Antitumor and antimetastatic actions by royal jelly in Lewis lung carcinoma-bearing mice

Yoshiyuki KIMURA,[*a)] Takeshi TAKAKU,[b)] Hiromichi OKUDA[c)]

[a)]*Second Department of Medical Biochemistry and* [b)]*Central Research Laboratory, School of Medicine, Ehime University, Shigenobu-cho, Onsen-gun, Ehime 791-0295, Japan, and* [c)]*Department of Environmental and Symbiotic Sciences, Prefectural University of Kumamoto, Tsukide 3-1-100, Kumamoto-City, Kumamoto 862-8502, Japan.*

(Received March 19, 2003. Accepted June 17, 2003.)

Abstract

Royal jelly is a secretion of the cephalic glands of nurse bees and it is the food of the larvae that will become queen bees. We found that royal jelly at a dose of 300 or 600 mg/kg significantly reduced the tumor weight and metastasis to the liver in mice implanted intrasplenically with highly metastatic Lewis lung carcinoma (LLC) tumors. To clarify the antitumor and antimetastatic activities of royal jelly, we examined its effects on Matrigel-induced angiogenesis *exo-vivo* model. Mice were subcutaneously inoculated with Matrigel supplemented with acidic fibroblast growth factor and heparin in the presence or absence of royal jelly. Royal jelly inhibited the Matrigel-induced angiogenesis. From these results, it seems likely that the antitumor and antimetastatic activities of royal jelly may be partly due to the inhibition of angiogenesis.

Key words royal jelly, antitumor activity, antimetastatic activity, antiangiogenic activity, mice.

Abbreviations aFGF, acidic fibroblast growth factor; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; LLC, Lewis lung carcinoma; TCA, trichloroacetic acid; PBS, phosphate buffered saline.

Introduction

Royal jelly is a secretion of the cephalic glands of nurse bees and it is the food of the larvae of queen bees. Queen bees live longer and are larger than nurse bees, which do not feed on royal jelly. It has been reported that many investigations of royal jelly were carried out to clarify its possible therapeutic use in a variety of human diseases, including leukemia.[1)] Royal jelly has been used for the prevention of a variety of human diseases in the worldwide as a health food. Recently, Okuda *et al.*[2)] reported that royal jelly inhibits catecholamine-induced lipolysis and stimulates lipogenesis from glucose in isolated rat fat cells, and that (*E*)-10-hydroxy-2-decenoic acid isolated from royal jelly as an insulin-like substance inhibits angiotensin converting enzyme activity. It has been reported that royal jelly and (*E*)-10-hydroxy-2-decenoic acid isolated from royal jelly have antitumor effects in tumor-bearing mice.[3-5)] It is well known that malignant cancer often gives rise to metastases to the lung, liver, bone, *etc.* To determine whether royal jelly prevents tumor metastasis, we examined the antitumor and antimetastatic actions of royal jelly using mice implanted intrasplenically with Lewis lung carcinoma (LLC), and investigated the mechanisms of the antitumor and antimetastatic actions of royal jelly.

Materials and Methods

Natural materials : Royal jelly was supplied by Attested Transaction Conference for Royal Jelly of All Japan (Tokyo, Japan). The voucher sample was stored at the Second Department of Medical Biochemistry, School of Medicine, Ehime University, Japan.

Materials : Matrigel® basement membrane (reduced growth factor) was obtained from Becton Dickinson Labware (Bedford, MA). Dulbecco's modified Eagle's medium (DMEM) was obtained from Nissui Pharmaceutical Ltd. (Tokyo, Japan) and used as culture medium. Antibiotic and antimycotic solutions were purchased from Sigma Chemical (St. Louis, MO). Fetal bovine

*To whom correspondence should be addressed. e-mail : yokim@m.ehime-u.ac.jp

serum (FBS) was purchased from ICN Biochemicals (Aurora, OH). Culture plates were purchased from Corning Glass Works (NY). Other chemicals were of reagent grade.

