

Cysteinyl Leukotriene Receptor Antagonists Inhibit Tumor Metastasis by Inhibiting Capillary Permeability

Masako Nozaki,^{1,2,3} Masanobu Yoshikawa,¹ Kunihiro Ishitani,² Hiroyuki Kobayashi,^{1,3} Kiyohiro Houkin,⁴ Kohzoh Imai,⁵ Yoichiro Ito^{3,6} and Takamura Muraki^{3,7}

¹Department of Clinical Pharmacology, Tokai University School of Medicine, Kanagawa, Japan

²Higashi Sapporo Hospital, Sapporo, Japan

³Inflammation Research Center Co. Ltd., Sapporo, Japan

⁴Department of Neurosurgery, Sapporo Medical University School of Medicine, Sapporo, Japan

⁵Sapporo Medical University School of Medicine, Sapporo, Japan

⁶Biochemistry and Biophysics Center, National Heart, Lung, and Blood Institute, National Institutes of Health, Maryland, USA

⁷Department of Pharmacology, Tokyo Women's Medical University School of Medicine, Tokyo, Japan

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We explored the possibility of the cysteinyl leukotriene receptor antagonists, pranlukast and montelukast, preventing tumor cell migration through both cerebral and peripheral capillaries. To study tumor cell migration through brain capillaries, male Fisher rats were cannulated via the cisterna magna under pentobarbital anesthesia. RCN9 cells labeled with a fluorescent marker PKH67 were intravenously administered following arachidonic acid administration into the subarachnoid space, and specimens of the central nervous system were collected every 30 min for 8 h. Arachidonic acid increased the fluid volume with elevated white blood cell and RCN9 cell counts. When given 2 h before arachidonic acid administration, pranlukast, but not montelukast, reduced the fluid volume and inhibited white blood cell and RCN9 cell extravasation through the brain capillary. In addition, a Lewis lung carcinoma metastasis model in mice was used to study the inhibitory effect of pranlukast and montelukast against cancer cell extravasation through general capillaries. When mice were given food containing either pranlukast or montelukast, immediately after paw amputation, tumor metastasis was prevented by both drugs, and their survival was prolonged. These results show that pranlukast can inhibit tumor cell migration through both the brain and peripheral capillaries, whereas montelukast inhibits tumor cell migration only in the peripheral capillaries. (Keio J Med 59 (1) : 10–18, March 2010)

Keywords: pranlukast, montelukast, cysteinyl leukotriene receptor antagonist, metastasis inhibition, capillary permeability

Introduction

Metastasis is the primary cause of death in most cancer patients. Nevertheless, an anti-tumor metastasis agent is not yet available for clinical use.

It is known that selectins, integrins, cadherins, and CD44 play an important role in hematogenic tumor me-

tastasis. Since it has been reported that the adhesion of cancer cells to vascular endothelial cells is a key step in tumor cell extravasation,^{1,2} many studies have focused on the inhibition of the binding of sialyl Lewis x and sialyl Lewis a antigen with E- and P-selectins on the activated endothelium by using competitive ligand inhibitors, metabolic carbohydrate mimetics, or neutralizing

monoclonal antibodies. However, these efforts have not yet produced any fruitful results. Kannagi³ suggested that the inhibition of E-, P-, and L-selectin might block tumor cell extravasation.

Cysteinyl leukotriene receptor antagonists such as pranlukast, montelukast, and zafirlukast are well known inhibitors of slow-reacting substance of anaphylaxis (SRSA), a mixture of LTC₄, LTD₄, and LTE₄, and hence, useful for treating asthma and allergy by suppressing both capillary permeability and white blood cell (WBC) extravasation. This fact led us to consider that these cysteinyl leukotriene receptor antagonists might also inhibit tumor metastasis because cell extravasation is a common event in inflammation and tumor metastasis.

In our present study, 2 animal models are proposed to investigate cell extravasation during the process of inflammation and tumor metastasis in rats and mice. *In vivo* morphological studies revealed useful information on the process of tumor cell extravasation. Using these animal models, our studies were mainly focused on demonstrating the anti-metastatic action of 2 leukotriene receptor antagonists, i.e., pranlukast and montelukast. Possible clinical applications of these drugs are also discussed.

Materials and Methods

Tumor cell lines and culture conditions

A rat colon cancer cell line (RCN9) was obtained from Riken BRC Cell Bank, Tsukuba, Japan, and a mouse Lewis lung carcinoma (LLC) cell line was purchased from Health Science Research Resources Bank, Osaka, Japan. Cells were cultured at 37°C under 5% CO₂ in PRMI-1640 (SIGMA, St. Louis, MO, USA) supplemented with 10% fetal bovine serum, 2 mM L-glutamine (Gibco, Grand Island, NY, USA), 100 units penicillin, and 100 µg/ml streptomycin (Gibco).

