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# How platelet aggregation affects B16BL6 melanoma cell trafficking

Chieko Koike[1,a], Manabu Watanabe[a], Atsushi Isoai[b], Hiromichi Kumagai[b], Hideo Tsukada[c], Tatsuro Irimura[d], Shoji Okada[a], Naoto Oku[a,*]

[a]*School of Pharmaceutical Sciences, University of Shizuoka, 52-1 Yada, Shizuoka 422-8526, Japan*
[b]*Asahi Glass Co., Ltd., Research Center, Kanagawa-ku, Yokohama 221, Japan*
[c]*Hamamatsu Photonics, Central Research Laboratory, Hamakita, Shizuoka 434, Japan*
[d]*Faculty of Pharmaceutical Sciences, The University of Tokyo, Bunkyo-ku, Tokyo, Japan*

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**Abstract In blood-borne metastasis, intravasated metastatic tumor cells are thought to localize at the target site via a series of processes involving platelet aggregation, adhesion to endothelium, and invasion through the basal membrane. In the present study, we examined how platelet aggregation contributes to the trafficking of metastatic tumor cells in vivo by use of an inhibitor of platelet aggregation. Highly invasive B16BL6 melanoma cells were labeled with [2-$^{18}$F]2-fluoro-2-deoxy-D-glucose and injected into mice to determine cell trafficking non-invasively by positron emission tomography. Both platelet aggregation inhibitor cyclo(RSarDPhg), which could not inhibit metastasis, and metastatic inhibitor cyclo(GRGDSPA) suppressed the accumulation of B16BL6 cells in the lung by about 12%, suggesting that platelet aggregation partly affects cell trafficking but not to a great extent, and that platelet aggregation is not the essential step for B16BL6 cell arrest in targets.**

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*Key words:* Positron emission tomography; Tumor metastasis; Cell trafficking; Platelet aggregation; RGD

## 1. Introduction

Complex multiple processes are known to be responsible for the formation of metastasis of blood-borne tumor cells. However, the contribution of each event such as platelet aggregation, invasion, and adhesion to extracellular matrices (ECM) to metastatic cell trafficking has not been fully understood because of a lack of methodology to determine cell trafficking non-invasively. Using positron emission tomography (PET), we recently developed a non-invasive method to analyze real-time metastatic tumor cell trafficking [1]. In the present study, the effect of inhibitors of metastatic processes, namely, one for platelet aggregation and the other for invasion, on tumor cell trafficking was investigated. A previous study showed that cyclic RGD-Phenylglycine had a strong inhibitory activity toward tumor cell-induced platelet aggregation, although it had no anti-metastatic effect nor inhibited adhesion to ECM [2]. We employed a cyclic octapeptide dimer of R-Sarcosine-D-Phenylglycine (RSarDPhg), cyclo(RSarDPhg), in the present study instead of cyclic RGD-Phenylglycine. Another inhibitor, cyclo(GRGDSPA) [3], is active toward metastatic cell adhesion to basal membranes. It contains the cell adhesion recognition domain of fibronectin, i.e. the RGD sequence. This peptide caused a significant reduction in metastasis, as well as in cell adhesion to fibronectin and vitronectin in vitro at a low dose in comparison with that induced by linear GRGDSPA [3]. Here, we determined the effect of these peptides on trafficking of the highly lung-metastatic and invasive B16BL6 melanoma. The results indicate how tumor cell-induced platelet aggregation contributes to metastatic cell trafficking.

