Possible role of immune surveillance at the initial phase of metastasis produced by B16BL6 melanoma cells

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Abstract The relationship among the real-time trafficking of lung metastatic B16BL6 cells, metastatic potential, and the injected number of the cells was examined, since the smaller the number of tumor cells injected, the more clearly the immune defense may be observed. When 1×10^6 or 1×10^5 B16BL6 cells were injected into mice via the tail vein, both numbers of cells accumulated in the lung at a similar rate: there was an approximately 10-fold difference in the number of accumulated cells between the two doses. Elimination from the lung was not dependent on the cell number but on the proportion of accumulated cells. However, the injection of 1×10^4 cells resulted in lung accumulation less than one-tenth of that obtained with 1×10^5 cell injection. Metastasis was observed when 1×10^5 or 1×10^6 B16BL6 cells were injected, but not after injection of 1×10^4 cells. To clarify the roles of the immune defense system at the initial phase of metastasis, we challenged macrophage-depleted mice with 1×10^4 tumor cells. Treatment of mice with 2-chloroadenosine prior to the tumor cell challenge cancelled the suppression of not only metastasis but also the lung accumulation. Furthermore, the administration of 2-chloroadenosine following the tumor cell challenge had little effect on the metastatic potential. These results suggest that the immune surveillance whose action was obvious at the low dose of challenged tumor cells functions strongly at the initial phase but not at the advanced stages of the metastatic process, and that macrophages play an important role in the suppression of metastasis.

Key words: Metastasis; Immune surveillance; Macrophage; B16BL6; Positron emission tomography; Tumor cell trafficking

1. Introduction

Metastasis occurs through a complex cascade of events including dissociation of cancerous cells from the primary site, intravasation, adhesion to the vascular endothelium of the target organ, extravasation, and growth at the colonization site [1–3]. On the other hand, host resistance mechanisms for limiting the dissemination and rapid progressive outgrowth of tumor cells may come into play to prevent metastasis. For example, even after surgical removal of the primary tumor, substantial numbers of viable tumor cells are detected in the circulation whereas this finding does not correlate well with subsequent development of detectable metastases [4]. It has also been empirically shown that a certain number of metastatic tumor cells is required for obtaining metastatic foci, and metastasis is not observed after inoculation of the smaller number of cells in experimental metastasis models. Therefore, development of metastases may be the end result of a sequential process including the ability of the metastatic cells to circumvent the immune surveillance of the host. Macrophages and NK cells are thought to be the first line of defense in target organs such as the liver and lung against invading metastatic tumor cells [5], and come into play following the immune reaction involving specific T and B lymphocytes. However, it is not clear when macrophages are able to exclude the metastatic tumor cells in the course of metastasis.

Due to technological developments, we are now able to obtain positron-labeled compounds of more than 1 GBq/ml for labeling tumor cells. Thus now we can examine real-time tumor cell trafficking non-invasively with as few as 10^4 cells after positron labeling of the cells by positron emission tomography (PET). In this study, we investigated the trafficking of lung metastatic B16BL6 cells in relation to the number of injected cells, since the smaller the number of tumor cells inoculated, the more clearly the immune defense system may work. We observed that fast elimination of metastatic tumor cells from the target tissue, lung, resulted in suppression of metastatic potential when a small number of the cells was injected. In addition, to clarify the roles of immune surveillance against metastasis, we investigated the metastatic potential and trafficking of tumor cells in mice treated with 2-chloroadenosine (2-ClAd). This compound is known to exert a lethal effect on mouse adherent peritoneal cells in vitro without affecting mouse lymphocytes and polymorphonuclear cells [6], and is known to enhance lung metastasis of M109 tumor cells after i.p. injection [7]. The results indicate the importance of immune surveillance at the early phase of metastasis.

