

Antimetastatic Activity of Acteoside, a Phenylethanoid Glycoside

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We examined the antimetastatic effect of acteoside, a phenylethanoid glycoside widely distributed in the plant kingdom, on lung metastasis using a mouse model injected with B16 melanoma cells intravenously. Male C57BL/6 mice were injected intravenously with 2×10^5 of B16 melanoma cells, while acteoside at a dose of 50 mg/kg was administered intraperitoneally every other day from 13 d before B16 melanoma cell injection until all mice had succumbed to the metastatic tumor burden in the lung. Administration of acteoside prolonged survival time significantly and the average survival time was 63.3 ± 3.4 d compared with 52.1 ± 2.5 d in control mice. This result suggests that acteoside showed suppressive effect on lung metastasis of B16 melanoma cells.

Key words acteoside; phenylethanoid glycoside; metastasis; B16 melanoma

The ability to produce metastasis is a property of malignant tumor cells. The process of metastasis follows a series of sequential steps that include motility, invasion, survival in the circulation, adhesion, extravasation, proliferation, and angiogenesis. Malignant cells are finally released from the primary tumor and disseminate to distant sites *via* lymphatic and/or circulatory systems, and halt in distant lymph nodes or in the microvasculatures of secondary sites. The emergence of metastasis in organs distant from the primary tumor is the most devastating aspect of cancer.

Excessive production of reactive oxygen species (ROS), which are generated by tumor, inflammatory or endothelial cells, play a fundamental role in a wide variety of disease processes. In tumor metastasis, ROS are also known to play important roles: increasing expression of P-selectin by exposure of pancreatic tumor cells to ROS,¹ and increasing expression of very late antigen-4 on B16 melanoma cells also in response to hydrogen peroxide.² These cell-adhesion molecules are involved in the recruitment of metastatic tumor cells to a target organ. In addition, treatment of Walker 256 carcinosarcoma with hydrogen peroxide enhances a secretion of matrix metalloproteinase which is involved in the degradation of extracellular matrix to extravasate from or intravasate into blood vessel.³ Rat peritoneal macrophages, macrophage-like cell lines, U937 and RAW264.7 cells produce vascular endothelial growth factor, which is an angiogenic factor known to be necessary for inducing angiogenesis to maintain metastasized tumor proliferation,⁴ when treated with hydrogen peroxide. On the other hand, scavenging of ROS leads to a reduction in metastasis, as evidenced by the facts that catalase or superoxide dismutase effectively suppresses metastasis in an experimental metastasis model,^{5–8} and that ascorbic acid-2-*O*-phosphate-6-*O*-palmitate exerts antimetastatic activity by increasing intracellular anti-oxidant activity.⁹ Taken together these results, ROS seem likely to affect various processes of metastasis, resulting in the development of metastasis.

ROS are known to be generated by endothelium, macrophages, or fibroblasts following exposure to pro-inflammatory mediators such as endotoxin, interleukin-1 (IL-1), and tumor necrosis factor- α (TNF- α).^{2,10} ROS are also able to activate a nuclear transcription factor, NF- κ B, which induces the inflammatory cytokines IL-1, IL-6, and TNF- α .¹⁰ Thus,

the suppression of cytokine production by anti-inflammatory agents appears to reduce metastasis effectively.

Acteoside is a well-studied phenylethanoid glycoside and is widely distributed in the plant kingdom. Many studies have shown that acteoside has various kinds of biological activities.^{12–21} Among those so far reported, its anti-oxidant activity,^{22,23} a modulating activity of nitric oxide (NO) production,^{12,24} and cytotoxicity against various tumor cells^{25–27} are believed to inhibit tumor metastasis. However, there is no report its antimetastatic effect. In the present study, we evaluated the antimetastatic effect of acteoside on lung metastasis using a mouse model injected with B16 melanoma cells.²⁸

MATERIALS AND METHODS

Animals Male C57BL/6 mice were obtained from Charles River (Hino, Japan). They were housed in a temperature-controlled room (at 23 ± 2 °C) with lighting from 6 a.m. to 6 p.m. under specific pathogen-free conditions. The humidity was automatically maintained at $50 \pm 10\%$. They were fed a commercial diet (CE-2; Nippon Crea Co. Ltd., Shizuoka, Japan) and water *ad libitum*. All the mice were 8 weeks old when the tumor cells were intravenously injected.

Tumor Cells B16 melanoma cells (C57BL/6 mice, melanoma) were obtained from the Health Science Research Resources Bank (Sennan, Osaka, Japan) and maintained in MEM-Eagle's salts medium with non-essential amino acid (Irvine Scientific Co., Santa Ana, CA, U.S.A.) supplemented with 10% heat-inactivated fetal bovine serum, 100 U/ml penicillin and 100 μ g/ml streptomycin (Life Technologies Inc., Grand Island, NY, U.S.A.).

Pulmonary Metastasis Model Male C57BL/6 mice injected intravenously with B16 melanoma cells (2×10^5 cells/200 μ l saline) were allowed to live until they succumbed to the metastatic tumor burden in the lung. Acteoside, which was kindly provided by Tsumura Co. Ltd. (Tokyo), was dissolved in saline, filtered through a 0.22 μ m filter, and then administered intraperitoneally at a concentration of 50 mg/kg every other day from 13 d before B16 melanoma cell injection until all mice succumbed. This dose was determined according to the IC₅₀ (125 μ g/ml) of acteoside for cytotoxicity against B16 melanoma cells in *in vitro* assay.