Cells : Highly metastatic, drug-resistant mouse Lewis lung carcinoma (LLC) cells were obtained from Riken Gene Bank (Tukuba, Japan) and maintained in DMEM supplemented with 10% FBS, penicillin (1 x 10^3 U/ml), streptomycin (100 μg/ml) and amphotericin B (0.25 μg/ml).

Animals : Female C57BL/6 J strain mice (6 weeks old) were obtained from Clea Japan (Osaka, Japan). They were acclimatized for 1 week before the experiments in a room maintained at 25 ± 1℃ with 60% relative humidity and given free access to nonpurified diet (8 g water, 51.3 g crude carbohydrate, 24.6 g crude protein, 5.6 g crude lipid, 3.1 g crude fiber, 6.4 g mineral mixture and 1 g vitamin mixture per 100 g diet; Oriental Yeast Ltd; Osaka Japan) and water. The room was illuminated for 12 h/d starting at 0700 h. Animals were treated according to the ethical guidelines of the Animal Center, School of Medicine, Ehime University. The experimental protocol was approved by the Animal Studies Committee of Ehime University.

Measurements of antitumor and antimetastatic activities in LLC-bearing mice : Cultures of LLC cells were harvested by trypsinization, washed and suspended at 5 x 10^5 cells/ml in DMEM supplemented with 10% FBS containing 1 mg/ml Matrigel. Matrigel was used to prevent the cell suspension from leaking out of the spleen. Solid-type LLC was prepared by intrasplenic implantation of 1 x 10^5 cells (0.2 ml) into the spleen of C57BL/6 female mice on day 0. Royal jelly (300 or 600 mg/kg) was administered orally once (at 0700 h) daily for 20 consecutive days, starting 12 h after implantation of the tumor cells. Sham-operated mice (normal) and LLC-implanted mice (control) were given distilled water alone on the same schedule. On day 21, blood was obtained via venipuncture in mice with pentobarbital anesthesia, and then spleen, thymus, lung and liver were removed and weighed for evaluation of antitumor and antimetastatic activities and side effects. The blood samples were chilled in test tubes containing heparin, and the number of red cells, leukocytes and hemoglobin content were measured using a Coulter Counter (Japan Scientific Instruments Ltd. Tokyo, Japan). The number of tumor colonies in the liver was counted manually.

Histological examination : All liver tissues were fixed in 10% buffered formalin for at least 24 h, and then divided in five blocks, and then progressively dehydrated in solutions containing an increasing percentage of ethyl alcohol (70, 80, 95 and 100 %). Following that, they were cleared in Histoclear, embedded in paraffin under vacuum, sectioned at 5-μm thickness, deparaffinized, and stained with Harris hematoxylin and eosin. After the same cross sections were selected from 5 plates per one sample, 4 different microscopic fields (x 20 magnification) per plate were photographed, and the photomicrograph images were stored in a computer. The total area of tumor metastatic colonies in each photograph (x 20 magnification) was measured using Adobe Photoshop (Adobe, Tokyo, Japan).

Measurement of Matrigel-induced neovascularization : *Exo-vivo* Matrigel-induced neovascularization was assayed according to the method of Passaniti *et al.*[6] Briefly, female C57BL/6 mice were each injected subcutaneously with 0.5 ml of Matrigel containing 1 ng of acidic fibroblast growth factor (aFGF) and 64 U of heparin per ml in the presence or absence of royal jelly (800 μg/ml). The mice were killed on day 5 with an overdose of pentobarbital, and the gels were removed and weighed. The distilled water (1 ml) was added in the gels, and then the gels were sonicated at 4℃ and centrifuged at 2000 x g and 4℃ for 10 min. The hemoglobin content in the supernatant was determined using Hemoglobin-Test kits (Wako Pure Chemical Co., Osaka, Japan).

Data and statistical analyses : All values are expressed as mean ± standard error of the mean (S.E.M.). Data were analyzed by one-way ANOVA, and then differences in means among groups were analyzed using Fisher's protected LSD multiple comparison test (significantly different at $p<0.05$).