## 2. Materials and methods

### 2.1. Cells

B16BL6 melanoma cells, a highly lung-metastatic subline of B16 cells, were cultured in Dulbecco's modified Eagle and Ham's F12 (1:1) medium supplemented with 10% fetal calf serum (JRH Biosciences, Lenexa, KS) under a humidified atmosphere of 5% $CO_2$ in air. For PET experiment, the cells were labeled with [2-$^{18}$F]2-fluoro-2-deoxy-D-glucose ([2-$^{18}$F]FDG) by the method described previously [4]. In brief, B16BL6 cells were washed with glucose-free medium and incubated with [2-$^{18}$F]FDG for 30 min at 37°C. After removal of the free [2-$^{18}$F]FDG, the cells were detached from the plate with EDTA solution to obtain a single cell suspension. The size of single cells determined after EDTA treatment was $17.6 \pm 1.6$ μm. Cells of less than 15 passage levels were used in all experiments.

### 2.2. Adhesion assay

Adhesion of the melanoma cells to fibronectin was determined as described previously [3]. In brief, fibronectin was coated onto tissue culture wells for 30 min at 37°C, and BSA was added to each well. Melanoma cells ($1 \times 10^5$) were added to each well and incubated for 1 h at 37°C in the presence or absence of various concentrations of peptides. Adhering cells after having been washed with PBS were trypsinized and counted. Thus, fifty percent inhibition dose was calculated.

### 2.3. Platelet aggregation assay

Platelet aggregation assay was performed as described previously [2]. In brief, human platelet-rich plasma was obtained from healthy volunteers. The plasma (200 μl) was incubated for 2 min in a cuvette under constant stirring in an aggregometer (SSR Engineering Co. Model PAT-2A) after the addition of 25 μl of PBS or peptides diluted with PBS. Then, 25 μl of ADP solution was added, and the change in light transmittance was monitored for 10 min. Thus, fifty percent inhibition dose was calculated.

### 2.4. Metastasis assay

B16BL6 melanoma cells ($1 \times 10^5$ cells/100 μl) were co-injected with 100 μl of peptide solution (1 mg peptides) or 100 μl of PBS into syngeneic 7-week-old C57BL/6 female mice (six per group, Japan SLC, Inc.) via a tail vein. Fourteen days after the injection, the lungs were excised, and the number of metastatic colonies on the lung surface was counted.

*Corresponding author. Fax: +81 (54) 264 5705.
E-mail: oku@momo1.u-shizuoka-ken.ac.jp

[1]Present address: Children's Hospital and Harvard Medical School, Department of Surgery, Boston, MA, USA.

*Abbreviations:* PET, positron emission tomography; ECM, extracellular matrices; [2-$^{18}$F]FDG, [2-$^{18}$F]2-fluoro-2-deoxy-D-glucose; ROI, region of interest

