Inhibition of Metastatic Tumor Growth in Mouse Lung by Repeated Administration of Polyethylene Glycol-Conjugated Catalase: Quantitative Analysis with Firefly Luciferase-Expressing Melanoma Cells

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ABSTRACT

Purpose: To develop a novel and effective approach to inhibit tumor metastasis based on controlled delivery of catalase, we first evaluated the characteristics of the disposition and proliferation of tumor cells. Then, we examined the effects of polyethylene glycol-conjugated catalase (PEG-catalase) on tumor metastasis. On the basis of the results obtained, PEG-catalase was repetitively administered to completely suppress the growth of tumor cells.

Experimental Design: Murine melanoma B16-BL6 cells were stably transfected with firefly luciferase gene to obtain B16-BL6/Luc cells. These cells were injected intravenously into syngeneic C57BL/6 mice. PEG-catalase was injected intravenously, and the effect was evaluated by measuring the luciferase activity as the indicator of the number of tumor cells.

Results: At 1 hour after injection of B16-BL6/Luc cells, 60 to 90% of the injected cells were recovered in the lung. The numbers decreased to 2 to 4% at 24 hours, then increased. An injection of PEG-catalase just before inoculation significantly reduced the number of tumor cells at 24 hours. Injection of PEG-catalase at 1 or 3 days after inoculation was also effective in reducing the cell numbers. Daily dosing of PEG-catalase greatly inhibited the proliferation and the number assayed at 14 days after inoculation was not significantly different from the minimal number observed at 1 day, suggesting that the growth had been markedly suppressed by the treatment.

Conclusions: These findings indicate that sustained catalase activity in the blood circulation can prevent the multiple processes of tumor metastasis in the lung, which could lead to a state of tumor dormancy.

INTRODUCTION

Tumor metastasis is the major cause of death in cancer patients. It can be roughly divided into the following steps: tumor cell dissociation, invasion, intravasation, distribution to distant organs, arrest in small vessels, adhesion to endothelial cells, extravasation, invasion of the target organ and proliferation (1). Adhesion of circulating tumor cells to capillary endothelial cells is a crucial event in the retention of tumor cells in a specific organ (2). Initial interactions between tumor cells and endothelium activates both tumor cells and endothelial cells through cytokines, free radicals, bioactive lipids, and growth factors, leading to the increased expression of adhesion molecules, which strengthens the initial adhesive bonds (3, 4). Reactive oxygen species (ROS), such as hydrogen peroxide (H2O2), superoxide anion and hydroxyl radical, are well known regulators of such adhesion molecules (5–7).

In most cases, the lung is the first organ that tumor cells detached from primary tumors encounter, making it a major site for tumor metastasis. We have shown that an experimental pulmonary metastasis of colon carcinoma cells in mice can be effectively inhibited by polyethylene glycol-conjugated catalase (PEG-catalase; ref. 8). The number of metastatic colonies on the lung surface was significantly lower in mice treated with PEG-catalase than in untreated (saline-injected) mice. However, the mechanism of this inhibition is not clear because ROS are involved in various metastatic processes, such as adhesion (5, 6), invasion (9–11), and proliferation (12). Counting visible metastatic colonies on the tissue surface is not sensitive enough to evaluate these early processes of tumor metastasis. In an attempt to circumvent this problem, radiolabeled tumor cells are sometimes used to trace their disposition in vivo, but cell death as well as the release of radiolabeled compounds from cells make it very difficult to analyze the disposition of tumor cells. Furthermore, tumor growth cannot be evaluated by this approach.

Labeling of cells with any protein by introducing its gene has been applied to studies of tumor metastasis. This technique is very promising in evaluating tumor metastasis because the protein introduced can be tumor cell-specific. Thus far, several authors have already used this kind of experimental system to...
show the growth of tumor cells in vivo (13, 14) and metastasis by in vivo imaging (14–17). However, there have been few investigations of the early processes of tumor metastasis, such as the embolization, adhesion, and invasion, using these reporter gene-labeled tumor cells. These processes cannot be evaluated by in vivo imaging because of a lack of sensitivity and its less quantitative nature. Reporter gene assays can solve these problems if tumor cells are stably transfected at a high level of specific activity. In addition, the reporter gene expression should be constant under a variety of conditions to use the activity as a scale for the number of tumor cells in vivo.