2. Materials and methods

2.1. Cell culture and determination of metastatic potential

B16BL6 cells, a highly lung metastatic subline of murine B16 melanoma, were cultured in Dulbecco's modified Eagle's medium (DME) and Ham's F12 (1:1) medium (DME/F12M) supplemented with 10% fetal bovine serum (JRH Biosciences, Lenexa, KS, USA) under a humidified atmosphere of 5% CO_2 in air. The metastatic potential of these cells was determined after injection of 1×10^4, 1×10^5, or 1×10^6 cells/0.2 ml via the tail vein of 8-week-old male C57BL/6 mice (five per group, Japan SLC Inc., Hamamatsu, Japan). Coloniza-

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Abbreviations: PET, positron emission tomography; [2-^{18}F]FDG, [2-^{18}F]2-fluoro-2-deoxy-D-glucose; ROI, region of interest; 2-ClAd, 2-chloroadenosine

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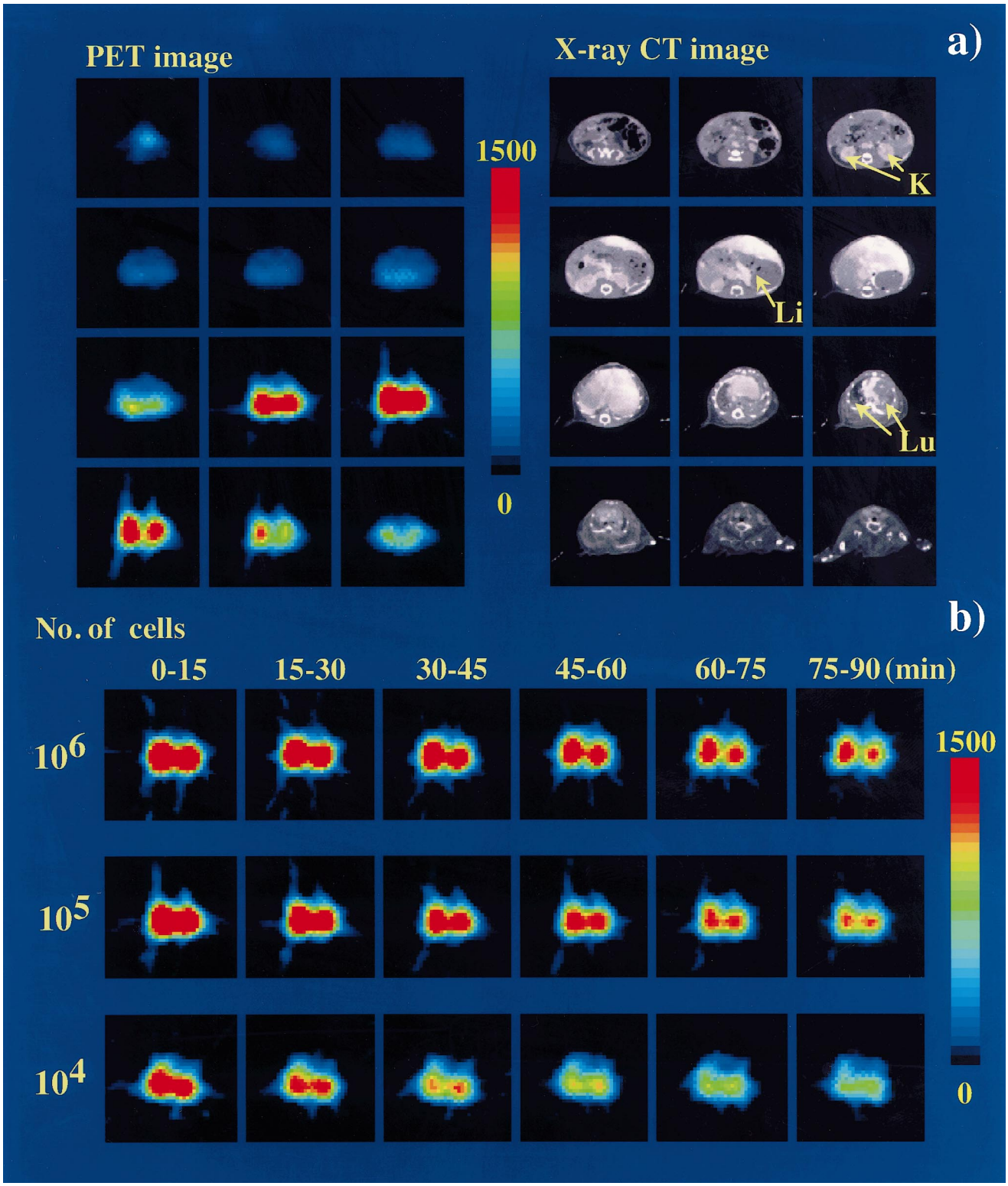