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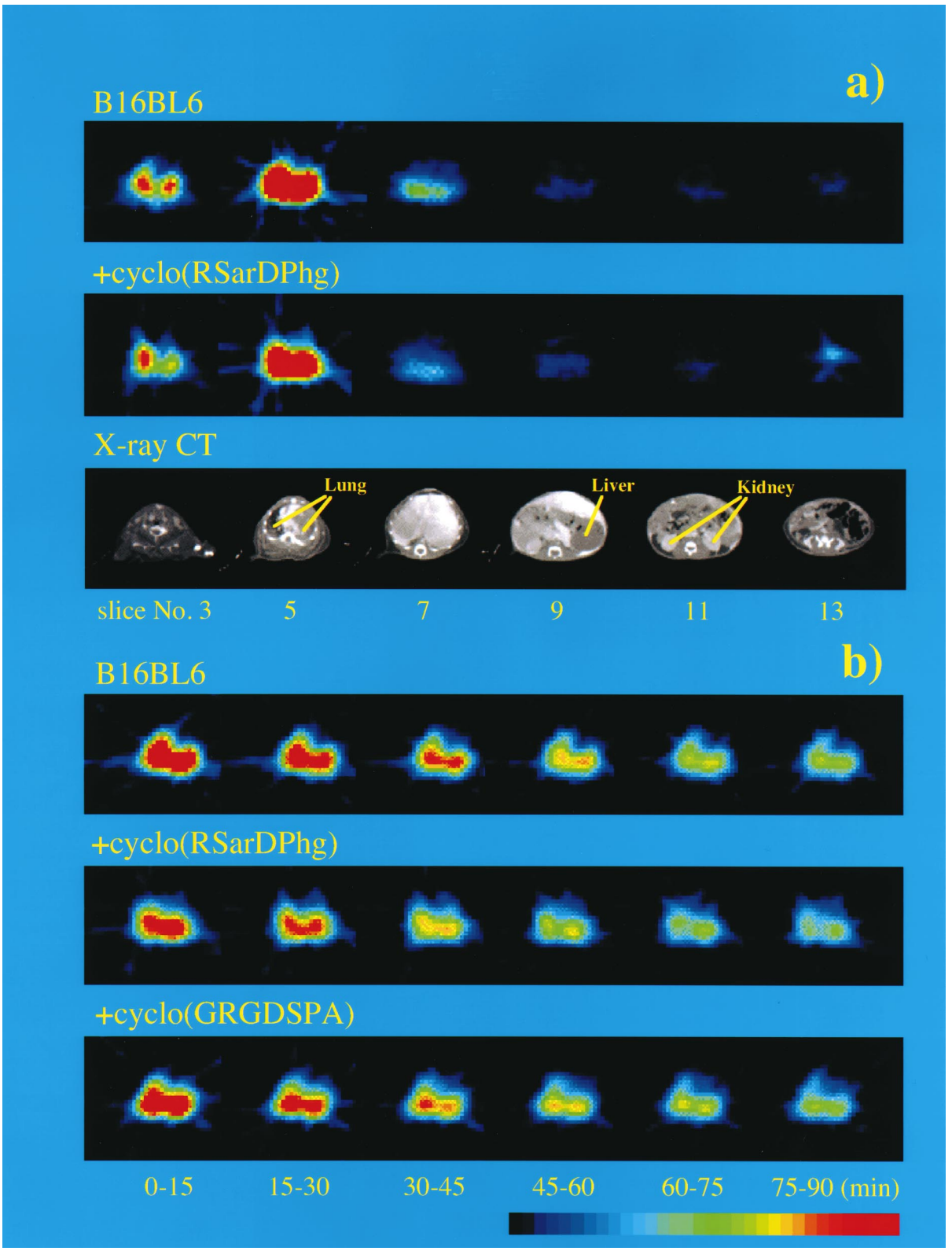


Fig. 1. PET images of B16BL6 melanoma cell accumulation in mice in the presence of cyclo(RUDPhg) or cyclo(GRGDSPA). a: Accumulation of [2-$^{18}$F]FDG-labeled B16BL6 cells in mice after co-injection with 1 mg of cyclo(RSarDPhg) (middle panel) or injection of cells only (upper panel). Imaging by PET was done as described in Section 2. Images show the accumulation of the cells in lungs, liver, and kidney during 30 min after the administration of them. The upper and middle panels show PET images of mice injected with [$^{18}$F]FDG-labeled B16BL6 cells, and the lower one shows X-ray CT images of a mouse of similar age presented as a reference for the PET images. b: Accumulation of [2-$^{18}$F]FDG-labeled B16BL6 cells in lungs of mice treated or not with cyclo(RSarDPhg) or cyclo(GRGDSPA), each at 1 mg. Images show the status of the accumulation of the cells every 15 min till 90 min after the injection of them. The three panels show PET images of lungs of mice injected with [$^{18}$F]FDG-labeled B16BL6 cells (upper), or the cells plus cyclo(RSarDPhg) (middle) or cyclo(GRGDSPA) (lower).

