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Wf-536 prevents tumor metastasis by inhibiting both tumor motility and angiogenic actions

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Abstract

The signaling pathway of Rho and Rho-associated coiled-coil forming protein kinase (ROCK) is involved in tumor metastasis. In the present study, we investigated the suppressive effect of a novel inhibitor of ROCK, Wf-536 [(+)-(*R*)-4-(1-Aminoethyl)-*N*-(4-pyridyl) benzamide monohydrochloride], on spontaneous tumor metastasis in vivo and analyzed its action on tumor cell motility and angiogenesis to clarify its action mechanism. Wf-536 (0.3–3 mg/kg/day) was found to inhibit Lewis lung carcinoma (LLC) metastasis and LLC-induced angiogenesis in orally treated mice; in vitro, it inhibited both invasion and migration by LLC cells and invasion, migration, and formation of capillary-like tubes on Matrigel by endothelial cells, without cytotoxicity or anti-proliferative action in either cell type. We conclude that Wf-536 has tumor anti-metastatic activity which may depend on inhibition of tumor motility and angiogenesis. The findings support its further clinical development as an anti-metastatic agent.

Keywords: Angiogenesis; Endothelial cell; Invasion; Migration; Tumor metastasis; Wf-536

1. Introduction

Tumor metastasis is a multistep event involving detachment of tumor cells from the primary site and growth at the colonization site (Liotta et al., 1991; Nicolson, 1991). Within this multistep process, the invasive ability of metastatic tumor cells is a critical factor in intravasation, extravasation, and invasion through host stroma tissues, and is closely correlated with cellular motility.

At the same time, angiogenesis, the process that leads to the formation of new capillaries from preexisting blood vessels, is essential for tumor metastasis and solid tumor growth (Folkman, 1995). Angiogenesis consists of a sequence of events, including degradation and invasion of the basement membrane underlying the endothelial layer, migration and proliferation, and formation of new vascular capillaries by endothelial cells. Some of these functions are dependent on the motility and morphology of endothelial cells.

The signaling pathway which consists of Rho, a Ras-related small GTPase, and Rho-associated coiled-coil-containing protein kinase (ROCK) is considered to be pivotally involved in controlling the focal adhesions and the dynamics of stress fibers which regulate cell motility and morphology (Ridley and Hall, 1992; Nobes and Hall, 1995; Narumiya et al., 1997). Recent studies have shown that activation of ROCK is involved in invasion by tumor cells (Imamura et al., 2000; Somlyo et al., 2000), angiogenesis (Uchida et al., 2000), and their evolution to metastasis (Itoh et al., 1999), suggesting potential for prevention of tumor metastasis via inhibition of tumor motility and angiogenesis on the basis of ROCK inhibition.

We have developed a novel inhibitor of ROCK, Wf-536 [(+)-(*R*)-4-(1-Aminoethyl)-*N*-(4-pyridyl) benzamide monohydrochloride] (Fig. 1), which is around three times more potent in ROCK inhibition than its originator substance Y-27632 [(+)-(*R*)-*trans*-4-(1-Aminoethyl)-*N*-(4-pyridyl)

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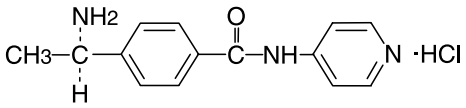

Fig. 1. Molecular structure of Wf-536.

cyclohexanecarboxamide dihydrochloride] (Uehata et al., 1997; Nakajima et al., 2001). Wf-536 inhibits metastatic activity in mouse tumor models, including C1300 neuroblastoma, B16BL6, and B16F10 melanoma (Fujii et al., 2001; Hayashi et al., 2001). However, no study has combined detailed clarification of action mechanism with investigation of the compound's effect.

In the present study, we investigated the effect of Wf-536 on tumor metastasis in vivo, and, to clarify its action mechanism in the prevention of tumor metastasis, examined its effect on the motile function of tumor cells in vitro and on tumor-induced angiogenesis in vivo; we also evaluated its effect in vitro on the angiogenic function of endothelial cells in terms of invasion, migration, and capillary-like tube formation.