Results

Antitumor and antimetastatic activities

The spleen weights of mice with intrasplenic implantation of LLC cells were significantly greater than those of sham-operated mice (normal mice) (Fig. 1). In control mice, the increase of the spleen weight was significantly inhibited by orally administered royal jelly at

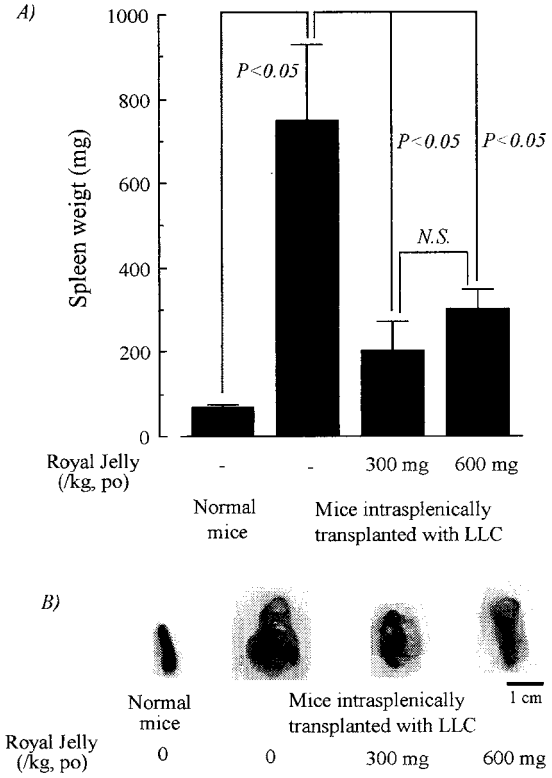

Fig. 1 Effect of royal jelly on intrasplenic tumor growth on day 21 in Lewis lung carcinoma (LLC)-bearing mice.
A): Solid-type LLC was prepared by intrasplenic implantation of 1 x 10^5 cells (0.2 ml) containing 1 mg/ml Matrigel into the spleen of C57BL/6 female mice on day 0. Sham-operated mice (normal) and LLC-implanted mice (control) were given distilled water for 20 days. LLC-implanted mice were administered 300 or 600 mg/kg of royal jelly orally once daily for 20 days.
Values are mean ± S.E.M. The sham-operated group (normal) consisted of 5 mice; the LLC-bearing group (control) and royal jelly-treated groups (300 or 600 mg/kg) consisted of 8 mice per group.
B): Photographs of the inhibition of primary tumor growth in the spleen by royal jelly.

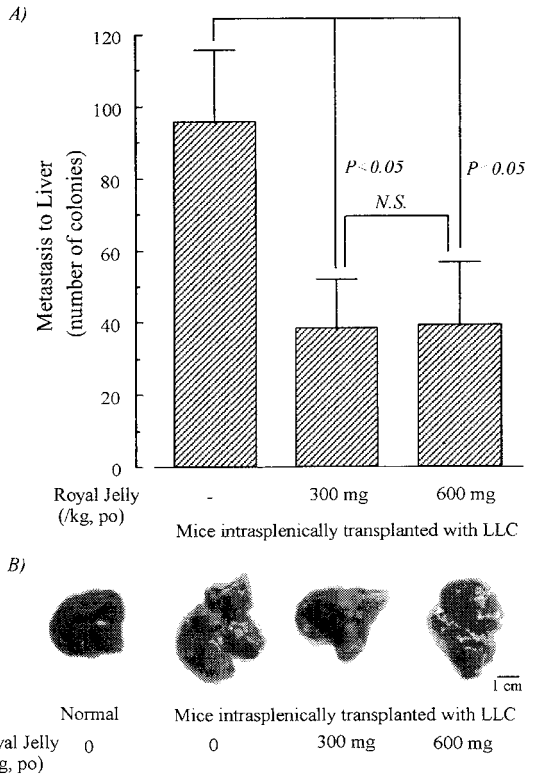

Fig. 2 Effect of royal jelly on LLC tumor metastasis to the liver on day 21 in LLC-bearing mice.
A): Values are mean ± S.E.M. The sham-operated group (normal) consisted of 5 mice; the LLC-bearing group (control) and royal jelly-treated groups (300 or 600 mg/kg) consisted of 8 mice per group.
B): Photographs of the inhibition of tumor metastatic colonies to the liver by royal jelly.