*2.5. PET analysis*

Melanoma cells labeled with [2-$^{18}$F]FDG ($7.5\times10^5$ cells in 150 μl DME/F12 medium, 555–925 kBq) were mixed with 1.5 mg (150 μl) inhibitory peptides or with control peptide composed of 20 amino acid residues derived from c-fos just prior to injection, and 200 μl of the mixed suspension was co-injected into 10-week-old C57BL/6 female mice via a tail vein under anesthesia induced with sodium pentobarbital. Thus, the final amounts of cells and peptides injected were $5\times10^5$ and 1 mg per mouse, respectively. Control mice were each injected with $5\times10^5$ cells in 200 μl of the above medium. The emission scan of PET was started immediately after injection of the cells and performed for 120 min with an animal PET camera (Hamamatsu Photonics, SHR-2000) having a resolution of 2.7 mm. PET images were simultaneously obtained in 14 slices with 3.25-mm apertures where the 6th slice was settled at the xiphisternum. The radioactivity in the form of coincidence gamma photons was measured and converted to Bq/cm$^3$ of tissue volume by calibration after correction for decay and attenuation. A time-activity curve was obtained from the mean pixel radioactivity in the region of interest (ROI) of the composed PET images. The injected dose in each experiment was normalized as 740 kBq throughout the whole study. After every PET measurement, animals were killed for removal of organs and counting of the radioactivity in these organs with an auto gamma counter to confirm the accumulation of cells in each organ. The ROI value of each organ correlated with the result of the direct counting of the radioactivity in each organ. The viability of the remaining cells (i.e. not injected) after administration of the cells was determined at 2 h after initiation of the PET scan by trypan blue dye exclusion, and was more than 90%.

*2.6. X-ray CT*

X-ray CT images of 10-week-old mouse were obtained with slice distances of 3.2 mm.

## 3. Results

Tumor cell-induced platelet aggregation is thought to play an important role in the metastatic pathway [5]. The precise time schedule for platelet aggregation is unknown, however. Therefore, we investigated the effect of cyclo(RSarDPhg), an inhibitor of tumor cell-induced platelet aggregation, on cell trafficking. Characteristics of peptides tested in this experiment are as follows: Fifty percent suppression doses of cell adhesion to fibronectin by cyclo(GRGDSPA) or by cyclo(RSarDPhg) were 10 μM and over 200 μM, and those of platelet aggregation induced by ADP were 9.0 μM and 0.1 μM, respectively. When inhibitory effect of the peptides on metastasis was evaluated by the reduction of lung colonization number as described in Section 2.4, cyclo(GRGDSPA) having the RGD sequence caused 54.7% reduction, possibly due to their inhibition of cell adhesion to the ECM, whereas cyclo(RSarDPhg) did not cause any reduction of tumor metastasis.

The effect of cyclo(RSarDPhg), as well as that of cyclo(GRGDSPA), on B16BL6 cell trafficking was analyzed by PET. Fig. 1a shows the typical images for the first 30 min of B16BL6 cell accumulation in mice after injection of the cells. As shown in the figure, B16BL6 cells accumulated mainly in lungs. B16BL6 cells also accumulated mainly in lungs when they were co-injected with either cyclo(RSarDPhg) (Fig. 1a) or cyclo(GRGDSPA) (data not shown). Since the metastatic cells mainly accumulated in lung tissue, time-dependent changes in the accumulation in the lungs was determined. Fig. 1b shows the cumulative images for every 15 min of B16BL6 cell accumulation in the lungs after injection of the cells. Cell accumulation in this organ decreased time dependently. The accumulation of B16BL6 cells in the lungs was slightly suppressed in the presence of both kind of peptides.