Animals

Male Fisher 344 rats (weighing approximately 400 ± 50g) and female BDF₁ mice (6 weeks old) were purchased from Sankyo Lab-Service Co. (Sapporo, Japan). Animals were maintained in a ventilated, temperature-controlled (24 ± 1°C) envelopment, on a bed of wood shavings with free access to water and food and were subjected to a 12-h light-dark cycle. The present experiments were in compliance with the guidelines for animal care followed at the Sapporo Medical University School of Medicine.

Chemicals

Sodium pentobarbital (Nembutal injection), a product of Abbott Laboratories (Abbott Park, Chicago, IL) and

PKH67 Green Fluorescent Cell Linker Kit, a product of Zynaxis Cell Science Inc., were purchased from Dainippon Sumitomo Pharmaceutical Co. (Osaka, Japan). Arachidonic acid (sodium salts) from porcine liver was obtained from Calbiochem (San Diego, CA, USA). Dextran (M.W. 190,000–230,000) was obtained from Nacalai Tesque Inc. (Kyoto, Japan), and 2-methylbutane was obtained from Kishida Chemical Co., Ltd. (Osaka, Japan). Malinol, a mounting agent, was obtained from Muto Pure Chemicals Co., Ltd. (Tokyo, Japan). Tissue-Tek OCT compound was obtained from Sakura Finetek (Tokyo, Japan). Sterile phosphate buffered saline (PBS), which contains 144 mg (=1.06 mM) of KH₂PO₄, 9,000 mg (=154 mM) of NaCl, and 795 mg (=5.60 mM) of Na₂HPO₄ in 1,000 ml of H₂O was obtained from Cambrex Charles City Inc. (IA, USA). ONON, containing 112.5 mg/capsules of pranlukast, was purchased from Ono Pharmaceutical Co. (Osaka, Japan). Singulair tablets (10 mg) that contain 10 mg/tablet of montelukast and are a product of Merck & Co. Inc. (White House Station, NJ, USA) were purchased from Banyu Pharmaceutical Co., Ltd. (Tokyo, Japan).

Instruments

A cryostat (Tissue Tek Cryo 2,000; Bayer Co., NY, USA), fluorescence microscopes (ProvisAX x 80; Olympus, Tokyo, Japan and Axiovert S 100; Zeiss, Germany), and a stereotaxic unit (Type SRS-6; Narishige Scientific Instrument Lab, Tokyo, Japan) were used.

Miscellany

Polyethylene tube (SP-10; I.D.: 0.28 mm; O.D.: 0.61 mm) was obtained from Natsume Seisakusho Co. (Tokyo, Japan).

The morphological clarification of tumor cell extravasation in vivo

1) RCN9 cell extravasation through the brain capillary

Rats were anesthetized using sodium pentobarbital (50 mg/kg i.p.). A femoral vein was exposed for the intravenous injection of cancer cells. The rats were then placed in a stereotaxic unit. An 18-gauge needle was inserted through the dura via the cisterna magna, and a cannula (SP-10) was introduced into the subarachnoid space (**Fig. 1A**). The cannula was fixed with cyanoacrylic glue to the surrounding peripheral tissue. Arachidonic acid (816 ng/2 µl of PBS) was administered through the cannula, and subsequently, specimens of the CNS (CNS specimens) were collected.

RCN9 cells that originated from the Fisher rats were stained with a fluorescent marker PKH 67 linker kit. These cells (between 1.88×10^4 and 2.43×10^4) were suspended in PBS, which amounts to approximately

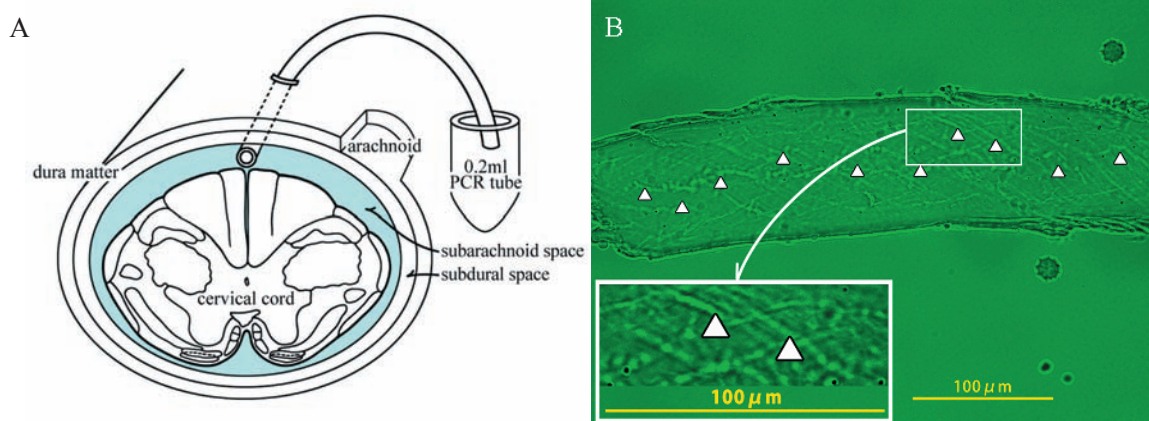


Fig. 1 An SP-10 polyethylene tube inserted into rat cisterna magna was used for administration of arachidonic acid and collection of cerebrospinal specimens (A). Capillary of pia mater was observed by fluoromicroscopy 30 min after intracerebrospinal injection of arachidonic acid in rats administered without (B) RCN9 cells. Arrowheads show the endothelial cell junction (B).