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Statistical Analysis Survival time was represented as mean \pm S.E. of 10 mice in each group. Statistical significance was determined by Mann–Whitney *U* test. *p* values less than 0.05 were considered significant.

RESULTS AND DISCUSSION

The effect of acteoside on pulmonary metastasis was examined in C57BL/6 mice following its every other day intraperitoneal administration from 13 d before B16 melanoma cell injection until all animal had succumbed. In the control group, all the mice had died by 63 d after the injection and the average length of survival was 52.1 ± 2.5 d (Fig. 1), while acteoside extended the survival time and two mice survived for 80 d. The average time of survival was 63.3 ± 3.4 d, indicating that acteoside significantly increased the survival of the B16 melanoma cell-injected mice ($p < 0.05$; Mann–Whitney *U*-test). In another of our studies using this model, 28 d after B16 melanoma cell injection the lung weight increased by 0.08 to 0.22 g and about 400 metastatic colonies were found in the lung (data not shown). Autopsy indicated that mice injected with the melanoma cells had succumbed to the lung metastasis, because there were no causative symptoms in the other organs. This result demonstrates that acteoside actually possesses the activity to suppress tumor metastasis.

When the mechanism underlying the antimetastatic effect of acteoside on the metastasis of B16 melanoma is speculated on, its anti-oxidant activity, inhibition of NO synthesis, and cytotoxic activity appear to be intimately related to the reduction of metastasis. Acteoside shows inhibitory activity on free radical-induced hemolysis of red blood cells induced by a peroxy radical initiator, 2,2'-azo-bis-(2-amidino-propane) and superoxide radical-scavenging activity represented by the inhibition of Cu^{2+} -induced low density lipoprotein oxidation.^{22,23} Although it is not obvious whether ROS is implicated in the process of metastasis in the pulmonary metastasis model mice injected with B16 melanoma cells, the antimetastatic activity shown by acteoside may be in part due to the antioxidant activity. When considering the relationship between nitric oxide as a radical and metastasis, an elevated eNOS expression promotes metastasis by maintaining a vasodilator tone in the blood vessels in and around the invading melanoma,^{29,30} and tumor-induced expression of host iNOS enhances melanoma metastasis and pleural effusion.³⁰ These suggest that the suppression of NO synthesis leads to the reduction of melanoma metastasis. In fact, acteoside has NO radical-scavenging activity at a relatively high concentration of 100–200 μM ,²⁴ indicating the possibility that this may contribute to the antimetastatic effect. We reported earlier that acteoside induced NO production by macrophages at a very low concentration of less than 1 μM .¹² NO, which is produced by iNOS of macrophages, is known to prevent tumor metastasis in the early stage of metastasis. Taken together, these results suggest that acteoside may exert an antimetastatic effect on tumor cell adhesion or invasion at the early stage of metastasis, if it increases NO production at a low concentration *in vivo*. However, as we have not determined the exact concentration of acteoside in the lung, a detailed study is required to elucidate the relationship between the effect of acteoside and NO.

Another important effect of acteoside may be cytotoxic ac-

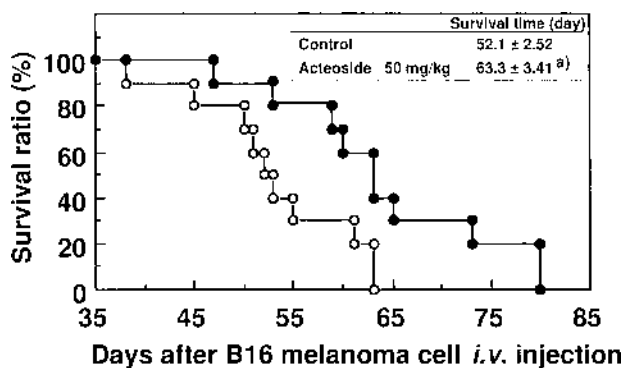


Fig. 1. Effect of Acteoside on Life Span of Mice Injected with B16 Melanoma Cells

Male C57BL/6 mice (8 weeks old) were injected intravenously with 2×10^5 mouse of cultured B16 melanoma cells and acteoside at a concentration of 50 mg/kg was administered intraperitoneally every other day from 13 d before B16 melanoma cell injection until all mice had succumbed to the metastatic tumor burden in the lung. Survival ratio is represented as the percentage of surviving mice. Survival time is mean \pm S.E. of 10 mice. \circ : control mice ($n=10$), \bullet : acteoside-treated mice ($n=10$). $p < 0.05$ vs. control group (Mann–Whitney *U* test).

tivity against tumor cells which is involved in the antimetastatic activity. We have so far reported that acteoside exhibits cytotoxicity against the tumor cells dRLh-84 (rat hepatoma), S-180 (sarcoma), P-388/D1 (mouse lymphoid neoplasma), and HL-60 (human promyelocytic lymphoma) cells.^{25–27} In fact, acteoside completely killed B16 melanoma cells (1×10^5 cells/ml) at a concentration of 320 μM (IC_{50} : ca. 200 μM) following 24 h incubation (data not shown). We are not able to exclude the possibility that the antimetastatic activity results from the cytotoxicity of acteoside, although whether its concentration in the blood reaches a level sufficient to show cytotoxicity has not yet been determined. We regard acteoside to be a possible antimetastatic agent and are now attempting to identify the mechanism by which it exerts antimetastatic activity against lung metastasis of B16 melanoma cells.

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