Fig. 1. In vivo trafficking of [2-^{18}F]FDG-labeled B16BL6 cells. a: [2-^{18}F]FDG-labeled B16BL6 cells (1×10^5 cells/0.2 ml) were injected via the tail vein of C57BL/6 mice. Biodistribution during 30 min after administration of the cells was imaged by PET, and corresponding X-ray CT images (depicted from [9]) are shown with a slice aperture of 3.25 mm. Twelve slices from the tail side (upper left) to the head side (lower right) are shown. The experiment was repeated twice, and similar results were obtained in separate experiments, although the images represent a typical result obtained from a single animal. Gradation was corrected to be comparable between all images. Li, liver; Lu, lung; K, kidney. b: B16BL6 cells (1×10^6 cells including 1×10^4 [2-^{18}F]FDG-labeled cells, 1×10^5 cells including 1×10^4 [2-^{18}F]FDG-labeled cells, and 1×10^4 [2-^{18}F]FDG-labeled cells) were injected via the tail vein. The emission scan of PET was started immediately after injection of the cells and was performed for 90 min. Every 15-min accumulation of B16BL6 cells in the lung is shown.

tion of B16BL6 cells in lungs and other tissues was determined at day 14 after injection. The animals were cared for according to the animal facility guidelines of the University of Shizuoka.

2.2. Preparation of cell suspensions for PET analysis

The cells were labeled with [2-^{18}F]2-fluoro-2-deoxy-D-glucose ([2-^{18}F]FDG) as described previously [8,9]. In brief, B16BL6 cells were washed with glucose-free medium and incubated with 1 GBq of [2-^{18}F]FDG for 30 min at 37°C. After the free [2-^{18}F]FDG had been washed away, the cells were removed from the plate with EDTA solution to obtain a single cell suspension. Preparation of cell suspension was as follows: 1×10^4 ^{18}F-labeled cells alone in 0.2 ml of medium or the cells were mixed with 9-fold and 99-fold excess of cold cells, resulting in total cell numbers of 1×10^5 cells and 1×10^6 cells in 0.2 ml of medium, respectively. Viability of the labeled cells was determined by the trypan blue dye exclusion method and was greater than 90% through the entire experiment.

2.3. PET analysis

The animal PET camera (Hamamatsu Photonics, SHR-2000) used in the experiment has an effective slice aperture of 3.25 mm and a resolution of 2.7 mm. After a 30-min transmission scan with 18.5 MBq of ^{68}Ge/^{68}Ga ring source, ^{18}F-labeled single cell suspension was injected into 8-week-old C57BL/6 male mice for B16BL6 cells via the tail vein under anesthesia with sodium pentobarbital. The emission scan of PET was started immediately after injection of the cells and performed for 90 min. The initial 60 frames were taken one every 1 min; and the next 12 frames, one every 2.5 min. Thus 72 frames were taken during a PET scan. PET images were simultaneously obtained in 14 slices where the mouse location was adjusted 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. Time–activity curves were obtained from mean pixel radioactivity in the region of interest (ROI) of the composed PET images where the injected dose was calibrated as 740 kBq. Each experiment was repeated at least twice, and similar results were obtained in repeated experiments, although each figure represents a typical result obtained from a single animal.

