Introduction

Malignant melanoma is a lethal disease occurring at the skin and laryngeal sites, and whose incidence is increasing year by year (1), especially in the countries of North-Western Europe and Australia (2, 3). Metastasis of this malignant cancer to other distant organs via hematogenous or lymphatic routes is most troublesome because melanoma metastases are more aggressive, resulting in shorter survival time, averaging several to fifteen months (4). In general, most anticancer agents exert little curative effects on micrometastases because metastasized cancer cells often remain dormant (5). Therefore, it appears difficult to develop the drugs that cure malignant melanoma and prevent its metastasis to other organs.

We previously reported that 2-aminophenoxazine-3-one (Phx-3), which is produced by the reaction of o-aminophenol and bovine hemoglobin solution (6), and which is referred to as questiomycin A (7), prevents the proliferation of human and mouse melanoma cells in vitro (8, 9), and is cytotoxic to mouse B16 melanoma cells implanted in mice, in vivo (9). Phx-3 is an oxidative phenoxazine, the essential component of Actinomycin D with strong anticancer activity, and exerts potential anti-cancer activity against various cancer cells (10 – 15). Kohno et al. (16) recently indicated that Phx-3/APO exerts strong anti-inflammatory effects by suppressing the production of nitric oxide (NO) and prostaglandin E2. In the light of the fact that the involvement of NO and prostaglandin E2 has been indicated in the mechanism for the metastasis of various cancer cells (17 – 19), it is

Abstract. 2-Aminophenoxazine-3-one (Phx-3) induced cellular apoptosis in mouse melanoma B16 cells as detected by DNA laddering and upregulated Fas expression in the cells in vitro. Next, the anti-metastatic effects of Phx-3 were investigated in C56BL/6 mice. When B16 melanoma cells were injected into the tail veins of mice, significant metastasis of the cells was indicated in the lungs, 14 days after treatment. In contrast, when 0.5 mg/kg Phx-3 was administered to mice through the tail veins, once simultaneously with or every three days after the administration of B16 melanoma cells, the number of metastasized pulmonary cells was extremely reduced. Moderate reduction of the number of metastasized pulmonary cells was indicated in the mice with a single dose of Phx-3 on day 3 after injection of the cells. However, when Phx-3 was administered in a single dose, 6 or 9 days after the injection of the cells, the number of metastasized pulmonary cells remained the same. The present results indicate that the metastasis of mouse B16 melanoma cells to the lung was significantly inhibited in mice administered Phx-3, which activated the intrinsic and extrinsic apoptotic pathways. The present study suggests that Phx-3 might be a potential anti-metastatic agent as well as an anticancer agent.

Keywords: 2-aminophenoxazine-3-one, pulmonary metastasis, mouse B16 melanoma cell
conceivable that Phx-3 might prevent metastatic events of cancer cells.

In this manuscript, we first examined whether Phx-3 might induce apoptosis of mouse B16 melanoma cells by activating the intrinsic and extrinsic apoptosis pathways. If so, Phx-3 might prevent the growth of metastasized cancer cells in vivo. We therefore investigated the anti-metastatic activity of Phx-3 against mouse B16 melanoma cells injected into mice and the possibility of this compound being an anti-metastatic agent.

Materials and Methods

Phx-3 and other reagents

Phx-3 was prepared by reacting o-aminophenol with bovine hemoglobin solution, according to the method described by Shimizu et al. (6). The chemical structure of Phx-3 is illustrated in Fig. 1. Phx-3 was dissolved in a mixture of dimethylsulfoxide (DMSO) and ethyl alcohol (3:1) as a vehicle to make a 50 mg/ml solution. This solution was diluted appropriately with an isotonic saline, and was administered to mice.

Cell line and culture conditions

Mouse B16 melanoma cells were obtained from the JCRB cell bank (JCRB0202). The cells (1 × 10⁵ cells) were cultured in Dulbecco’s minimum Eagle’s medium (DMEM) (Sigma, St. Louis, MO, USA) supplemented with 10% heat inactivated fetal bovine serum (FBS) (Cansera International Inc., Ontario, Canada) and penicillin/streptomycin (10,000 unit/ml; 10,000 μg/ml) (Invitrogen, Carlsbad, CA, USA) in a humidified incubator containing 5% CO₂ and 95% air at 37°C.