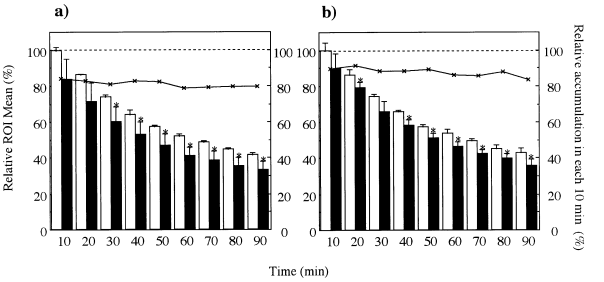


Fig. 2. Relative accumulation of $^{18}$F in lungs during every 10 min after injection of mice with [2-$^{18}$F]FDG-labeled B16BL6 cells mixed with cyclo(RSarDPhg) or cyclo(GRGDSPA). Time-activity curves of $^{18}$F in lungs of separate experiments ($n=4$) after injection of [2-$^{18}$F]FDG-labeled B16BL6 cells treated (closed bar) or not (open bar) with 1 mg of cyclo(RSarDPhg) (a) or 1 mg of cyclo(GRGDSPA) (b) were obtained. The average accumulation of $^{18}$F per min was calculated from every 10-min accumulation of $^{18}$F except immediately after injection in which case 2-min to 10-min accumulation of $^{18}$F was used. Percent accumulation of $^{18}$F was then calculated where the 100% accumulation was used as the first 10-min accumulation of $^{18}$F in the control mice. Relative accumulation of B16BL6 cells treated with the peptides against the control was also calculated for each point time after the injection (×). Significant difference against control; *, $P<0.05$.

We also examined the effect of synthetic peptide composed of 20 amino acids derived from the c-fos product on the trafficking, and this peptide did not affect the accumulation of B16BL6 cells in the lungs (data not shown). The FDG released by the corruption of cells in vivo is known to be distributed immediately to the liver and kidney but not to the lungs [1,4].

Fig. 2a summarizes the ROI mean of every 10 min obtained from four independent experiments and also shows the relative ROI mean for B16BL6 cells in the presence of cyclo(RSarDPhg) against that obtained for B16BL6 cells alone. The values did not change throughout each PET scan, suggesting that cyclo(RSarDPhg) suppressed lung accumulation of B16BL6 to some extent immediately after injection and that the suppressing activity did not change for at least 90 min. Fig. 2b shows the data of a similar experiment in which cyclo(RSarDPhg) was replaced with cyclo(GRGDSPA). Cyclo(GRGDSPA) also suppressed lung accumulation of B16BL6 to some extent and the suppressing activity did not change for at least 90 min.

## 4. Discussion

Tumor cell-induced platelet aggregation is considered to be involved in the metastatic pathway as follows: (1) tumor cell arrest in the vasculature; (2) promotion of tumor cell extravasation; (3) enhancement of tumor cell adhesion to extracellular matrix; and (4) stimulation of tumor cell proliferation. Intravasated metastatic tumor cells lead to local metastasis through induction of platelet aggregation, adhesion to the vessel endothelium, and invasion into local tissues. The rate of each of these processes, however, is not known. One reason is that there was previously no suitable non-invasive method to determine cell trafficking without changing the physiological conditions. We previously established a method that enables real-time analysis of cell movement in vivo from immediately after injection in a living animal by use of PET

[1,4]. In this current study, we investigated the effect of inhibitors of the processes of blood-borne metastasis on tumor cell trafficking.

Integrin αIIbβ3 is thought to be involved in tumor cell-induced platelet aggregation, and thus RGD-related peptides may inhibit tumor cell-induced platelet aggregation via binding to the integrin on platelets [6,7]. In the RGD sequence, the structure of the guanidium and carboxylate groups of Arg and Asp, respectively, with a restricted distance is thought to be important [8]. Interestingly, among the compounds having such a structure, cyclo(RSarDPhg) has inhibitory activity toward platelet aggregation but no anti-metastatic activity. Therefore, we investigated the effect of this peptide on the tumor cell trafficking. Cyclo(RSarDPhg) caused a slight reduction in the lung accumulation of B16BL6 cells. The evidence indicated that platelet aggregation is a fast event after intravasation of tumor cells.