3. Results

3.1. Trafficking of B16BL6 cells after injection of various numbers of the cells

The real-time trafficking of B16BL6 cells after injection via the tail vein of C57BL/6 mice was analyzed by PET (Fig. 1). PET images indicated that most of the B16BL6 cells accumulated in the lung, and that essentially none of cells accumulated specifically in heart, kidneys, spleen, or other tissues examined except liver, at which organ a small number of cells were accumulated. The results are consistent with the data previously reported [9]. The PET images indicated that the accumulation of cells in lung after the injection of 1×10^6 or 1×10^5 cells/0.2 ml was more intense than that after injection of 1×10^4 cells/0.2 ml, and that the cells were eliminated from the target organ faster in the latter case than in the former. The time–activity curve of the accumulation of B16BL6 cells in the lung is shown in Fig. 2. The results indicated that the accumulation of cells after injection with 1×10^6 and 1×10^5 cells/0.2 ml was more intense than that with 1×10^4 cells/0.2 ml starting immediately after the injection. Since 99- and 9-fold excesses of cold cells were present in the samples of 1×10^6 and 1×10^5 cell suspensions, respectively, the actual cell numbers for accumulation and elimination numbers for these two doses were 100- and 10-fold greater than those after injection with 1×10^4 cells (Fig. 2b).

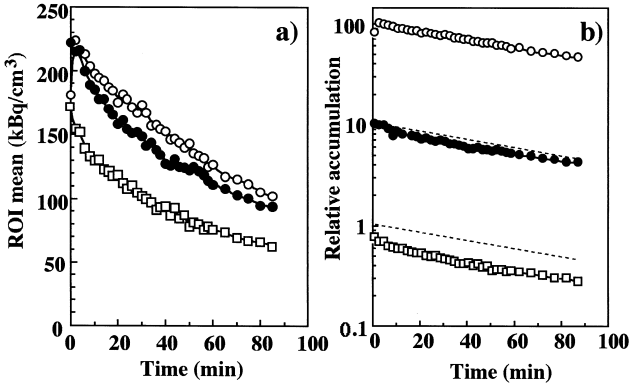

Fig. 2. Time–activity curves of ^{18}F accumulation in lung after injection of [2-^{18}F]FDG-labeled B16BL6 cells. In vivo trafficking of [2-^{18}F]FDG-labeled B16BL6 cells injected via the tail vein was examined as described in the legend to Fig. 1. a: Time–activity curves of ^{18}F accumulation in the lung were determined by PET analysis. ○, injected with 1×10^6 cells; ●, 1×10^5 cells; □, 1×10^4 cells. b: Relative cell accumulation calculated from panel a, where the highest accumulation after injection of 1×10^6 cells is given as 100. Dashed line shows 1/10 and 1/100 amounts of the relative accumulation of cells after injection of 1×10^6 cells. ○, injected with 1×10^6 cells; ●, 1×10^5 cells; □, 1×10^4 cells.

Table 1
Experimental metastasis produced by various numbers of B16BL6 cells

Cell number	Number of metastatic colonies	
	Lung	Liver
1×10^4 cells	0, 0, 0, 0, 0	0, 0, 0, 0, 0
1×10^5 cells	174, 173, 170, 165, 148	1, 0, 0, 0, 0
1×10^6 cells	>300, >300, >300, >300, >300	14, 11, 10, 8, 3

Various numbers of B16BL6 melanoma cells (1×10^4 cells/0.2 ml, 1×10^5 cells/0.2 ml or 1×10^6 cells/0.2 ml) were injected via the tail vein of male C57BL/6 mice ($n=5$), and the number of metastases in the lung and liver was counted 14 days after tumor inoculation.

3.2. Metastatic potential of B16BL6 cells in lung after injection of various numbers of the cells

To examine the correlation between accumulation of tumor cells in the target tissue and metastatic potential, we injected 1×10^6, 1×10^5, and 1×10^4 B16BL6 cells/0.2 ml into mice. Lung metastatic foci of B16BL6 cells were observed after injection at 1×10^6 or 1×10^5 cells (Table 1). In contrast, the injection of 1×10^4 cells/0.2 ml resulted in no metastatic foci at all, even though the number of cells injected was 10% of that in the case of the injection with 1×10^5 cells. Interestingly, injection of 1×10^6 cells caused not only lung metastatic foci but also liver metastatic ones. These results indicate that a certain critical number of metastatic tumor cells is initially required for establishing metastatic colonization in this exper-

Table 2
Effect of 2-ClAd treatment on metastasis of B16BL6 cells

Mice	Number of metastatic colonies	
	Lung	Liver
Non-treated	0, 0, 0, 0, 0	0, 0, 0, 0, 0
2-ClAd-pretreated	37, 11, 3, 3, 1	10, 4, 4, 0, 0
2-ClAd-posttreated	1, 0, 0, 0, 0	0, 0, 0, 0, 0

2-ClAd at 25 mg/kg was administered i.p. 2 days before and after the inoculation of B16BL6 cells (1×10^4 cells/0.2 ml) via the tail vein of C57BL/6 mice ($n=5$). The number of metastatic foci in the lung and liver was counted 14 days after tumor inoculation.