2. Materials and methods

2.1. Reagents and materials

Wf-536 [(+)-(*R*)-4-(1-Aminoethyl)-*N*-(4-pyridyl) benzamide monohydrochloride] was synthesized and purified by Mitsubishi Pharma (Osaka, Japan). The following materials were used: human umbilical vein endothelial cells, EGM-2 medium (Biowhittaker, Walkersville, MD), mouse Lewis lung carcinoma (LLC) cells (gene bank of the Institute of Physical and Chemical Research, Ibaraki, Japan), Dulbecco's modified Eagle medium (GIBCO BRL, Life Technologies, Rockville, MD, USA), fetal bovine serum (HyClone Laboratories, Logan, UT, USA), fatty acid-free bovine serum albumin (Nacalai Tesque, Kyoto, Japan), cell culture inserts (8-μm pore) and Matrigel (Becton Dickinson, Franklin Lakes, NJ, USA), Cell Counting Kit-8 (Dojindo Molecular Technologies, Kumamoto, Japan), hydroxy propyl methylcellulose (Mitsubishi Pharma), gelatin sponge (Spongel, Yamanouchi Pharmaceutical, Tokyo, Japan), and Diff-Quik kit (International Reagents, Hyogo, Japan).

2.2. Cell culture

Human umbilical vein endothelial cells were grown in EGM-2 medium containing 2% fetal bovine serum at 37 °C in a 5% CO_2 atmosphere and were used at passage levels between the fourth and the sixth. Mouse LLC cells were grown in Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum at 37 °C in a 5% CO_2 atmosphere.

2.3. Invasion and migration assay

For the invasion assay, cells (5×10^4) suspended in 200 μl of serum-free medium supplemented with 0.5% fatty acid-free bovine serum albumin were inoculated into cell culture inserts (8-μm pore) layered with 25 μg of Matrigel, a reconstituted basement membrane matrix. The inserts were then set on 24-well plates filled with 700 μl of serum-containing medium for chemo-attractive motile activity and incubated for 18 h in the presence of the test compound at various concentrations. After incubation, the cells on the upper surface of the insert were removed with a cotton swab, while those on its lower surface and on the bottom of the well were fixed and stained using a Diff-Quik kit. The number of infiltrating cells per insert was determined by counting with a microscope the number of cells in 10 1-mm^2 areas on the lower surface of the insert and on the bottom of the well. For the migration assay, unlayered culture insert was used and incubation time was 6 h (tumor cells) or 4 h (endothelial cells); other procedures were as for the invasion assay.

2.4. Capillary-like tube formation assay

Human umbilical vein endothelial cells (6×10^3) were suspended in 400 μl of EGM-2 medium containing 2% fetal bovine serum, plated into 48-well plates loaded with Matrigel basement membrane matrix, and incubated for 18 h in the presence of the test compound at various concentrations. After incubation, the medium was aspirated, the capillary-like tubes formed on the Matrigel were fixed and stained using a Diff-Quik kit, and their mean area was measured using a stereoscopic microscope (SMZ-10, Nikon, Tokyo, Japan) equipped with a CCD camera (DK-7001N, HITACHI, Tokyo, Japan) connected to an image analyzer

Table 1
Effect of Wf-536 on spontaneous metastasis of syngeneic tumor in mice

Dose (mg/kg, p.o.)	*n*	Number of colonies	Weight of primary site (g)	Body weight	
				Day 14 (% of day 0)	Day 28 (% of day 0)
vehicle	25	16.9 ± 3.8	6.220 ± 0.198	116.8 ± 1.0	134.5 ± 1.8
0.3	22	7.6 ± 1.5*	6.518 ± 0.211	115.3 ± 1.1	132.6 ± 1.8
1	23	4.9 ± 0.6**	6.695 ± 0.205	114.3 ± 1.3	130.2 ± 1.8
3	25	3.4 ± 0.5**	6.593 ± 0.205	114.5 ± 1.2	135.2 ± 1.7

LLC cells (1×10^5) were intramuscularly injected into the right hind leg and Wf-536 orally administered for 28 days from the day of tumor injection day. The number of pulmonary metastasized colonies was microscopically counted following lung fixation. The weight of the primary tumor was determined by subtracting the weight of the corresponding left leg from that of the tumor-bearing right leg. The body weight (g, mean ± S.E.M.) of each group at day 0 was 17.4 ± 0.1, 17.2 ± 0.1, 17.4 ± 0.1, and 17.4 ± 0.2, at vehicle, 0.3, 1, and 3 mg/kg administration group, respectively. Data represent means ± S.E.M.

