vivo, irinotecan was supposed to exhibit its pharmacological effect after metabolized to SN38. We thus preliminary checked the concentration of SN38 in serum and lung at 1 h after oral administration of irinotecan on the
Fig. 3 Irinotecan most effectively suppressed LM8 migration. a A clinically approved camptothecin analog, irinotecan most significantly reduced LM8 migration among six anti-cancer drugs clinically used for sarcoma treatment on the modified Boyden chamber migration assay ($n = 3$). Bars SD. * $P < 0.05$, ** $P < 0.01$ versus control. b Dose dependent effects of irinotecan (left) and SN38 (right) on migration and proliferation were measured on the modified Boyden chamber migration assay and WST-8 system, respectively. Irinotecan attenuated LM8 migration in a dose-dependent manner and at lower doses than suppressed LM8 proliferation ($n = 3$). IC$_{50}$ of migration was 25.5 nM, while IC$_{50}$ of proliferation was 136.6 nM. SN38 also reduced LM8 migration at lower doses than proliferation ($n = 3$). IC$_{50}$ of migration: 7.7 nM and IC$_{50}$ of proliferation: 333.3 nM. Bars SD.

The last day of the administration period (20 mg/kg/day × 5 days/week × 3 weeks). The concentrations in serum were 250 nM and in lung 50 nM. As shown in Fig. 3b, the serum and lung concentrations of SN38 indicated that they were enough to inhibit LM8 migration in vivo. There was no body weight loss by the administration. We then prepared a primary tumor resection model, in which pulmonary metastasis was not obvious at the time of primary tumor resection but became apparent at 3 weeks after resection. The difference was not admitted in the tumor size in the treatment group and the control group. Oral administration of irinotecan started after primary tumor resection (20 mg/kg/day × 5 days/week × 3 weeks) (Fig. 5a). Irinotecan showed substantial decrease in pulmonary metastasis. In the control group, histological images showed large metastatic foci that infiltrated into lung parenchyma, whereas metastatic foci were much smaller and solitary in the treatment group (Fig. 5b, left). The number of metastatic foci in lungs and the lung wet weight per mouse were significantly lower in the treatment group (Fig. 5b, right). We next examined the effect of irinotecan in tail vein injection model (Fig. 5c). Irinotecan also reduced the number of metastatic foci in lungs and the lung wet weight per mouse in this experimental metastasis model (Fig. 5d). We also performed time course analysis of pulmonary metastasis suppression induced by irinotecan using the IVIS imaging system (Supplementary Figure 6a). Pulmonary metastasis was accelerated from 3 weeks after luciferase-expressing LM8 (LM8Luc) cells were subcutaneously injected. On days 31 and 35, the volume of pulmonary metastasis in the treatment group was significantly lower but continued to increase (Supplementary Figure 6b), which was confirmed by comparing photon counts (Supplementary Figure 6c). The volume of primary tumors in the treatment group was also significantly smaller, but continued to increase during oral administration of irinotecan (Supplementary Figure 6d).
Fig. 4 Irinotecan inhibited the Cdc42 activity, migration and focal adhesion dynamics selectively in LM8 cells. a Two-hour pre-exposure of irinotecan (15 μM) reduced the Cdc42 activity and migration of LM8 cells, but did not affect those of Dunn cells (migration assay; n = 3). Bar SD. * P < 0.05, ** P < 0.01 versus control. b Irinotecan (15 μM) altered the stress fiber and focal adhesion formation in LM8 cells (left). Integrin β1 was internalized in the perinuclear area at 2 h after administration of irinotecan (right, arrowheads). Bar 20 μm. c Irinotecan (15 μM) decreased autophosphorylation of FAK at Tyr397. The upper panel shows a representative blot. The lower panel shows quantitative analysis of relative Tyr397-phosphorylated FAK/total FAK ratio calculated by densitometry (n = 2). Bar SD. * P < 0.05 versus control.

Discussion

Previous studies showed a correlation between single cell migration and metastasis [25–28]. During single cell migration, cells with ameboid and mesenchymal migration have different morphologies and signaling pathways to move forward. Ameboid migration is generally independent of MMP activity and is driven by Rho-ROCK dependent actomyosin-based cortical contraction, and cells using ameboid migration have more rounded shape. In vivo imaging revealed that metastatic breast cancer cells preferred single cell migration in Rho-ROCK dependent fashion [26, 29, 30]. We also reported the critical role of Rho-ROCK signaling pathway on cancer cell motility and invasion, and Y-27632 suppressed peritoneal invasion of hepatoma cells [17, 21, 31]. On the other hand, cells with mesenchymal migration show an elongated morphology and extended protrusions. Integrin-based adhesions and strong traction are the driving forces for movement in addition to ECM degradation by MMPs [11, 12, 32]. Several reports suggested that other signaling pathways than Rho-ROCK participated in mesenchymal cell migration. Nobes et al. reported that Y-27632 promoted the wound closure of primary rat embryo fibroblasts [33]. Wilkinson et al. showed that Cdc42-MRCK (myotonic dystrophy kinase-related Cdc42 binding kinase) signaling cooperated with ROCK in cells migrating with mesenchymal mode and sufficient for the phosphorylation of myosin light chain (MLC) [34]. We had reported that Y-27632 promoted rhBMP-2 induced ectopic bone