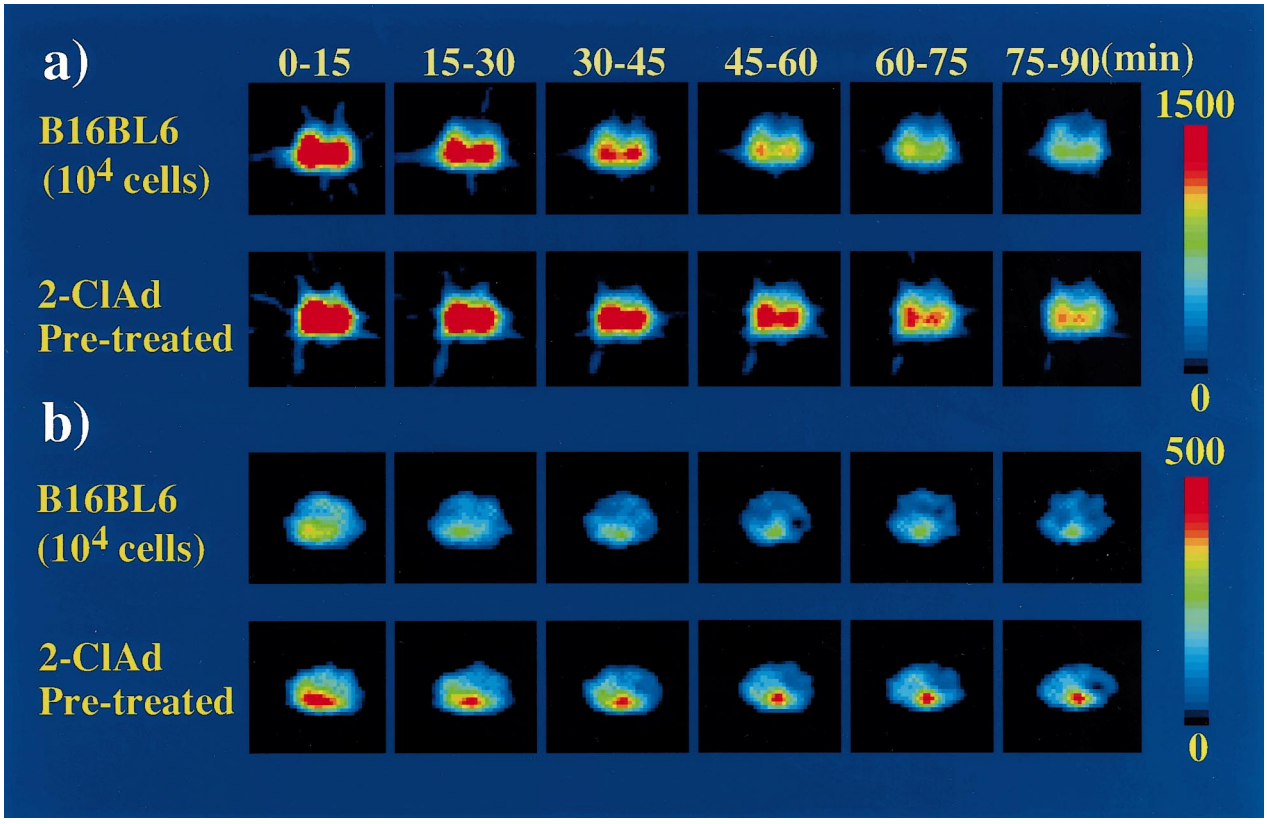

Fig. 3. In vivo trafficking of B16BL6 cells in mice pretreated with 2-ClAd. Eight-week-old BALB/c mice were i.p. pretreated with 25 mg/kg of 2-ClAd or phosphate-buffered saline (PBS). Two days later the mice were injected with [2-^{18}F]FDG-labeled B16BL6 cells (1×10^4 cells/0.2 ml) via the tail vein. The emission scan of PET was performed for 90 min. Every 15-min accumulation of B16BL6 cells in the lung (a) and liver (b) is shown.

imental metastatic model: the critical number may be between 10^4 and 10^5 in the present metastatic model.