1/100th of the number of neutrophils in the body of each rat, were intravenously administered immediately before arachidonic acid injection to the subarachnoid space. RCN9 cells in the CNS specimen were examined under a fluorescent microscope (Axisovert S 100), and the cells with a flat, square shape were counted.

2) RCN9 cell extravasation in the general capillary

RCN9 cells were intravenously administered immediately before intradermal injection of 0.1 ml of 0.01% dextran into the rat's hind paw; this induces edema without WBC extravasation. The fresh tissues of the paw were harvested 15–30 min after dextran injection and carefully embedded into OCT compound in a plastic mold, while taking care to avoid trapping air bubbles. The mold was covered with a piece of flat metal, soaked in 2-methylbutane, and frozen with liquid nitrogen for 30 seconds. Then, 10- μ m-thick frozen sections were obtained using the cryostat, mounted on the slides, dried on the heating plate for 30 seconds and finally fixed with a mounting agent (malinol). These frozen sections of the rat paw were examined for the presence of RCN9 cells under a fluorescent microscope (ProvisAX x 80).

The pharmacological studies evaluating cysteinyl leukotriene receptor antagonists in the arachidonic acid-induced inflammation and tumor metastasis

Pranlukast (450 mg/kg) or montelukast (10 mg/kg) was administered orally 2 h before arachidonic acid injection into the subarachnoid space. Equipotent doses of each cysteinyl leukotriene receptor antagonist were chosen taking into account the daily dose recommended for clinical use. RCN9 cells were intravenously administered immediately before arachidonic acid injection. A CNS specimen was collected every 30 min after arachidonic acid injection and the volume of CNS specimen,

WBC counts, and RCN9 cell counts were measured for a period of 8 h.

Effect of cysteinyl leukotriene receptor antagonists on a spontaneous metastasis model of LLC cells

LLC cells (5×10^5) were inoculated into the footpad of BDF₁ mice for a passage culture. When the diameter of tumors reached 10 mm, the tumor-cell-inoculated foot was amputated under ether anesthesia. Then, the mice were given powdered food containing pranlukast (0 mg/kg, 7.5 mg/kg, 41.25 mg/kg, 225 mg/kg, 450 mg/kg, and 675 mg/kg) or montelukast (0 mg/kg, 0.178 mg/kg, and 10 mg/kg). These doses were adjusted mg/kg/day by changing the drug concentration in the powdered food. The survival rate was evaluated 4 months after amputation and used as an indicator for anti-metastatic effect of these leukotriene receptor antagonists on peripheral spontaneous metastasis.

Statistical analysis

All data were summarized, and we analyzed the total volume of the CNS specimen and counts of WBC and RCN9 cells collected over an 8-h experimental period. Comparison between the means of multiple groups were analyzed by the Mann-Whitney's *U*-test using SPSS 16.0J for Windows (SPSS Inc. Chicago, IL, USA) in pharmacological studies for evaluating cysteinyl leukotriene receptor antagonists on arachidonic acid-induced inflammation and tumor metastasis.

Effect of cysteinyl leukotriene receptor antagonists on a spontaneous metastasis model of LLC cells was analyzed by plotting Kaplan-Meier survival curves⁴ with log-rank and Wilcoxon test using PRISM ver.5 software package (GraphPad Software, Inc., La Jolla, CA, USA). Significance was set at $P < 0.05$.