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Fig. 5 Continuous oral administration of irinotecan inhibited pulmonary metastasis in syngeneic mouse models. 

a For the primary tumor resection model, a total of 19 mice were divided into two groups: (i) untreated controls (10 mice); and (ii) treated with irinotecan at 20 mg/kg po×5/wk. On day 1, 1 × 10⁷ LM8 cells were transplanted subcutaneously into each mouse. Primary tumor nodules were surgically removed and oral administration of irinotecan was started on day 14. All mice were sacrificed on day 35. 

b Histological images of pulmonary metastasis are shown (HE staining, bar 100 μm). In the control group, LM8 cells formed large nodules and invaded into lung alveoli (left, top). In the treatment group, alveoli architecture was preserved and tumor nodules were small (left bottom). The number of metastatic foci in lungs and the lung wet weight per mouse were significantly lower in the treatment group (right). Median, quartiles and highest and lowest values are indicated on box-and-whisker plots. * P < 0.05, ** P < 0.01 versus control. 

c For the tail vein injection model, a total of 9 mice were divided into two groups: (i) untreated controls (four mice); and (ii) treated with irinotecan at 20 mg/kg/day 5 days per week for 2 weeks (five mice). On day 1, 1 × 10⁶ LM8 cells were transplanted intravenously into each mouse. All mice were sacrificed on day 21. The number of metastatic foci in lungs and the lung wet weight per mouse were significantly lower in the treatment group. Median, quartiles and highest and lowest values are indicated on box-and-whisker plots. * P < 0.05 versus control.
formation with increased recruitment of mesenchymal cells [35]. We also confirmed that Y-27632 increased the migration of a mesenchymal cell line, ST2 (data not shown). Thus, we speculated that acquisition of mesenchymal mode of migration was critical in pulmonary metastasis of osteosarcoma, which was mesenchymal cell origin.

In the current study, we investigated biological features of highly metastatic osteosarcoma focusing on the mode of migration. LM8 and Dunn cells expressed little cadherins [36], which indicated that these cells originally moved in single cell migration mode. As summarized in Table 1, LM8 cells acquired striking filopodia and showed higher levels of Cdc42 activation compared to Dunn cells, which were promoted by ECMs. Integrin β1 localization at the cell periphery in LM8 cells also became apparent with ECM, and blocking of integrin β1 decreased Cdc42 activity. FAK autophosphorylation was also enhanced in LM8 cells. As we previously reported, LM8 secreted MMP-2, while Dunn did not [6]. The increase in migration by Y-27632 and dependency on Cdc42 activity with above mentioned features indicated that LM8 cells showed a strong tendency using mesenchymal mode of migration, compared to Dunn cells.

We could not confirm the promotion of pulmonary metastasis by activated motility with mesenchymal migration in vivo. We established several clones from Dunn cells which had accelerated migration by overexpression of wild type Cdc42, V12Cdc42 mutant, and in vitro selection through Boyden chamber migration assays. However, these clones exhibited different morphology with LM8 cells and the dependency on Rho-ROCK signaling pathway, as Y-27632 decreased their migration. These clones could not metastasize to lungs in vivo metastasis experiments (data not shown). We speculated that activated motility was not enough, but the mesenchymal mode of migration was necessary for pulmonary metastasis of osteosarcoma, and that the balance between Rho-ROCK and Cdc42 signaling pathways was finely tuned in LM8 cells. The key molecules which induced the mesenchymal migration in osteosarcoma cells remained to be elucidated.

The pharmacological effects of irinotecan involved in inducing cell cycle arrest and apoptosis through interacting with topoisomerase I [37–39]. The current study suggested that irinotecan also inhibited cell migration at the lower concentration than suppressing proliferation. The inhibitory effect of irinotecan on cell migration might contribute to the prevention of pulmonary metastasis at the steps exiting circulation and forming secondary metastasis foci, in addition to cytotoxic effect. Moreover, the inhibitory effect on migration induced by irinotecan was seen earlier (2 h) than that on proliferation (6 h by FACS analysis). This suggested that putative cytoplasmic molecules other than topoisomerase I were targets of irinotecan to suppress above mentioned biological features. These molecules had potential to effectively inhibit the mesenchymal migration and metastasis of osteosarcoma.

In conclusion, mesenchymal mode of migration was characteristic of LM8 cells, and associated with the metastatic ability of mouse osteosarcoma. Activated signaling networks linking ECMs, integrin β1, FAK and Cdc42 enhanced the mesenchymal migration, and was a potential target for suppressing pulmonary metastasis of osteosarcoma. The inhibition of these biological features by irinotecan, in addition to its cytotoxic effects, might be effective in preventing pulmonary metastasis.

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