a dose of 300 or 600 mg/kg (Fig. 1). Royal jelly (300 or 600 mg/kg) significantly reduced the number of tumor cell colonies that metastasized to the liver compared with the number in control mice (Fig. 2). Antitumor and antimetastatic effects of royal jelly significantly did not differ at doses between 300 mg/kg and 600 mg/kg of royal jelly.

Body, liver, lung and thymus weights

Royal jelly had no effect on final body, lung or liver weights in control mice compared with those in normal mice (Table I). In control mice, the thymus weight was significantly lower than that in normal mice. Royal jelly

Table I Effects of royal jelly on the weights of body, thymus, lung and liver in LLC-bearing C57BL/6 mice[1,2]

	Body (g)	Thymus (mg)	Lung (mg)	Liver (g)
Sham-operated mice				
(Normal) (n=5)	20.5 ± 0.44	83.92 ± 10.59*	147.21 ± 8.79	1.24 ± 0.10
LLC-bearing mice				
(Control) (n=8)	20.9 ± 0.86	55.24 ± 8.00	159.70 ± 13.62	1.59 ± 0.18
+ Royal jelly				
(300 mg/kg) (n=8)	21.5 ± 0.85	58.13 ± 3.88	159.27 ± 5.30	1.58 ± 0.12
(600 mg/kg) (n=8)	20.9 ± 0.65	51.83 ± 6.24	145.11 ± 5.05	1.48 ± 0.08

[1] Royal jelly was administered orally for 20 days, starting 12 h after implantation of the LLC cells. Sham-operated mice (normal) and LLC-implanted mice (control) were given distilled water alone on the same schedule.
[2] Each value represents the mean ± S.E.M., n=5-8. *Significantly different from control mice, $p<0.05$.

Table II Effects of royal jelly on the number of red cells and leukocytes and the hemoglobin content in LLC-bearing C57BL/6 mice [1,2]

	Red cells (x 10^4/μl)	Leukocytes (μl)	Hemoglobin (g/100 ml)
Sham-operated mice			
(Normal) (n=5)	779 ± 9*	2650 ± 150	12.1 ± 0.12*
LLC-bearing mice			
(Control) (n=8)	495 ± 98	4333 ± 531	7.50 ± 1.54
+ Royal jelly			
(300 mg/kg) (n=8)	645 ± 84	3400 ± 423	9.68 ± 1.30
(600 mg/kg) (n=8)	644 ± 59	4067 ± 750	9.60 ± 0.94

[1] Royal jelly was administered orally for 20 days, starting 12 h after implantation of the LLC cells. Sham-operated mice (normal) and LLC-implanted mice (control) were given distilled water alone on the same schedule.
[2] Each value represents the mean ± S.E.M., n=5-8. *Significantly different from control mice, $p<0.05$.

Table III Effect of royal jelly on the area of metastatic tumors in the liver in LLC-bearing C57BL/6 mice [1,2]

	Area of metastatic tumor in liver[3] (mm^2/filed)
Sham-operated mice (Normal) (n=5)	0 ± 0*
LLC-bearing mice (Control) (n=8)	11.1 ± 2.26
+ Royal jelly	
(300 mg/kg) (n=8)	5.46 ± 2.41
(600 mg/kg) (n=8)	4.33 ± 1.99*

[1] Royal jelly was administered orally for 20 days, starting 12 h after implantation of the LLC cells. Sham-operated mice (normal) and LLC-implanted mice (control) were given distilled water alone on the same schedule.
[2] Each value represents the mean ± S.E.M., n=5-8. *Significantly different from control mice, $p<0.05$.
[3] After the same cross sections were selected from 5 plates per one sample, 4 different microscopic fields (x 20 magnification) per plate were photographed, and the photomicrograph images were stored in a computer. The total area in each photograph (x 20 magnification) was measured using Adobe Photoshop (Adobe, Tokyo, Japan). The area of metastatic tumors in the liver was measured by histological observation after staining with Harris hematoxylin and eosin.