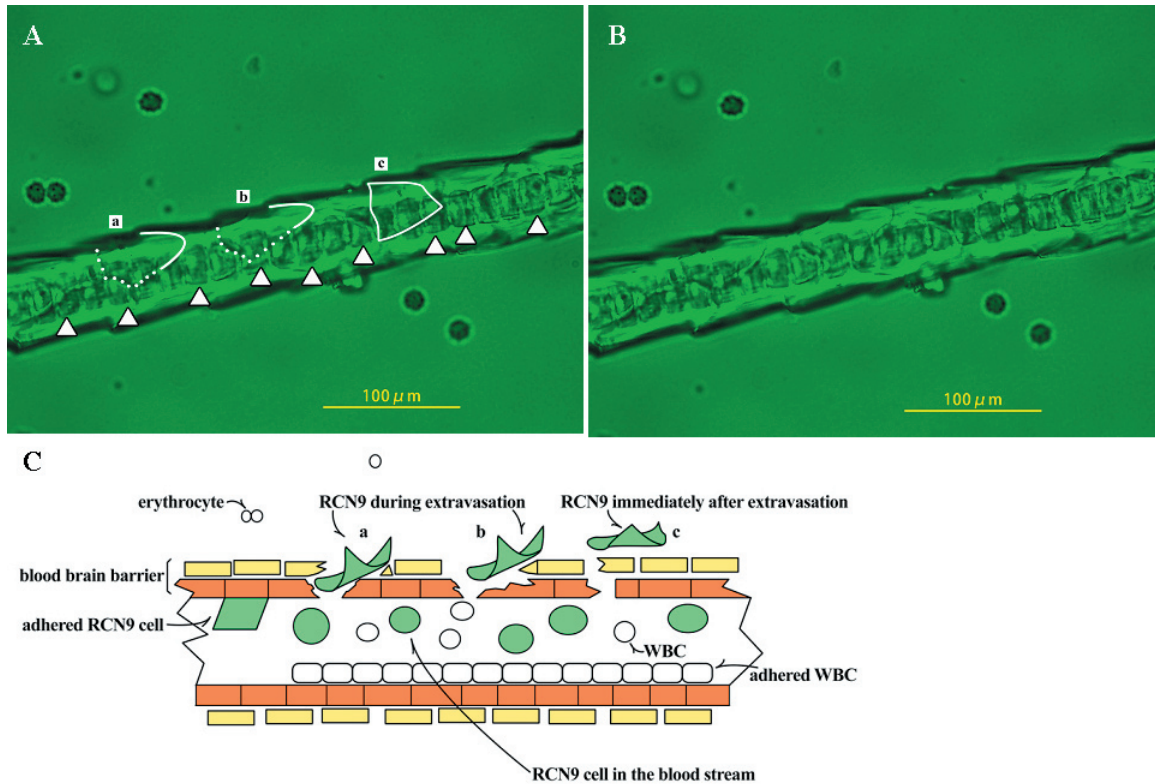


Fig. 2 Images of the process of RCN9 extravasations (A and B) and their schematic presentation (C). Contour of RCN9 cells is traced (A).

Results

Morphological studies

1) RCN9 cell extravasation through the brain capillary

The morphological changes in the brain capillaries were studied by intravenous injection of the fluorescent marker solution free of RCN9 cells and subsequent injection of arachidonic acid into the subarachnoid space (**Fig. 1B**). A piece of the brain capillary was obtained in the CNS specimen within 30 min of arachidonic acid injection. Arrowheads pointing towards the irregular gaps indicate the openings of the blood brain barrier caused by arachidonic acid. Arachidonic acid induced RCN9 cell extravasation in the brain capillary (**Fig. 2A** and **2B**). **Figure 2C** was the schematic presentation for the image of **Figure 2A**. The process of transmigration of cancer cells from the brain capillaries was revealed morphologically by comparing the abovementioned images. The outlines of cancer cells at the inside and the outside of the brain capillary are traced by the dotted and the solid lines, respectively (**Fig. 2A**), on the original picture (**Fig. 2B**). RCN9 cells (a and b) in **Figure 2A** and **2C** are shown escaping from the brain capillary. RCN9 cell c in **Figure 2A** and **2C** has extravasated. As observed in the CNS specimen, the RCN9 cell after extravasation is flat and square shaped. Dark square-shaped substances

shown with arrowheads in **Figure 2A** are neutrophils adhered to the endothelial cells. **Figures 3A** and **3B** are representative images of RCN9 cells obtained within 30 min after extravasations from the brain capillary in the CNS specimen.

2) RCN9 cell extravasation through the general capillary

The morphological changes of cancer cells were examined in the general capillary in the rat paw edema induced by dextran. **Figure 4A** shows RCN9 cells attached to the inside of the peripheral capillary. Arrowheads show RCN9 cells that were adhered to the gaps of endothelial cells. The shape of the RCN9 cells had changed from spherical to flat and square before transmigration through the junctions of endothelial cells. **Figure 4B** shows RCN9 cells (shown by arrowheads) during the transmigration through the general capillary; however, neither white blood cell adhesion nor the transmigration in the peripheral capillary is induced by dextran (**Fig. 4A** and **4B**).