* $P < 0.05$ vs. vehicle.

** $P < 0.01$ vs. vehicle.

(LUZEX-F, NIRECO, Tokyo, Japan). The rate of capillary-like tube formation was expressed as follows:

Total area (mm^2) of capillary - like tubes formed /area measured (11.5 mm^2).

2.5. Proliferation assay

Cells (2×10^5) were plated in the wells of a collagen-coated six-well plate in medium containing fetal bovine serum and incubated for 24, 48, 72, and 96 h in the presence of the test compound at various concentrations. The compound and medium was exchanged every 48 h. After the incubation, medium was removed, cells were washed with PBS, and harvested. Then the number of viable cells was determined per well by the trypan blue exclusion assay.

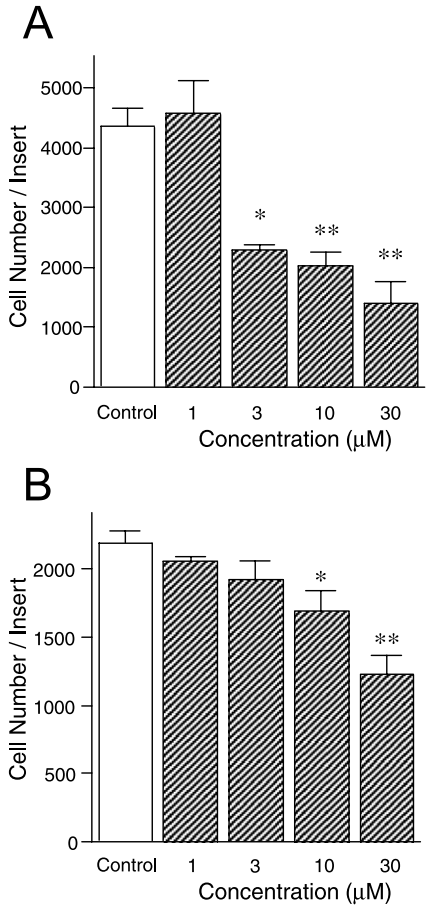

Fig. 2. Effect of Wf-536 on motile function of tumor cells. LLC cells (5×10^4) were incubated for 18 h on a culture insert layered with Matrigel for invasion (A), or for 6 h on an unlayered insert for migration (B), in the presence of the test compound at the indicated concentrations. The medium in the lower compartment contained 10% FBS for chemotactic activity. After incubation, the number of infiltrating cells per insert was determined. Each column represents the mean ± S.E.M. of three measurements in quadruplicate experiments. Statistical significance of differences calculated by Dunnett's test. $^{**}P<0.01$ and $^{*}P<0.05$ vs. control.

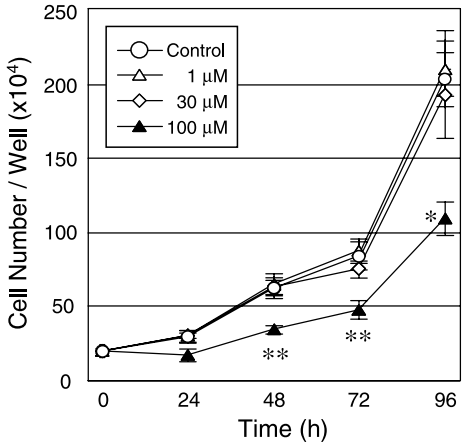

Fig. 3. Effect of Wf-536 on proliferation of tumor cells. Each value represents the mean ± S.E.M. of four observations. Note that no significant inhibition of tumor cell proliferation was observed at test compound concentrations of between 1 and 30 μM. Statistical significance of differences calculated by Dunnett's test. $^{**}P<0.01$ and $^{*}P<0.05$ vs. control.