In the present study, we first developed clones of murine melanoma B16-BL6 cells by transfecting firefly luciferase gene. The characteristics of the transfectant, B16-BL6/Luc cells, were examined in vitro as well as in vivo, and we found that the transfectant can be used to examine the disposition and proliferation of tumor cells in vivo. Therefore, the processes of tumor metastasis were examined in mice by measuring the luciferase activity in the lung after intravenous administration of the cells. Finally, the effects of catalase and PEG-catalase on early as well as later processes of tumor metastasis were evaluated in this system. To our knowledge, this is the first report showing that repeated injection of PEG-catalase almost completely suppresses the growth of metastatic tumors in the lung. These findings suggest that tumor dormancy may be induced by continuous administration of PEG-catalase.

MATERIALS AND METHODS

Animals. Male C57/BL6 (6-week-old) mice were purchased from the Shizuoka Agricultural Cooperative Association for Laboratory Animals (Shizuoka, Japan). Animals were maintained under conventional housing conditions. All animal experiments were conducted in accordance with the principles and procedures outlined in the United States NIH Guide for the Care and Use of Laboratory Animals. The protocols for animal experiments were approved by the Animal Experimentation Committee of Graduate School of Pharmaceutical Sciences of Kyoto University.

Chemicals. DMEM and HBSS were obtained from Nissui Pharmaceutical (Tokyo, Japan). Fetal bovine serum was obtained from Biowhittaker (Walkersville, MD). Bovine liver catalase (C-100, 40,000 units/mg) was purchased from Sigma (St. Louis, MO). A product of PEG [2,4-bis (o-methoxypolyethylene glycol)-6-chloro-s-triazin] was obtained from Seikagaku Corporation (Japan), and PEG-catalase and inactivated catalase were synthesized and their enzymatic activity measured as reported previously (18). All other chemicals were of the highest grade commercially available.

Tumor Cells. Murine melanoma B16-BL6 tumor cells (19), obtained from the Cancer Chemotherapy Center of the Japanese Foundation for Cancer Research (Tokyo, Japan), were grown in DMEM supplemented with 10% heat-inactivated fetal bovine serum, 0.15% NaHCO3, 100 units/mL penicillin, and 100 μg/mL streptomycin at 37°C in humidified air containing 5% CO2. To establish cell lines stably expressing firefly luciferase, B16-BL6 cells were transfected with plasmid DNA encoding firefly luciferase under the control of cytomegalovirus immediate early promoter (20) complexed with Lipo-fectamine2000 (Life Technologies, Inc.-Invitrogen). Then the cells were treated with medium containing 1 mg/mL G418 (Geneticin, Sigma) and single colonies of G418-resistant cells were picked up and examined for their luciferase activity as described below. On the basis of the luciferase activity, a clone was selected and its growth rate in vitro was compared with that of B16-BL6 cells. In addition, 1 × 104 cells of B16-BL6 or B16-BL6/Luc were injected into the lateral tail vein of mice. At 2 weeks after injection, the number of metastatic colonies on the lung surface was counted.

Disposition and Proliferation of B16-BL6/Luc Cells after Intravenous Injection in Mice. Into the lateral tail vein of mice were injected 1 × 103, 1 × 104, 1 × 105, 5 × 105 or 1 × 106 B16-BL6/Luc cells in 0.1 mL HBSS. At 1, 24 hours, 3, 7, and 14 days after tumor injection, mice were killed and the lung was excised, weighed, and the luciferase activity in the tissue was measured.