3.3. *Effect of 2-ClAd treatment on metastatic potential and tumor cell trafficking*

Since macrophages in the circulation or resident in the specific organs play an important role in immune surveillance against metastasis, we investigated macrophage-mediated resistance to metastasis by use of macrophage-depleted animals. Table 2 shows the metastatic potential of B16BL6 melanoma cells in relation to the timing of 2-ClAd treatment and tumor challenge. The most dramatic increase in metastasis was observed in mice treated with 2-ClAd prior to the tumor inoculation. This observation is consistent with the previous study reported by Zhang and coworkers [10]. In addition, metastatic foci could be detected in the liver even after the injection of 1×10^4 cells. In contrast, administration of 2-ClAd 2 days after the tumor inoculation had little effect on the colonization caused by B16BL6 cells.

On the basis of the results shown above, we employed PET analysis to determine the real-time trafficking of the tumor cells in 2-ClAd-pretreated mice. As shown in Fig. 3, the accumulation of B16BL6 cells in the lungs of 2-ClAd-pretreated mice was higher than that in the non-treated ones after the cells had been injected via the tail vein. In addition, the accumulation of the cells in liver was also increased for 2-ClAd-pretreated mice. The lung accumulation pattern of 1×10^4 cells injected into the 2-ClAd-pretreated mice was similar to that of 1×10^6 cells injected into non-treated mice shown in Fig. 2 (Fig. 4). Therefore, these results indicate that macrophages may influence tumor metastasis through affecting tumor cell trafficking at the initial phase of hematogenous dissemination, presumably by their ability to eliminate directly the tumor cells from the circulation in the blood or at the capillary beds in the target organ.

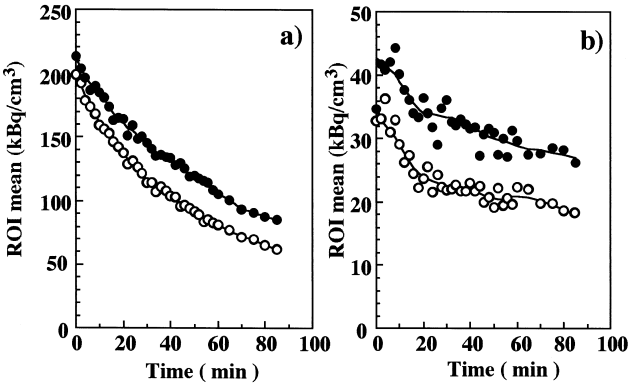

Fig. 4. Time–activity curves of ^{18}F accumulation in lung and liver after injection of [2-^{18}F]FDG-labeled B16BL6 cells into mice pretreated with 2-ClAd. Trafficking of [2-^{18}F]FDG-labeled B16BL6 cells was examined by PET after injection of the cells into i.p. pretreated with 25 mg/kg of 2-ClAd (●) or PBS (○) as described in the legend to Fig. 3. The time–activity curves of ^{18}F in the lung (a) and liver (b) are shown.

4. Discussion

In the early stage of blood-borne metastasis, metastatic tumor cells intravasating from the primary site may adhere to and extravasate through the vascular endothelium of the target organ [11–13], and this adhesion process is thought to be a prerequisite for metastasis establishment. The interaction of metastatic cells with the endothelium may be initiated via the interaction between selectin molecules and their ligands such as sialylglyco-conjugates [14,15]. During circulation and the adhesion of tumor cells, host cells, such as lymphocytes, NK cells, and monocytes, which are important for the disruption of tumor cells [16–19], may interact with them. Therefore, the ability of the metastatic cells to circumvent the immune surveillance of the host organ is important for the establishment of metastases.