The pharmacological studies evaluating cysteinyl leukotriene receptor antagonists in increased capillary permeability and WBC extravasation in the inflammation induced by arachidonic acid

Arachidonic acid (816 ng/2 μl) injection into the sub-

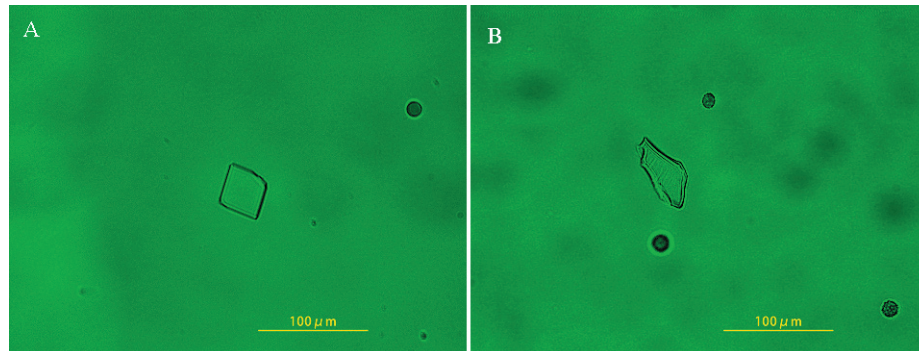


Fig. 3 Representative images of RCN9 cells (A and B) in the CNS specimen within 30 min after extravasations.

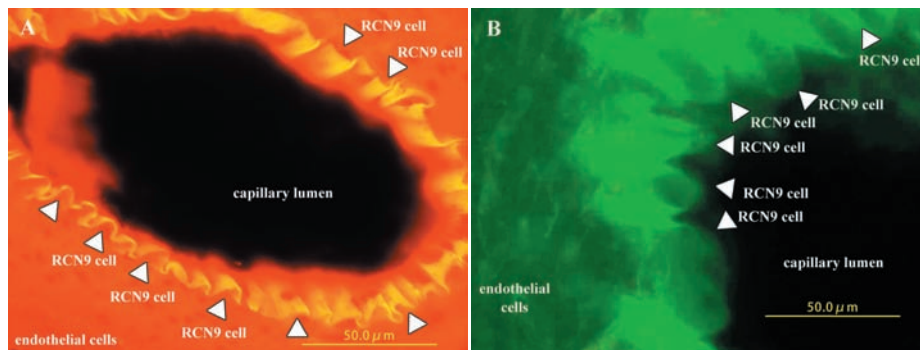


Fig. 4 Histological section of dextran-induced edema in rat foot pad. Arrowheads show RCN9 cells. The general capillary is shown with (A) or without orange color filter (B).

arachnoidal space immediately increased capillary permeability, which continued throughout an 8-h experimental period (**Fig. 5A**). On the other hand, WBC counts started to increase 30–60 min after arachidonic acid injection, maintaining its high level until the end of the experimental period (**Fig. 5A**). Remarkably, pranlukast inhibited both increased capillary permeability (**Fig. 5B** and **6A**) and WBC extravasation (**Fig. 5B** and **6B**) induced by arachidonic acid in the brain capillary, whereas montelukast exerted no such inhibitory effect under otherwise identical experimental conditions (**Fig. 5C**), instead, it caused an enhancement of both capillary permeability and WBC extravasation (**Fig. 6A**)

The effect of cysteinyl leukotriene receptor antagonists on RCN9 cell extravasation induced by arachidonic acid

When RCN9 cells were intravenously injected immediately before arachidonic acid to the subarachnoidal space, RCN9 cell extravasation was observed in the CNS specimen immediately after arachidonic acid injection (**Fig. 5D**). The RCN9 cell counts peaked at 30 min after arachidonic acid injection and then decreased sharply (**Fig. 5D**). Both, increased capillary permeability and RCN9 cell extravasation were inhibited by pretreatment with pranlukast (450 mg/kg) (**Fig. 5E** and **6A**), but not

with montelukast (10 mg/kg) (data not shown).

The effect of leukotriene receptor antagonist on a spontaneous metastasis model of Lewis lung carcinoma cells

The lung metastasis experiment was performed to determine the survival rate of mice for up to 4 months after paw amputation. Lung cancer metastasis was proven by gross examination of the lung after the death of mice. All the mice that did not receive the cysteinyl leukotriene receptor antagonist died within 1 month. The survival rates of mice treated with pranlukast (7.5–675 mg/kg) (**Fig. 7A** and **Table 1**) and montelukast (0.178–10 mg/kg) (**Fig. 7B** and **Table 1**) treatments were as high as 66–100%, although they were not dose dependent.

Discussion

A quantitative method to determine increased capillary permeability of the brain microvasculature and extravasation of both WBCs and tumor cells is described in this paper. This method includes subarachnoidal injection of arachidonic acid followed by collection of CNS effluent from the cisterna magna. We assume that the CSF effluent consists mainly of inflammatory exudates from the brain capillary. It provides reliable data because collect-