Effect of Catalase Derivatives on the Number of B16-BL6/Luc Cells in Mice. Experimental pulmonary metastasis was induced by injecting 1 or 5 × 105 B16-BL6/Luc cells in 0.1 mL of HBSS into the lateral tail vein of C57/BL6 mice. Saline (untreated, control group), catalase, PEG-catalase, or inactivated catalase was injected in the lateral tail vein at a dose of 1,000 catalase units unless otherwise indicated. At 24 hours after tumor injection, mice were killed, and the lung was excised, weighed, and the luciferase activity in the tissue was measured.

Separately, the effects of catalase on tumor metastasis at later periods were examined by measuring the luciferase activity of the lung at 7 days after tumor injection. Experimental pulmonary metastasis was induced by injecting 1 × 105 B16-BL6/Luc cells as described above. Then, saline or PEG-catalase was injected just before, and 1 or 3 days after tumor injection.

Multiple Dosing of PEG-Catalase. Experimental pulmonary metastasis was induced by injecting 1 × 106 B16-BL6/Luc cells in 0.1 mL of HBSS into the lateral tail vein of C57/BL6 mice. Saline (untreated, control group) or PEG-catalase was injected daily into the lateral tail vein at a dose of 1,000 catalase units/injection. At 2 weeks after tumor injection, mice were killed, and the lungs were excised, weighed, and the luciferase activity in the tissues was measured.

Separately, the effects of PEG-catalase on the survival of mice with lung metastases were examined. Experimental pulmonary metastasis was induced by injecting 1 × 105 B16-BL6/Luc cells as described above. Then, saline, PEG-catalase (1,000 catalase units), or bovine serum albumin (BSA; amount of protein equivalent to PEG-catalase) was injected daily into the lateral tail vein until 30 days after tumor injection.

Measurement of Luciferase Activity. The cells or tissues were homogenized with a lysis buffer [0.05% Triton X-100, 2 mmol/L EDTA, 0.1 mol/L Tris (pH 7.8), and subjected to three cycles of freezing (liquid N2 for 3 minutes) and thawing (37°C, 3 minutes), followed by centrifugation at 10,000 × g for 10 minutes. Ten microliters of the supernatant was mixed with 100 μL of luciferase assay buffer (Picagene, Toyo Ink, Tokyo, Japan), and the light produced was immediately measured with a luminometer (Lumat LB 9507, EG & G Berthold, Bad Wildbad, Germany).

Statistical Analysis. Differences were statistically evaluated by one-way ANOVA followed by the Student-Newmann-
Keuls multiple comparison test and Kaplan-Meier analysis with a log-rank test to determine survival, and the level of statistical significance was \( P < 0.05 \).

**RESULTS**

**Characteristics of B16-BL6/Luc Cells.** A number of B16-BL6/Luc colonies were obtained with different levels of luciferase activity. A colony expressing high luciferase activity was selected, and the characteristics of the cells were examined. There was no significant difference in the growth rates of B16-BL6 and B16-BL6/Luc cells *in vitro* (data not shown). In addition, they were microscopically identical. When injected into the tail vein of mice, both types of cell induced comparable numbers of metastatic colonies at 14 days (5 ± 1 for B16-BL6 cells and 6 ± 2 for B16-BL6/Luc cells, \( P > 0.05 \)). These results indicate that the characteristics of B16-BL6 cells are hardly altered during the procedure for preparing B16-BL6/Luc cells.

The luciferase activity was proportional to the number of cells [relative light units (RLU)/cell] over a wide range from 100 RLU to 10,000,000 RLU. Treatment of the cultured B16-BL6/Luc cells with \( \text{H}_2\text{O}_2 \) (10 \( \mu \text{mol}/\text{L} \)) or catalase (10,000 units/mL) for up to 7 hours hardly altered the luciferase activity of the cells (data not shown). The expression level of luciferase in the clone was stable for up to 1 year.

The luciferase activity was proportional to the number of cells (data not shown). In other organs such as the liver and spleen (data not shown). At 24 hours, however, only 2 to 4% of the injected cells were detected in the lung. Thereafter, when \( \geq 1 \times 10^5 \) cells were injected, the number of tumor cells in the lung increased with time. However, the growth of the tumor cells was rather slow when small numbers of cells were injected. When mice were injected with \( 1 \times 10^3 \) cells, the number of cells in the lung hardly changed with time up to 14 days.