In the present study, we examined the trafficking of B16BL6 cells after injection via the tail by PET analysis. The analysis reflects physiological phenomena under actual blood flow conditions where the fluid mechanical forces are taken into account in the microcapillary environment [9,20]. As shown in Fig. 1, accumulation of ^{18}F-labeled cells in lung, the target organ, after injection of 1×10^6 cells including 1×10^4 ^{18}F-labeled cells was quite similar to that after injection of 1×10^5 cells including 1×10^4 ^{18}F-labeled cells. These results indicate that the amount of tumor cells accumulated in a target organ does not depend on the number of the cells injected but on the proportion of the cells: when 10-fold cells were injected, about 10-fold cells accumulated. Similar phenomena are usually observed in cell adhesion assays in vitro: the percent adhesion of certain tumor cells to endothelial cells or extracellular matrix components is little dependent on cell number. A correlation between the number of tumor cells injected and the accumulation of the cells in the target organ was only observed when 1×10^6 and 1×10^5 cells were injected, and the accumulation rate decreased markedly after injection of 1×10^4 ^{18}F-labeled cells.

Since the effect of host cells against metastatic tumor cells may be more obvious when the number of metastatic cells is small enough to be attacked, we investigated the metastatic potential in relation to the number of challenging tumor cells in the experimental metastatic model. When 1×10^6 or 1×10^5 metastatic tumor cells were injected, apparent metastasis was observed. In contrast, metastasis was not demonstrated after injection of 1×10^4 cells: if one-tenth of the population of the case 1×10^5 cells injected, some metastatic foci would have been detectable. These data suggest that the formation of metastases requires a certain number of tumor cells for avoidance of the host defense mounted by cells such as NK cells and monocytes, and that host immune surveillance influences the metastatic spread of tumors during the initial phase of metastasis.

The presumption of tumor cell trafficking during metastasis has not been fully confirmed. In addition, it is unclear when macrophages can influence the metastatic tumor cells in the process of metastasis under actual blood flow conditions. Thus, we next examined the effect of the immune defense system such as the function of macrophages on tumor cell trafficking in relation to tumor challenge and the time of 2-ClAd treatment. As shown in Table 2, the most drastic increase in metastasis occurred when mice were treated with 2-ClAd 2 days prior to tumor inoculation. This result also correlated well with the trafficking of the cells: PET analysis demonstrated a marked increase in the accumulation of B16BL6 cells in the lungs of mice pretreated with 2-ClAd. In addition, 2-ClAd pretreatment caused the enhanced accumulation of the cells in liver, which phenomenon is consistent with the liver colonization of B16BL6 cells shown in Table 2. In contrast, administration of 2-ClAd following tumor inoculation had little or no effect on metastasis. This fact may be explained as follows: once tumor cells have invaded the parenchyma of the target organ, macrophages would have less influence on the tumor cells. Thus, macrophages could eliminate metastatic tumor cells from the bloodstream or from the target organ only in the early phase of the metastatic process. Moreover, it is known that tumor cells grow and secrete transforming growth factor β and prostaglandin E2 in the advanced stages of the metastatic process, both of which are known to reduce the cytotoxic action of macrophages in the tumor area [5].

Our present data demonstrated that the drastically lower metastatic potential of 1×10^4 cell injection in comparison with that of the higher numbers of injected cells correlated with the accumulation of the cells in the target organ as determined by the PET analysis. This result might be attributable to the removal of this smaller number of tumor cells by the immune defense system such as the function of macrophages. The cytotoxic/cytostatic activity of macrophages is attributed to the up-regulation of inducible nitric oxide synthase and the production of nitric oxide (NO) [21–23], phagocytic actions [24], and the action mediated by membrane-bound tumor necrosis factor α [25]. However, the elimination of tumor cells seemed to be quite fast compared with the reported time course of in vitro cell lysis. Hence, NO may not be responsible for the early elimination of tumor cells by macrophages in vivo.

Taken together, the data presented here allow us to conclude that immune surveillance against metastatic tumor cells functions strongly at the initial phase of the metastatic process but not at the advanced stage, and that macrophages, at least, may play an important role in this immune surveillance.

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