2.6. Metastasis in vivo

All animal studies were performed in accordance with the procedures outlined in the Guidelines for Animal Experimentation of Mitsubishi Pharma, which was complied from the Guidelines for Animal Experimentation of the Japanese Association for Laboratory Animal Science. The protocols were approved by the Institutional Animal Care and Use Committee.

The assay of spontaneous LLC metastasis in mice was performed using a previously described method (Prontera et al., 1999) with some modifications. LLC cells (1×10^5) suspended in 50 μl of phosphate buffered saline were

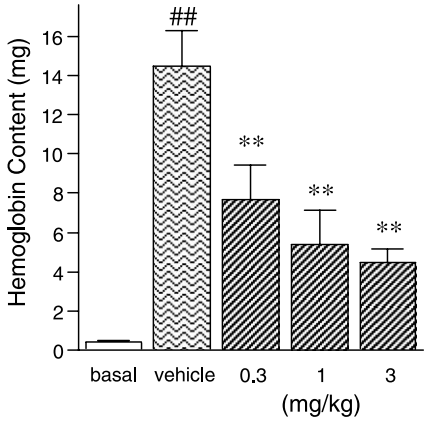

Fig. 4. Effect of Wf-536 on in vivo angiogenesis. Gelatin sponges containing LLC cells (1×10^6) were subcutaneously implanted into the backs of the mice and each compound administered orally for 28 days from the day of implantation. At 28 days after implantation, the gelatin sponge was excised and the hemoglobin content of the sponge was determined. Each column represents the mean ± S.E.M. ($n=15$). $^{\#\#}P<0.01$ vs. basal (cell-free sponge implanted) (Student's t-test); $^{**}P<0.01$ and $^{*}P<0.05$ vs. vehicle-treated (cell-loaded sponge implanted) (Dunnett's test).

intramuscularly injected into the right hind leg of 6-week old male C57BL/6 mice (Charles River Japan, Shiga, Japan), lightly anesthetized with ether. Wf-536 was suspended in 0.5% w/v hydroxy propyl methylcellulose in distilled water for oral administration and orally administered for 28 days (0.3, 1, and 3 mg/kg/day) from the day of tumor cell injection. The mice were then killed with ether and the lungs and both legs were removed. The number of metastasized pulmonary colonies was microscopically counted following fixation of the lungs with Bouin's