**Disposition and Proliferation of B16-BL6/Luc Cells in Mouse Lung.** The lung excised from untreated mice showed no significant luciferase activity (<100 RLU/10 \( \mu \text{L} \) of sample). The addition of B16-BL6/Luc cells to lung tissues proportionally increased the luciferase activity according to the number of the cells (data not shown), indicating that the luciferase activity of the tissue can be used as an indicator of the number of cells. On the basis of these findings, the luciferase activity measured was converted to the number of B16-BL6/Luc cells in the lung. The regression line gave a constant of 50 RLU/cell for the quantification of the number of tumor cells. Measuring the luciferase activity of lung homogenates mixed with B16-BL6/Luc cells showed that \( \geq 60 \) cells were enough for the detection of B16-BL6/Luc cells (Fig. 1).

Figure 2 shows the number of B16-BL6/Luc cells in the lungs of mice after intravenous injection of different numbers of cells. At 1 hours, about 60 to 90% of the luciferase activity derived from the injected cells was recovered in the lung. Little luciferase activity was detected in other organs such as the liver and spleen (data not shown). At 24 hours, however, only 2 to 4% of the injected cells were detected in the lung. Thereafter, when \( \geq 1 \times 10^5 \) cells were injected, the number of tumor cells in the lung increased with time. However, the growth of the tumor cells was rather slow when small numbers of cells were injected. When mice were injected with \( 1 \times 10^3 \) cells, the number of cells in the lung hardly changed with time up to 14 days.

**Effect of Catalase Derivatives on the Number of B16-BL6/Luc Cells in Mouse Lung at 24 hours.** It was found that the number of tumor cells was minimal at 24 hours after injection of B16-BL6/Luc cells. Then, we investigated whether catalase derivatives were able to reduce the number of tumor cells as early as 24 hour after the intravenous injection of \( 1 \times 10^5 \) B16-BL6/Luc cells (Fig. 3A). An intravenous injection of catalase at a dose of 1,000 units/mouse tended to reduce the number of the tumor cells in the lung from \( 1.5 \times 10^5 \) cells to \( 1.1 \times 10^5 \) cells, but the difference was not significant. However, PEG-catalase had a greater inhibitory effect on the number of the tumor cells in the lung than catalase; only \( 0.50 \times 10^5 \) cells were detected at 24 hours after tumor injection (\( P < 0.05 \) compared with the saline-treatment or catalase-treatment group).

In a separate set of experiments, PEG-catalase was injected at doses ranging from 100 to 10,000 units/mouse. The lowest dose of PEG-catalase was also effective in reducing the number of tumor cells in the lung (Fig. 3B). The number of the cells in the lung was proportional to the dose of PEG-catalase, suggesting that the detoxification of \( \text{H}_2\text{O}_2 \) inhibits the tumor cell survival in the lung. Inactivated catalase injected at a dose equivalent to 10,000 units catalase had no effect on metastasis.

**Effect of Catalase on the Number of B16-BL6/Luc Cells in Mouse Lung at 7 Days.** As shown in Fig. 2, the tumor cells in the lung were in a logarithmic growth phase at 24 h or later.
Therefore, at any time point around 24 h after injection, the tumor cells that survived appeared to adhere already to the endothelial cells and be ready for invasion and proliferation. To examine the effect of catalase on these tumor metastatic processes, PEG-catalase was injected at 1 or 3 days after tumor injection and the number of the tumor cells was measured at 7 days (Fig. 4). An intravenous injection of PEG-catalase at a dose of 1,000 units/mouse significantly reduced the number of tumor cells in the lung in both cases ($P < 0.005$ compared with the saline treatment group). Furthermore, compared with the results of Fig. 2, the number of the cells in the lung of PEG-catalase–treated mice was not significantly different from that observed at 24 hours after tumor injection ($P > 0.05$). In addition, no metastatic colonies were seen under a dissecting microscope (Fig. 5B). Therefore, this suggests that the growth of tumor cells in the lung is almost completely inhibited by a daily injection of PEG-catalase.