Cyclo(RSarDPhg) has no anti-metastatic potential, whereas cyclo(GRGDSPA) does. Therefore, the metastasis of B16BL6 melanoma is not inhibited by the inhibition of platelet aggregation alone. In other words, tumor cell-induced platelet aggregation may not be an essential step for establishment of metastasis, at least in the case of B16BL6 melanoma metastasis, as it was previously suggested [2]. The result of B16F10 melanoma cell movement determined by in vivo videomicroscopy also indicated that platelet did not appear to play any role in the cell arrest [9]. Some previous reports, however, showed that tumor cell-induced platelet aggregation is critical for metastasis establishment. In fact, tumor cell-induced platelet aggregation-inhibiting agents [10], peptides [11], or antibody [12,13] have been known to suppress tumor metastasis. Similarly, lipopolysaccharide-induced platelet starvation reduced the metastatic potential of murine fibrosarcoma [14]. Therefore, it is possible that platelet aggregation plays an important role in establishment of metastasis of some tumors, but not essential to some other tumors such as B16BL6. It is also possible that 1 mg cyclo(RSarDPhg) is not enough to suppress platelet aggregation in vivo. However, this is unlikely because cyclo(RSarDPhg) slightly but significantly reduced tumor cell arrest in the target tissues, suggesting that the peptide actually affected cell trafficking.

The RGD sequence is known as a recognition domain of fibronectin, and is also a ligand for various integrins such as α5β1, αvβ3, αIIbβ3, α2β1, αvβ1, αvβ5, αvβ6, and α3β1 [15–17]. RGD-related peptides are also noted for their anti-metastatic effect. The anti-metastatic action of RGD and related peptides is thought to be based on inhibition of adhesion and that of angiogenesis. Interestingly, cyclo(GRGDSPA) inhibited lung accumulation of B16BL6 to the similar extent as cyclo(RSarDPhg) did. Since the platelet aggregation-inhibiting activity of cyclo(RSarDPhg) is about 90-fold stronger than that of cyclo(GRGDSPA), cyclo(GRGDSPA) may inhibit lung accumulation by a different mechanism, although it is also possible that 1 mg/mouse of cyclo(GRGDSPA) is enough to reduce platelet aggregation and that the 12% reduction in B16BL6 accumulation in the lungs by either peptide was caused by the inhibition of platelet aggregation, since cyclization of RGD-related peptide increases the inhibitory activity towards platelet aggregation [3]. Alternatively, cyclo(GRGDSPA) may inhibit lung accumulation through suppression of the interaction between B16BL6 cells and endothelial cells of lung capillaries. In fact, a recent study showed that cyclic RGD inhibited the function of integrin α4β1, which ligand is on the endothelium [18]; and in another study, a different kind of RGD-related peptide, trimesyl$(GRGDS)_3$, which also had anti-metastatic activity, was not observed to affect tumor cell trafficking [19].

In spite of the strong inhibitory activity of cyclo(GRGDSPA) against metastasis, the lung accumulation of B16BL6 melanoma cells was only slightly affected by the compound. Therefore, the population of cells that undergo extravasation may be rather small in the early stage, and invasion may proceed mainly at a later stage. This is consistent with the data reported by Koop and co-workers that more than 80% of melanoma cells survived and extravasated in the chick embryo chorioallantoic membrane at 24 h after i.v. injection [20].

As shown in Fig. 2, B16BL6 cells that had accumulated in the lungs were released from this location time dependently. Part of this loss may have been due to disruption of the cells by immunity, although the release of [2-$^{18}$F]FDG from intact cells may also have contributed to the decrease in $^{18}$F in the lung tissue. High accumulation of metastatic cells in the lungs after injection may indicate the mechanical retardation of cell flow in the lungs because the lungs are the first organ through which i.v. injected cells pass. The difference in accumulation between that in the presence of metastasis inhibitors and that in the absence of them, however, suggests that some interactions between metastatic cells and target organ, rather than platelet aggregation, affect the cell trafficking.

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