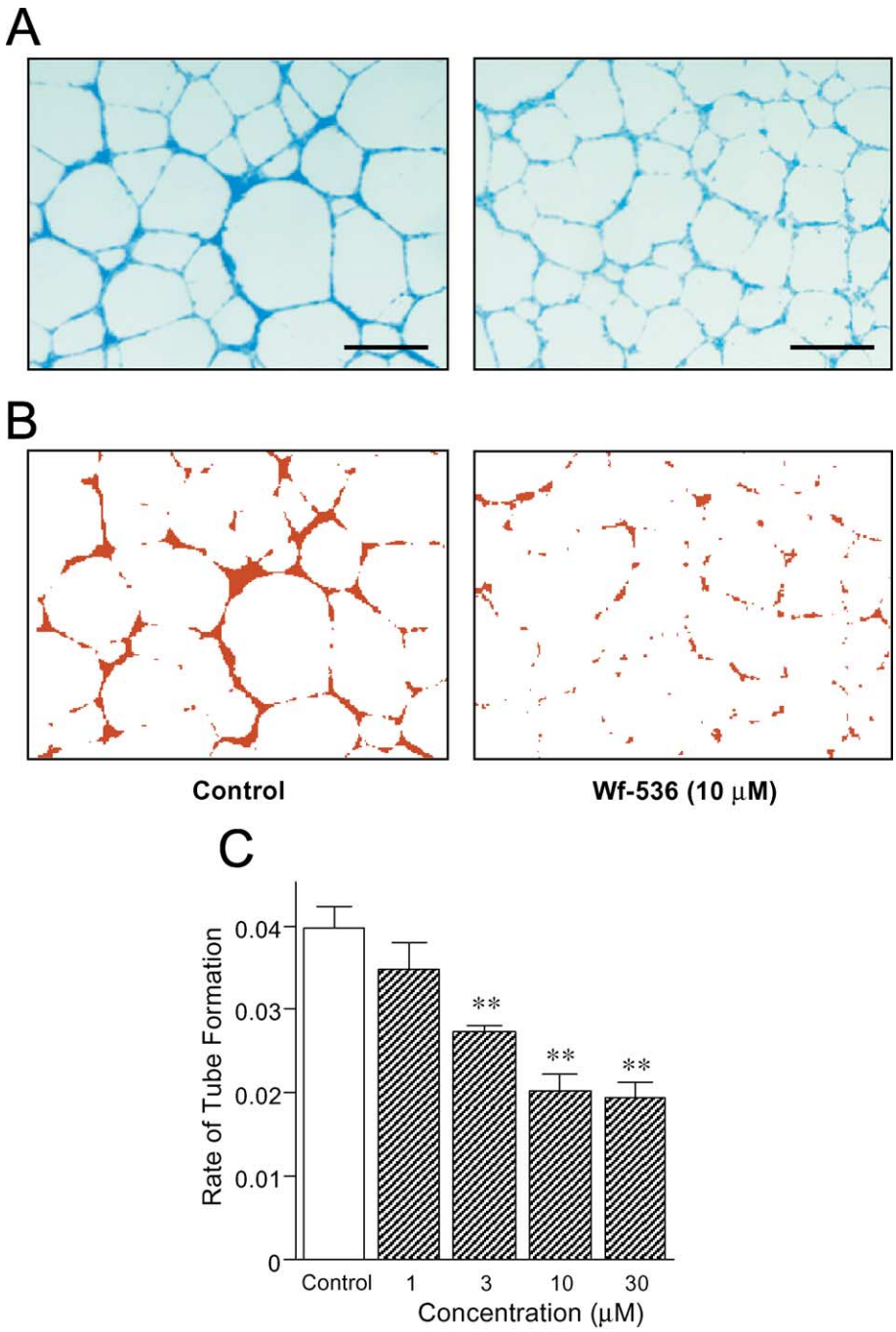

Fig. 5. Effect of Wf-536 on formation of capillary-like tubes by endothelial cells. Human umbilical vein endothelial cells (6×10^3) were incubated for 18 h in the presence of the test compound in a 48-well plate loaded with Matrigel, after which staining was performed with a Diff-Quik kit. The mean area of the tubes was measured using a stereoscopic microscope equipped with a CCD camera connected to an image analyzer. (A) Tube network on Matrigel, bar = 0.5 mm. (B) Image of tube network at same field after computer enhancement using LUZEX-F software. (C) Effect of Wf-536 on capillary-like tube formation in endothelial cells. Columns represent area of tubes formed in relation to total area measured (11.5 mm^2). Each value represents mean ± S.E.M. of three observations. Statistical significance of differences calculated by Dunnett's test. $^{**}P < 0.01$ vs. control.

solution. Primary tumor weight was determined by subtracting the weight of the corresponding left leg from the weight of each tumor-bearing right leg.

2.7. In vivo angiogenesis assay

A gelatin sponge (10 × 10 × 5 mm) was injected with LLC cells (1×10^6) suspended in 50 μl of phosphate-buffered saline and subcutaneously implanted into male C57BL/6 mice lightly anesthetized with ether. Wf-536 (0.3, 1, and 3 mg/kg/day) was orally administered for 28 days from the implantation day. Neovascularization was assessed by measuring the hemoglobin content of the gelatin sponge enlarged with granuloma tissue. Briefly, the mice were killed with ether and the sponge with granuloma tissue was excised and cut into several pieces. Hemoglobin was extracted from the sponge by soaking in 0.1 M ammonia solution (Davidson et al., 1985; Kusaka et al., 1991), and hemoglobin content was determined using a hemoglobin assay kit (Wako, Osaka, Japan). The validity of this method has been demonstrated (Hu et al., 1995; Majima et al., 1997).

2.8. Statistical analysis

The results are presented as means ± S.E.M.; *n* represents the number of experiments or the number of animals per group. Statistical analysis was performed using one-way analysis of variance (ANOVA) followed by Dunnett's test or Student's *t*-test. $P < 0.05$ was accepted as significant.