**DISCUSSION**

Although metastasis is a major target of cancer therapy, it is difficult to treat metastases effectively. One of the major reasons for this is that the tissue disposition of tumor cells in vivo is poorly understood even in animal models. Metastasis consists of a number of different processes, such as adhesion, invasion, proliferation, and angiogenesis. Therefore, any inhib-
now there has been little published information on the separate processes of tumor metastasis. Therefore, in this study, we first examined the tissue disposition of B16-BL6/Luc cells after intravenous injection and identified the characteristic features of the in vivo fate of the cells. Then, we applied this analytical system to elucidate the mechanism whereby PEG-catalase reduces the number of metastatic colonies in the lung.

There are several requirements for the transfected to use the luciferase activity in tissues as an indicator of the number of tumor cells. First, the expression level of luciferase should be high enough to ensure that a few cells can be detected in vivo. Second, the expression must be stable for a long period even after inoculation in mice. Furthermore, the expression should be independent of stimuli such as cytokines and ROS and of cell cycles. Finally, it is best that the characteristics of the transfected do not differ from those of the parent cell line. Before in vivo studies, we examined the characteristics of the transfected, B16-BL6/Luc cells. The luciferase activity of the cells was stable for at least 1 year and proportional to the cell number over a wide range. The expression of the luciferase in the cells was hardly affected by the growth phase, the addition of H2O2, or catalase. These properties are consistent with previous results showing that for plasmid DNA encoding firefly luciferase under the control of cytomegalovirus, immediate early promoter activity was independent of the cell cycle (21), and the expression level was scarcely affected after inoculation into mice (22). In addition, the half-life of firefly luciferase is very short (about 3 hours); therefore, the level of the luciferase protein in the cell would level off quickly. These properties enable us to use the luciferase activity in lung tissue as an indicator of the exact number of tumor cells.

In the experimental pulmonary metastasis model, B16-BL6 or B16-BL6/Luc melanoma cells were injected intravenously into syngeneic C57BL/6 mice. Therefore, the first step in the pulmonary metastasis is the arrest of the tumor cells in small vessels in the lung followed by their adhesion to endothelial cells (1, 23). As shown in Fig. 2, intravenous injection of B16-BL6/Luc cells resulted in the accumulation of 60 to 90% of the injected cells in the lung and few in other organs at 1 hour after tumor injection. Thereafter, the tumor cells in the lung fell to 2 to 4% of the injected cells at 24 hours. In contrast, when injected into the portal vein, about 60% of the injected B16-BL6/Luc cells were detected even at 24 hours after injection.3 These results of organ- or cell number-independent tumor cell arrest suggest that the initial step in the formation of metastatic colonies of B16 tumor cells is mediated by physical trapping of the cells within the microvasculature of organs rather than by a specific interaction between tumor cells and endothelial cells via adhesion molecules. The tumor cells arrested in the lung could be destroyed in the microvasculature by mechanical stress caused by respiration in the first 24 hours after tumor injection. Because the injected tumor cells decreased to 2–4% irrespective of the initial number of cells, this inefficiency could be because of the physical forces of contraction and relaxation of the lung tissue but not to immunity. It is also suggested that at 24 hours

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3 Unpublished data.
Inhibition of Metastatic Tumor Growth by Catalase