Detection of DNA laddering of mouse B16 melanoma cells caused by Phx-3

Mouse B16 melanoma cells (1 × 10⁶ cells) were placed on a 10 cm plate and cultured in DMEM supplemented with 10% heat inactivated FBS in a humidified incubator containing 5% CO₂ and 95% air at 37°C for 24h. The cells were then treated with 50 μM Phx-3, and incubated for an additional 72 h. Briefly, the supernatant was collected, transferred to a 15 ml glass tube, and centrifuged at 1,500 rpm for 3 min at room temperature (20). The pellets were used to analyze the DNA laddering. B-16 DNA was extracted using Gene Elute Mammalian Genomic DNA Miniprep Kit (Sigma), and subjected to electrophoresis in 2% agarose gel (containing 0.25 μg/ml ethidium bromide) for 30 min. The electrophoretic bands were visualized and photographed under transmitted ultraviolet light.

Effects of Phx-3 on expression of Fas in mouse B16 melanoma cells

Expression of Fas in mouse B16 melanoma cells was detected by a flow-cytometric method according to the method of Dhein et al. (21). Briefly, mouse B16 melanoma cells (1 × 10⁶) in each tube were stained with biotinylated anti-Fas IgG3 monoclonal antibody for 45min at 4°C. After washing with PBS, a second incubation step with streptavidin-phycoerythrin (Becton Dickinson, Heidelberg, Germany) was performed for 30 min at 4°C. Cells were washed with PBS and analyzed with a flow-cytometer using Cell Quest software (Becton & Dickinson Co., Franklin Lakes, NJ, USA). FITC IgG3 antibody was used as an isotype-matched non-binding antibody to control unspecific binding.

Animal

Five-week-old female C57BL/6 mice were purchased from Japan SLC (Shizuoka). The mice were housed in a pathogen-free environment at 25 ± 1°C and at 55 ± 5% of humidity. They were allowed free access to water and food pellets. All procedures were based on the Guiding Principles for the Care and Use of Laboratory Animals approved by the Japanese Pharmacological Society.

Effects of Phx-3 on the experimental pulmonary metastasis

Mouse B16 melanoma cells in the exponential growth phase were harvested by trypsinization and washed twice with PBS before injection. The cells (1 × 10⁶ cells in 100 μl PBS) were injected into the tail veins of mice for the PBS treated group (control group, n = 3) and for the groups treated with 0.5 mg/kg Phx-3 on different days (n = 3, each) (22, 23). The protocol for treating each mouse group with Phx-3 is presented in Table 1. Briefly, the protocol was as follows. A) Lung metastases were established by injecting a 100 μl suspension of mouse B16 melanoma cells with 0.5 mg/kg Phx-3 into the tail veins of mice on day 0, without further administration of Phx-3. The mice (group I, n = 3) were sacrificed after 14 days. B) Lung metastases were established by injecting mouse B16 melanoma cells without Phx-3 into the tail veins on day 0. A 100 μl solution containing 0.5 mg/kg Phx-3 was then injected into the tail veins of mice, in a single dose, on 3, 6, 9, or 11 day, or in consecutive doses on days 3, 6, 9, and 11 (group II to IV, n = 3 for each group). After 14 days, all mice were sacrificed, and their lungs were

![Chemical structure of 2-aminophenoxazine-3-one (Phx-3).](image)
Examined for visible metastasis. This study was conducted under the control of the Animal Research Committee, in accordance with the Guidelines on Animal Experiments of the Chiba Institute of Technology.

Estimating metastasis of tumor cells

The mice were anesthetized with pentobarbital and sacrificed at 14 days after the cell injection. Black nodules appearing in the lungs were macroscopically counted (22, 23).

Statistics

Data were analyzed using a two-tailed Student's t-test. A P value of less than 0.05 was considered to be statistically significant.