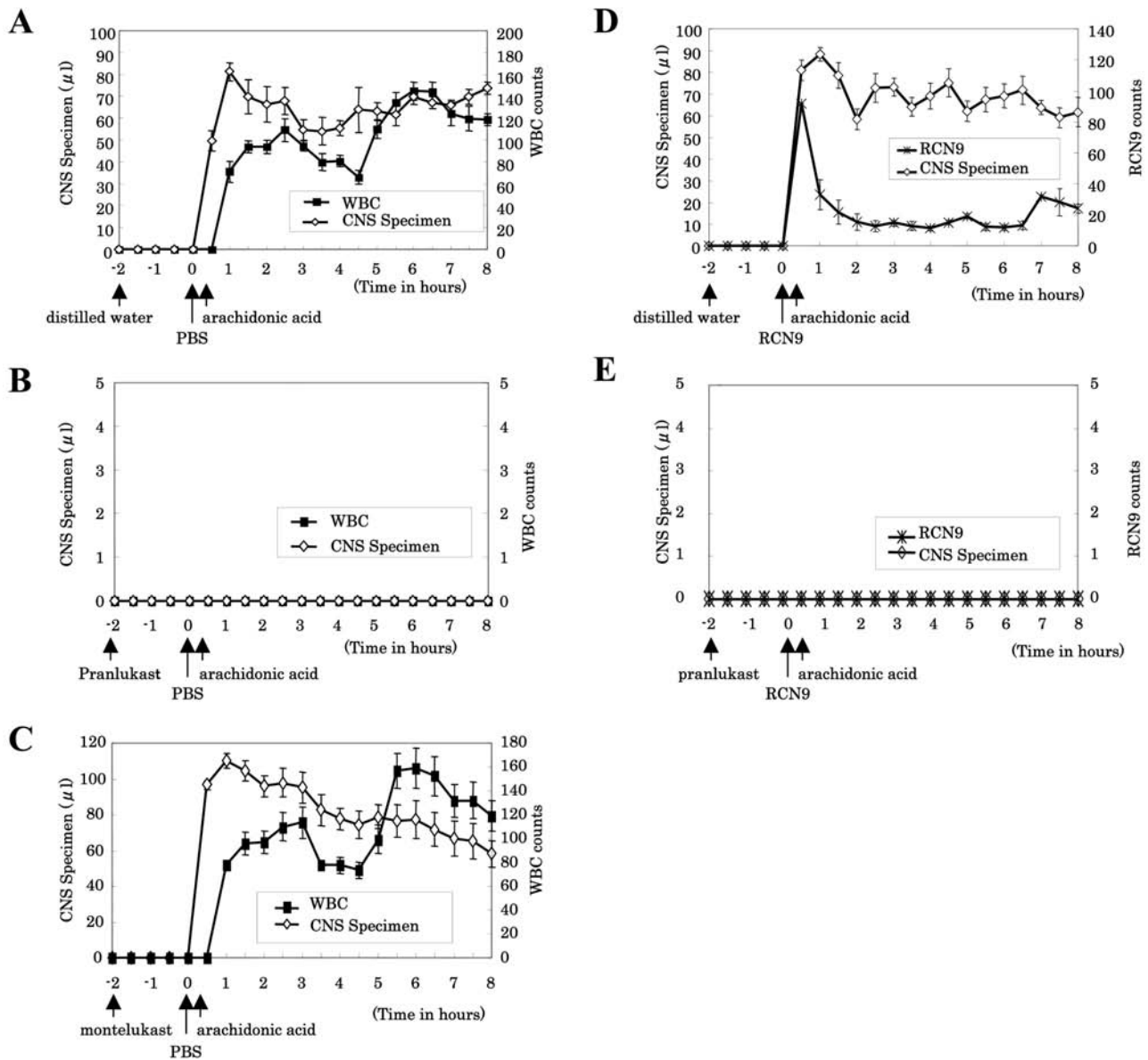


Fig. 5 Time-dependent effect of pranlukast and montelukast on the arachidonic acid-induced CNS specimen (μl) (\diamond), counts of WBC (\blacksquare) (A, B, C), and RCN9 cells ($*$) (D, E) are shown. Symbols represent the mean and the s.e.m. $n = 6$. Rats were pretreated with pranlukast (B, E), montelukast (C), or distilled water (A, D), and CNS specimen was collected every 30 min up to 8 h after arachidonic acid injection.

ing CNS specimens from the same set of animals at given time intervals can eliminate statistical variations that could arise while sampling between individual animals. This method was first applied to determine the phase that is inhibited by drugs during the process of inflammation and tumor metastasis. Furthermore, this method can be used as a tool to clarify the mode of action of the anti-inflammatory and/or anti-tumor metastatic drugs.

Our *in vivo* morphological studies on both the brain and general capillary revealed that cancer cells can pass through narrow gaps in between the capillary endothelial cells by flattening themselves. The ability of cancer cells

to change their shape from spherical to flat and square may facilitate transmigration through the gap between endothelial cells shortly after arachidonic acid administration; the transmigration of WBCs had a delay of over 30 min. The fact that cancer cell extravasation started immediately after a noxious stimulus such as arachidonic acid injection suggests the possibility of causing acute metastasis directly through the capillary rather than forming emboli as generally believed. Therefore, it may be necessary for cancer patients to start taking cysteinyl leukotriene receptor antagonist drugs before the surgery to inhibit this form of acute metastasis.