3. Results

3.1. Metastasis in vivo

We investigated the effect of oral administration of Wf-536 on the spontaneous metastasis of syngeneic mouse tumor cells (Table 1). Wf-536 significantly reduced the number of pulmonary metastatic colonies of LLC in a dose-dependent manner (0.3–3 mg/kg). The rate of colony-formation inhibition by Wf-536 was between 55% and 80%. In contrast, Wf-536 showed no significant effect on the weight of the primary tumor on day 28. During the drug treatment, we found neither body weight loss nor toxic death in the Wf-536-treated mice.

3.2. Metastatic motile function of tumor cells

We evaluated the effect of Wf-536 on the metastatic motile functions of tumor cells by examining its effect on LLC-cell invasion through a Matrigel layer and LLC-cell migration (Fig. 2).

Wf-536 at 3–30 μM showed significant and concentration-dependent inhibition of LLC-cell invasion (Fig. 2A), with inhibitory rate of between 48% and 68%. Wf-536 also significantly inhibited LLC-cell migration, with inhibitory rates of 23% and 44% at 10 and 30 μM, respectively (Fig. 2B).

To clarify whether the inhibitory action on motile functions could be due to a cytotoxic effect, Wf-536 was tested for its effect on LLC-cell proliferation (Fig. 3). Wf-536 showed no significant effect at 1–30 μM on the proliferation up to 96 h incubation, although the proliferation was decreased at 100 μM over 48 h.

3.3. Angiogenesis in vivo

We evaluated in vivo the inhibitory effect of Wf-536 on the angiogenesis induced by syngeneic LLC cells (Fig. 4). The hemoglobin content of the sponge containing LLC cells was approximately 50 times higher than that of the

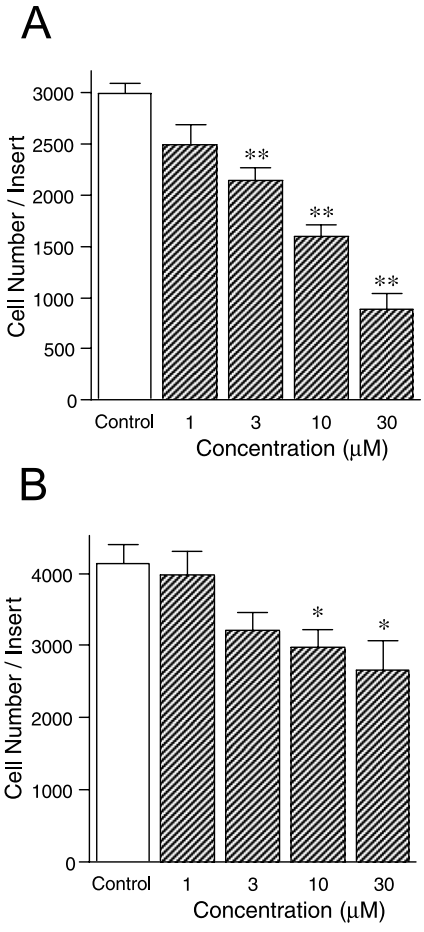

Fig. 6. Effect of Wf-536 on motile functions of endothelial cells: invasion through reconstituted basement membrane matrix (A) and migration (B). Human umbilical vein endothelial cells (5×10^4) were incubated for 18 h on a culture insert layered with Matrigel (invasion), or for 4 h on an unlayered insert (migration), in the presence of the test compound at the indicated concentrations. After the incubation, the number of infiltrating cells per insert was determined. Each column represents the mean ± S.E.M. of three determinations in quadruplicate experiments. Statistical significance of differences calculated by Dunnett's test. $^{**}P < 0.01$ and $^{*}P < 0.05$ vs. control.

sponge without LLC cells, indicating that angiogenesis was induced by the LLC cells in the sponge. Orally administered Wf-536 inhibited the increase in hemoglobin content: at Wf-536 doses of 0.3, 1, and 3 mg/kg, hemoglobin content was reduced by 48%, 63%, and 70%, respectively. Like LLC cells, mouse melanoma B16BL6 cells induced angiogenesis in vivo (hemoglobin content, mean ± S.E.M. (mg): 1.78 ± 0.07 and 20.54 ± 2.08, basal and tumor cell-loaded vehicle, respectively; $P<0.01$ by Student's t-test; $n=15$), an action which Wf-536 significantly inhibited (hemoglobin content: 14.77 ± 1.96; 1 mg/kg/day; $P<0.05$ vs. vehicle by Dunnett's test; $n=15$).