Results

Phx-3 extensively suppressed the viability of mouse B16 melanoma cells (9), so we first investigated whether Phx-3 may induce the apoptosis of this cell line in vitro. As depicted in Fig. 2, Phx-3 caused DNA laddering with an excellent fragmentation of n × 180 bp, when the cells were incubated with 50 μM Phx-3 for 72 h. These results suggest that Phx-3 induces apoptosis of mouse B16 melanoma cells through an intrinsic pathway associated with increased mitochondrial depolarization and activation of caspase-3 (24, 25), as has been demonstrated in a variety of cancer cells with Phx-3 (10 – 15).

In contrast, Fas, a member of a tumor necrosis factor / nerve growth factor receptor superfamily mediates apoptosis upon trimerization and through crosslinking to Fas ligand of surfaces of activated T cells and natural killer cells, leading to the activation of an extrinsic apoptotic pathway of cancer cells (26 – 28). Fas is upregulated in various cancer cells after treatment with anti-cancer agents such as doxorubicin and etoposide and is involved in determining sensitivity to chemotherapy (29, 30). Thus, we examined whether the expression of Fas in mouse B16 melanoma cells is upregulated 24, 48, and 72 h after the cells are treated with Phx-3 in vitro by using a flow-cytometric assay (Fig. 3). Phx-3 was found to upregulate the expression of Fas significantly in mouse B16 melanoma cells after 24, 48, and 72 h because the peak shifted to the right from that of the control without Phx-3. This result suggests that mouse B16 melanoma cells will be purged due to apoptotic cell death induced by Phx-3 through the extrinsic apoptotic pathway, which is associated with attacks of the activated T cells and natural killer cells (26, 27). Taken together, the results in Figs. 2 and 3 indicate that Phx-3 might exert anti-cancer activity through these intrinsic and extrinsic apoptotic pathways in vivo.

We previously demonstrated that the growth of mouse B16 melanoma cells implanted subcutaneously in mice was extensively suppressed by Phx-3 (0.5 mg/kg) (9), demonstrating that Phx-3 could elicit anticancer activity in vivo. However, it was unclear whether Phx-3 could
prevent the metastasis of these cancer cells to the distant organs. Mouse B16 melanoma cells are known to have metastatic potential (31) and are often trapped in the lung when injected into the tail veins of mice (22, 23). We therefore investigated whether pulmonary metastasis of B16 melanoma cells could be caused by injecting the cells into the tail vein and whether pulmonary metastasis in mice could be prevented by Phx-3.

As illustrated in Fig. 4, the extent of metastasis and its inhibition was assessed macroscopically by counting the metastatic nodules on the pleural surface of three lung lobules 14 days after injecting mouse B16 melanoma cells via the tail veins of mice, with or without Phx-3. A significantly increased number of black metastatic nodules was counted in the lungs of the positive control group. However, the number of black metastatic nodules was significantly reduced when the mice were treated with 0.5 mg/kg Phx-3 simultaneously with the injection of mouse B16 melanoma cells into the tail veins (single dose on day 0, in Fig. 4).

When 0.5 mg/kg Phx-3 was injected in a single dose three days after the injection of mouse B16 melanoma cells into the tail veins, the number of metastatic nodules was moderately reduced. Such reduction of the number of metastatic nodules was not indicated in the lungs of mice that had received a single dose of Phx-3, six or nine days after the injection of the cells. The number of visible black metastatic nodules in the lung was reduced to the levels of mice treated with Phx-3 simultaneously with mouse B16 melanoma cells when 0.5 mg/kg Phx-3 was administered consecutively on days 3, 6, 9, and 11.