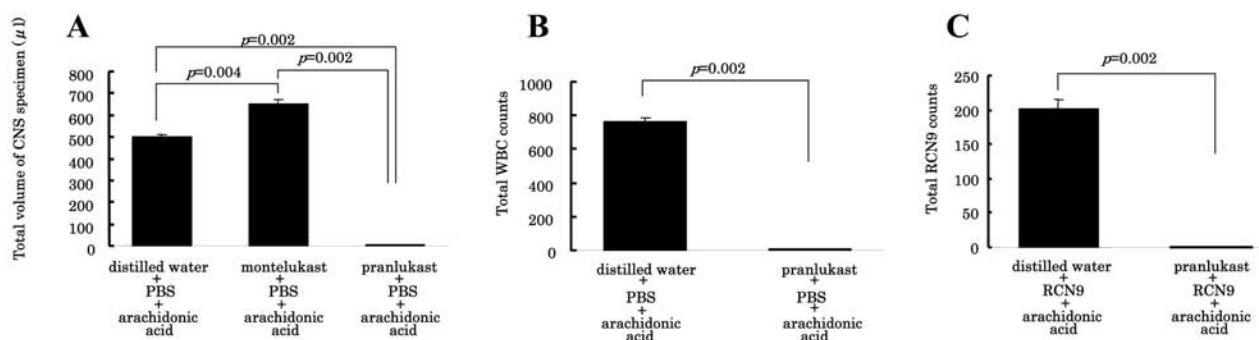


Fig. 6 The summary of the results of pharmacological effects of cysteinyl leukotriene receptor antagonists on inflammation and tumor cell migration induced by arachidonic acid in the brain capillary. Total volume of cerebrospinal specimen (A) and counts of WBC (B) and RCN9 cells (C) examined over an 8-h experimental period are calculated from Figure 3(A–E). Bars represent the mean and the s.e.m. $n = 6$.

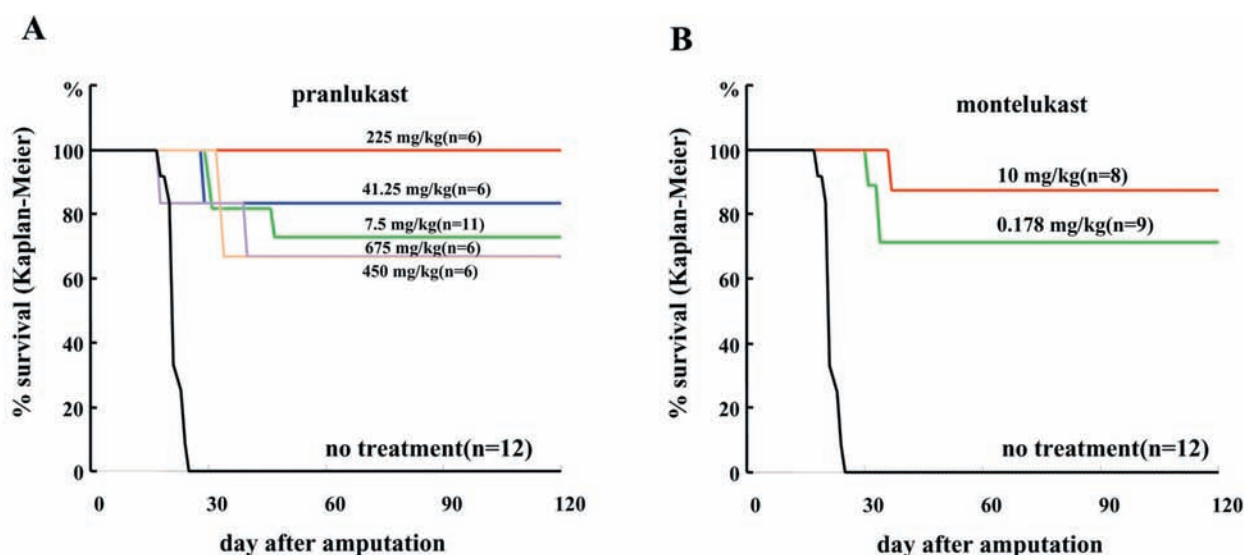


Fig. 7 Effect of pranlukast (A) and montelukast (B) on the Kaplan-Meier survival curve of a spontaneous metastasis model in mouse for 4 months after amputation of the paw inoculated with Lewis Lung Carcinoma cells. Log rank test and Wilcoxon test are shown as inset. $n = 6-12$.

Our morphological studies do not focus on the effect of cysteinyl leukotriene receptor antagonists on dextran-induced cancer cell extravasation since dextran-induced paw edema is not recognized as an inflammation because of a lack of WBC extravasation. The only reason of using dextran-induced edema is to demonstrate how cancer cells change their shape during transmigration through the general capillary without the effects of other factors such as WBC extravasation.