3.4. Angiogenic functions of endothelial cells

We evaluated the effect of Wf-536 on the angiogenic functions of endothelial cells by examining its effect on tube formation, invasion, and migration by human umbilical vein endothelial cells.

Capillary-like tubes were formed by endothelial cells on the Matrigel layer in the wells (Fig. 5A and B), a process which Wf-536 inhibited in a concentration-dependent manner, with statistically significant effects at concentrations of 3 to 30 μM (Fig. 5C). The effect appeared to plateau at 10 μM, with an inhibitory rate of approximately 50%.

Next, we investigated the effect of Wf-536 on the motile functions of endothelial cells, that is, invasion and migration. Wf-536 at 3–30 μM showed significant and concentration-dependent inhibition of invasion by endothelial cells, with inhibitory rate of between 29% and 71% (Fig. 6A). Wf-536 at 10 and 30 μM inhibited endothelial cell migration by 28% and 36%, respectively (Fig. 6B). Wf-536 at 1–30 μM showed no significant effect on endothelial cell proliferation up to 96-h incubation (Fig. 7), although Wf-536 inhibited the proliferation of endothelial cells at 100 μM over 48 h.

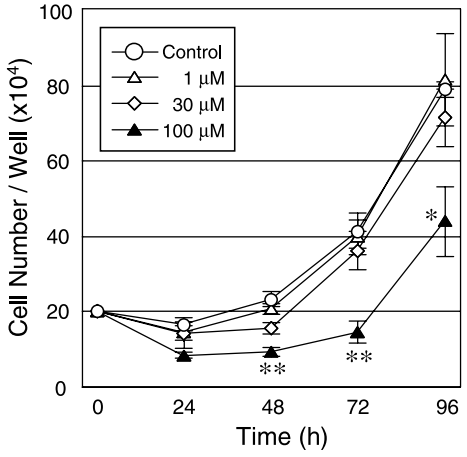

Fig. 7. Effect of Wf-536 on proliferation of endothelial cells. Each value represents the mean ± S.E.M. of four observations. Note that no significant inhibition of endothelial cell proliferation was observed at test compound concentrations of between 1 and 30 μM. Statistical significance of differences calculated by Dunnett's test. $^{**}P<0.01$ and $^{*}P<0.05$ vs. control.

4. Discussion

The present study demonstrated that oral administration of Wf-536 inhibits the spontaneous pulmonary metastasis of tumor cells and tumor-induced angiogenic response in vivo. Wf-536 significantly inhibited invasion and migration by tumor cells and invasion, migration, and formation of capillary-like tubes by endothelial cells, without affecting cell proliferation in vitro. The present data suggest that Wf-536 prevents tumor metastasis by inhibiting both the metastatic motility of tumor cells and tumor-induced angiogenesis.

ROCK has been demonstrated to be associated with invasion, migration, and morphological change in many tumor cells (Imamura et al., 2000; Manning et al., 2000; Nath et al., 2000; Somlyo et al., 2000; Hayashi et al., 2001). Wf-536 shows enzymatic ROCK inhibition with IC_{50} values of 0.57 and 0.39 μM for ROCK-I and ROCK-II, respectively (Nakajima et al., 2001). In the present study, Wf-536 inhibited tumor metastasis in vivo (Table 1). Moreover, Wf-536 inhibited invasion and migration of metastatic tumor cells (Fig. 2) without cytotoxic or anti-proliferative effect (Fig. 3). These findings suggest that the inhibitory action of Wf-536 on tumor motile functions is dependent on ROCK inhibition and that its suppressant effect on tumor metastasis in vivo involves anti-invasive action.