Even under normal conditions, ROS are continuously produced as by-products of metabolism by enzymes such as superoxide dismutase, xanthine oxidase, and NADPH oxidase (25). Several antitumor drugs as well as radiation also generate ROS through reductive activation and redox recycling (25–27), and it has also been reported that ROS are involved in various processes of tumor metastasis, such as adhesion, invasion and proliferation (5, 6, 9–12). Therefore, the scavenging of ROS by antioxidant enzymes can be an effective approach for inhibiting tumor metastasis. We have proposed a hypothesis that the elimination of H$_2$O$_2$ at the site where tumor metastasis occurs reduces this and we have examined the effect of increasing the plasma half-life of catalase on the inhibition of an experimental pulmonary metastasis (8). We found that intravenous injection of PEG-catalase greatly decreased the number of colonies in the lung 2 weeks after tumor injection and, in a separate set of experiments, we have shown that targeted delivery of catalase to hepatocytes greatly inhibited experimental hepatic metastasis in mice (28). These results indicate that the detoxification of H$_2$O$_2$ is a promising approach for inhibiting metastasis. However, these results did not identify which metastatic processes are inhibited by the catalase derivatives. To address this question, we measured the number of tumor cells in the lung at 1 hour, 1 and 7 days after injection of tumor cells and examined the effects of catalase derivatives on the metastatic processes.

Administration of PEG-catalase just before tumor injection, which is the same protocol as the one used in a previous study (8), reduced the number of tumor cells in the lung at 24 hours after tumor injection, indicating that PEG-catalase inhibits the early steps of metastasis. Additional studies are needed to identify whether changes in the expression of adhesion molecules are involved in the inhibitory effect of catalase derivatives in the lung. PEG-catalase was effective in reducing the number of tumor cells at day 7, even when intravenously injected at 1 or 3 days after tumor injection. As shown in Fig. 2, the tumor cells in the lung were in a growth phase at the times when PEG-catalase was injected. Therefore, these results indicate that scavenging of H$_2$O$_2$ by PEG-catalase can inhibit the growth of metastatic tumor cells. It has already been reported that H$_2$O$_2$ can accelerate proliferation (12); therefore, the suppressive effect of catalase derivatives would be attributable to direct inhibition of the proliferation of tumor cells. As shown in Fig. 5B, a single injection of PEG-catalase just before tumor injection reduced the number of metastatic nodules but scarcely affected the diameter of the nodules. After triple injection of PEG-catalase (just before, 1 and 3 days after tumor injection), the number of metastatic colonies did not markedly differ from that produced by a single (just before tumor injection) or double (just before and 1 day after tumor injection) injection, although the diameter of the colonies was found to be small. These findings indicate that the first injection of PEG-catalase reduces the number of surviving tumor cells, and the subsequent injections inhibit the proliferation of the surviving tumor cells. Therefore, it is suggested that PEG-catalase inhibits not only the survival or adhesion of tumor cells but also their invasion and proliferation.

Thus, it has been shown that catalase derivatives effectively inhibit not only the early steps of metastasis, but also the later steps. Then, we tried to effectively suppress metastatic tumor growth by inducing tumor dormancy. As shown in Fig. 5A, daily injection of PEG-catalase greatly reduced the number of tumor cells detected at 14 days in the lung. The number of tumor cells scarcely increased compared with that detected at 24 hours after tumor injection. Therefore, the tumor cells within the lung tissue may be in a dormant state after repetitive injection of PEG-catalase. This significant inhibition of growth was effective in increasing the survival of the tumor-bearing mice. However, the mice treated with PEG-catalase died sequentially after stopping the injection, suggesting that the tumor cells of micrometastases start to proliferate when the supply of catalase is stopped. In previous publications (29, 30), “dormancy” was used to refer to individual tumor cells that were thought to persist “symbiotically” for long periods, but subsequently could be stimulated to exhibit malignant growth. Therefore, the results of the present study indicate that repeated administration of PEG-catalase can induce tumor dormancy and prolong the survival period.
In conclusion, we have developed a quantitative method to analyze tissue disposition of tumor cells and found that scavenging of H₂O₂ by PEG-catalase can effectively inhibit not only the early steps of metastasis, such as embryolization, adhesion, or survival, but also in later steps, such as invasion or proliferation. Continuous supply of catalase activity within the blood circulation by multiple dosing of PEG-catalase greatly suppressed the growth of tumor cells in metastatic foci. This reduction of metastatic tumor growth by PEG-catalase offers potentially a very effective approach to the antitumor therapy of a variety of tumors.

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