This is the first report showed that pranlukast, but not montelukast, is able to inhibit increased capillary permeability and extravasation of both WBCs and tumor cells through the brain capillary. As the blood brain barrier has continuous tight junctions, drug penetration into the brain must depend on transcellular rather than paracellular transport. Therefore, it is necessary to overcome this limitation of the blood brain barrier to deliver anti-in-

flammatory agents and/or anti-tumor metastasis agents that otherwise do not cross the blood brain barrier, and for this reason, there is no drug available for the clinical treatment of encephalic inflammation and brain tumor.⁵ Drugs that open the blood brain barrier would cause extravasation of blood components from the brain capillary to the CNS because of the large difference between blood pressure (80–140 mm Hg) and intracranial pressure (7–15 mm Hg), thereby giving rise to adverse physical conditions in the patients. Since cysteinyl leukotriene receptors are present at the outside of the brain capillary, cysteinyl leukotriene receptor antagonists that do not, or only minimally, cross the blood brain barrier⁶ should have no effect on the brain capillary. In fact, the present study demonstrated that montelukast, which is known as a pure cysteinyl leukotriene receptor antagonist, failed to inhibit the extravasation of both WBCs and cancer cells

Table 1 Summary of statistics of Kaplan-Meier survival curves

dose(mg/kg)	log rank test	Gehan-Breslow Wilcoxon test
pranlukast		
7.50	$p < 0.0001$	$p < 0.0001$
41.25	$p < 0.0001$	$p < 0.0006$
225.00	$p < 0.0001$	$p < 0.0006$
450.00	$p < 0.0001$	$p < 0.0006$
675.00	$p < 0.0001$	$p < 0.0001$
montelukast		
0.175	$p < 0.0001$	$p < 0.0001$
10.00	$p < 0.0001$	$p < 0.0001$

in the brain capillary. The results of our study suggest that arachidonic acid-induced capillary permeability might be further increased by pretreatment with montelukast because of an unknown mechanism. This unexpected finding should be confirmed by future studies.

The inhibitory effect of pranlukast against both inflammation and tumor metastasis through the brain capillary might be attributed to some particular mode of action via peripheral and/or the interior of the blood vessel, including the endothelial cells and the capillary lumen. Pranlukast might block VEGF^{7,8} and/or glycoprotein in the blood vessels, because over 98.9% of pranlukast binds to plasma proteins after oral administration in rats.⁹ Therefore, it is possible that pranlukast inhibits not only tumor metastasis but also tumor growth.

Because of the effect of leukotriene receptor antagonists on a spontaneous metastasis model of the LLC cells, both montelukast and pranlukast treatments produced increased survival rates, suggesting that these leukotriene receptor antagonists may inhibit peripheral tumor metastasis. Since the inhibitory effect of pranlukast and montelukast on the permeability in the general capillary have been well documented and used for the treatment of asthma and allergy,^{10–16} the inhibitory effect on the general capillary was not tested in the present study.

Hoff, *et al.* reported that the adhesion of cancer cells to vascular endothelial cells is considered to be a key step in tumor cell extravasation,^{1,2} Kobayashi, *et al.*¹⁷ clarified that metastasis is inhibited by the prevention of cancer cell adhesion to the endothelial cells because of the blocking E-selectin expression, and Kannagi³ proposed that inhibiting the expression of P-, E-, and L-selectins induces a blockade of metastasis. We speculate that pranlukast suppresses expression of all selectins on the vascular endothelial cells, thereby inhibiting tumor cell adhesion and extravasation.

While the process of tumor metastasis and inflammation, once started, is progressive and consecutive, enhanced capillary permeability is a prerequisite, enabling cell extravasation through the capillary. Hence, suppressing the capillary permeability with pranlukast is considered to be the most effective means of preventing these pathological events. Because the mediators that increase

capillary permeability are same to those that activate E- and P-selectin on the vascular endothelial cells pranlukast might block not only leukotriene receptor but also the mediators.

Cimetidine was first reported as an anti-tumor metastatic drug in 1979.¹⁸ Matsumoto¹⁹ reported that cimetidine had a beneficial effect on survival in colorectal cancer patients, the inhibitory effect of cimetidine (100 mg/kg administered intramuscularly) on increased capillary permeability was studied using our rat model and was found to be potent. It suggests that cimetidine might be useful in inhibiting acute metastasis during surgeries for colorectal cancer patients. Although no severe adverse effects were described in their study, long-term use of cimetidine has, in general, adverse side effects, such as inhibition of P450 system, headache, and gynecomastia. In contrast pranlukast has long been used for treating asthma chronically with little adverse effects.²⁰

The clinical applications of pranlukast, as suggested by the present study, include prevention of systemic tumor metastasis and treatment of brain edema, secondary injuries in cerebral circulation failure, and systemic inflammation such as sepsis.

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