Wf-536 inhibited in vitro the angiogenic functions of endothelial cells, namely, capillary-like tube formation (Fig. 5), invasion (Fig. 6A), and migration (Fig. 6B). Wf-536 significantly inhibited tumor cell-induced angiogenesis in vivo under daily administration at doses of 0.3–3 mg/kg (Fig. 4), the same doses as had been found to inhibit tumor metastasis (Table 1). Since ROCK regulates the formation of stress fibers and the activation of focal adhesion of endothelial cells, the Rho-ROCK signaling pathway would appear to play an important role in angiogenesis (Uchida et al., 2000). The anti-angiogenic action of Wf-536 appears therefore to be involved in its inhibitory effect on tumor metastasis and to depend on the modulation of angiogenic functions by blockade of ROCK in endothelial cells.

Although the endothelia of many organ systems remain in a quiescent, nonproliferative phenotype under normal conditions during adult life, the motile and proliferative activities of endothelial cells are increased at points where angiogenesis has been induced (Folkman and Shing, 1992; Schwartz and Liaw, 1993). Angiogenesis is believed to be induced by appropriate stimulation of endothelial cells by angiogenic factors. Many angiogenic factors activate the intracellular signaling molecules located in the downstream of the stimulation, which regulate various cellular physiological functions including cell proliferation. For example, angiogenic factors such as vascular endothelial growth factor (VEGF) or sphingosine-1-phosphate induce endothelial cell migration, mitogenesis, and survival (Ilan et al., 1998; Hisano et al., 1999). Specifically, VEGF induces the tyrosine phosphorylation of VEGF receptor-2, and sphing-

osine-1-phosphate induces the migration and spread of endothelial cells. In both cases, the contribution of Rho has been proved (Gingras et al., 2000; Okamoto et al., 2000).

In the initiation of angiogenesis, one of the effectors of Rho, ROCK is on the major signal-transduction pathway in endothelial cells, which is responsible for cellular motility rather than proliferation (Uchida et al., 2000). In the present study, Wf-536 was effective in vivo in the inhibition of angiogenesis induced by B16BL6 as well as by LLC (Fig. 4), and in endothelial cells which inhibited capillary-like tube formation, invasion, and migration at 1–30 μM (Figs. 5 and 6), without cytotoxicity or affecting proliferation (Fig. 7). These findings suggest that the anti-angiogenic effect of Wf-536 is specific to endothelium activated with angiogenic development regardless of which tumor and that Wf-536 may have no effect on normal endothelial cell survival and proliferation. They also suggest that, of the many endothelial cell functions essential for angiogenesis, Wf-536 may specifically regulate the motile and the morphological functions.

Wf-536 inhibited tumor-induced angiogenesis in mice implanted with the tumor-bearing gelatin sponge (Fig. 4). In the beginning of this assay system, tumor cells are completely separated from the host tissues, including endothelium, by the gelatin sponge. In advance of the tumor growth in this system, the implanted tumor cells generate formation of vessels by inducing the angiogenic actions of the host endothelial cells. As shown in the experiments of endothelial cells in vitro, Wf-536 specifically inhibits the critical steps of angiogenesis, such as invasion, migration and capillary-like tube formation. These facts suggest that Rho-ROCK signaling pathway is mainly involved in the initial processes of angiogenesis associated with tumor growth.

The prevention of angiogenesis is believed to bring about an inhibitory effect on tumor growth. In the present study, however, Wf-536 showed no effect on the weight of the primary tumor site in mice subcutaneously injected with tumor cell suspension (Table 1). It appears that Wf-536 did not sufficiently present anti-angiogenic effect in this model. In a preliminary experiment when tumors were implanted subcutaneously into the dorsal scapular region, Wf-536 treatments for 14 days showed sign of a slight decrease in the tumor volume with reduction rate of 24% (tumor volume at day 14, mean ± S.E.M. (mm^3): 218.57 ± 23.19 and 165.32 ± 26.32, vehicle and Wf-536 3 mg/kg/day treatment, respectively; no significance by Student's *t*-test; $n = 10$ and 9, respectively). We speculate that this inhibitory action may be dependent on the anti-angiogenic action of Wf-536. The difference of the effect of Wf-536 between these in vivo assay models may be dependent on the site, the method of tumor implantation, and the period of the experiment.

In conclusion, the anti-angiogenic activity and inhibitory activity on tumor cell motile functions observed in Wf-536 in the present study suggest that this compound prevents tumor metastasis by inhibiting angiogenesis as well as tumor motility, and indicate its potential for future clinical development.

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