Figure 5 summarizes the number of black metastatic nodules in the lungs of mice with or without 0.5 mg/kg Phx-3 treatment on different days, as presented in the photos of Fig. 4. In the positive control group, there were 127 ± 56 (n = 3) black metastatic nodules. The number of nodules was extremely reduced in the mice treated with 0.5 mg/kg Phx-3, simultaneously with mouse B16 melanoma cells (group I, 12 ± 2; 91% reduction compared with the positive control, \( P < 0.08 \)). Moderate reduction of the visible black metastatic nodules was indicated in group II mice that were treated with Phx-3 on day 3 (76 ± 22; 40% reduction of the positive control, \( P < 0.3 \)). The number of metastatic nodules in mice treated with 0.5 mg/kg on day 6 (group III) or day 9 (group IV) after the injection of the cells was almost the same as that of the positive control (134 ± 14 for group III; 115 ± 59 for group IV). When a 0.5-mg/kg dose of Phx-3 was administered to mice 3, 6, 9, and 11 days after the injection of the cells, the number was extremely reduced (group V, 21 ± 9; 84% reduction compared with the positive control, \( P < 0.08 \)).

The result for group I may indicate that the early events of metastasis, including invasion, adhesion, and growth, could be significantly hindered by Phx-3, although the detailed mechanism remains unclear. The results for group V, where Phx-3 was administered consecutively on day 3, 6, 9, and 11, suggest that the middle and later
events of metastasis including growth and angiogenesis of the mouse B16 melanoma cells in mice, could be hindered by Phx-3. In this case, the apoptosis of cells could be caused by the activation of intrinsic and extrinsic apoptotic pathways in the presence of Phx-3, as indicated in Figs. 2 and 3.

Discussion

Kohno et al. (16) indicated that Phx-3 exerts strong anti-inflammatory effects by suppressing the production of NO and prostaglandin E2. Since the involvements of NO and prostaglandins have been indicated in the metastatic mechanism including the invasion and angiogenesis of a variety of cancer cells (17 – 19), it is reasonable that Phx-3 would prevent such critical events during metastasis. The mechanism by which Phx-3 inhibits metastasis of B16 melanoma cells is unclear, although we consider that the proapoptotic action of Phx-3 against mouse B16 melanoma cells as demonstrated in Figs. 2 and 3 might be crucial to the mechanism as well.

Metastasis of cancer cells is one of the most difficult problems arising during the proliferation of cancer, and even during cancer therapy, thus contributing to the poorer prognosis of cancer. No curative agents for human malignant melanoma are known except for dacarbazine, the only compound that was approved for the treating malignant melanoma, with 15% – 25% improvement rate (32). Furthermore, there are no agents for completely preventing the metastasis of this malignant cancer. We previously found potential anticancer activity of Phx-3 at a dose of 0.5 mg/kg on mouse B16 melanoma cells implanted in mice. In the present study, we demonstrated that the same dose of Phx-3 significantly reduced the metastasis of mouse B16 melanoma cells in mice (Figs. 4 and 5). These results suggest that Phx-3 has both anticancer and anti-metastatic activity against malignant melanoma.

Anzai et al. (7) first found Phx-3 in 1951 in streptomycetes from the soil. Phx-3 was indicated to exert antimicrobial effects only on mycobacterium tuberculosis and was therefore named questiomycin A. Since then, no further biological effects of this compound have been examined because its antimicrobial activity seemed to be negligible. Eckert and Eyer (33) demonstrated that o-aminophenol, a precursor of Phx-3 injected to dogs caused little nephrotoxicity because o-aminophenol was converted to Phx-3 in the circulating blood of the animals. We also previously indicated that Phx-3 is less toxic to normal cells such as lymphocytes (15) and non-toxic to mice (9). Recently, Kohno et al. (16) found that the compound responsible for the anti-inflammatory effects of edible brown mushrooms is APO (equivalent to Phx-3) and demonstrated that this phenoxazine compound exerted no obvious adverse effects when administered orally at doses up to 1,500 mg/kg. Such minimal toxic effects of Phx-3, in addition to potential anticancer and anti-metastatic activity of this compound, may be beneficial in completely preventing cancer metastasis through quantitative and continuous doses.

In conclusion, our data indicate that Phx-3 prevents pulmonary metastasis of B16 melanoma cells at a dose of 0.5 mg/kg in vivo, possibly through apoptosis mechanisms associated with the intrinsic apoptotic pathway and with the upregulation of Fas (CD95/APO-1), which regulates the extrinsic apoptotic pathway. These findings suggest that Phx-3 could be useful as an anti-metastatic agent for treating malignant melanoma that is intractable to chemotherapy.

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References