(300 and 600 mg/kg) had no effect on the reduction of the thymus weight in LLC-bearing mice (Table I).

Number of leukocytes, red cells and hemoglobin content in blood

The number of leukocytes in control mice tended to be greater than that in normal mice, although the difference was not significant ($p=0.0623$). In contrast, the number of red cells and the hemoglobin content in control mice were significantly lower than those in normal mice (Table II). Thus, it was found that the intrasplenic implantation of LLC cells caused anemia. The numbers of leukocytes and red cells and hemoglobin content were not affected by the oral administration of royal jelly for 20 consecutive days in LLC-bearing mice (Table II).

Histology of liver

As shown in Figure 3, metastasis to the liver was caused by intrasplenic implantation of LLC cells. The area of metastatic tumors in the liver in royal jelly-treated mice was smaller than that in control mice (Fig. 3 and Table III).

Matrigel-induced angiogenesis (exo-vivo)

The gels that formed after subcutaneous implantation of Matrigel without aFGF and heaprin were readily

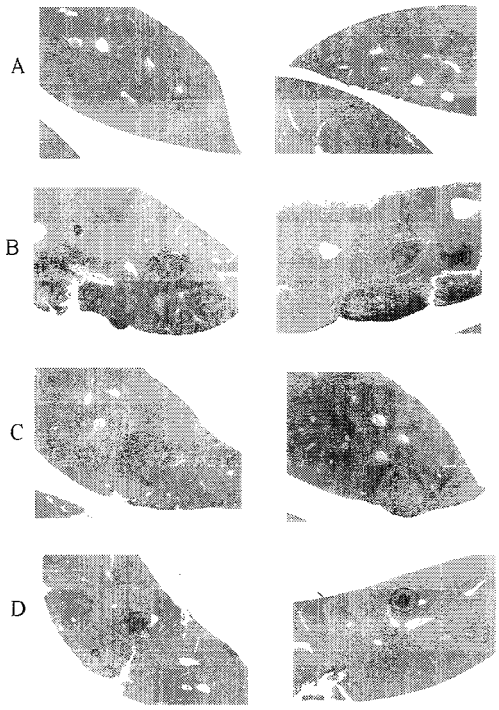

Fig. 3 Effects of royal jelly on the histology of LLC cells metastasizing to the liver on day 21 in LLC-bearing mice.
Photographs of livers from sham-operated mice (A), LLC-implanted mice (B), and LLC-implanted mice administered 300 mg (C) or 600 mg/kg (D) of royal jelly orally for 20 days.

Table IV Effects of royal jelly on the weight and hemoglobin content of the gels 5 days after implantation into mice of Matrigel supplemented with acidic fibroblast growth factor (aFGF) and heparin [1,2]

Treatment	Matrigel weight (mg)	Hemoglobin content (mg/Matrigel)
Matrigel alone	107.4 ± 12.83*	11.4 ± 1.95*
Matrigel + aFGF (1 ng/ml) +heparin (64 U/ml) (Control)	266.2 ± 67.88	23.2 ± 6.13
Matrigel, aFGF, heparin + royal jelly (800 μg/ml)	120.8 ± 14.66*	11.2 ± 1.20*

[1] C57BL/6 mice were injected subcutaneously with 0.5 ml of Matrigel supplemented with 1 ng/ml aFGF and 64 U/ml heparin in the absence or presence of royal jelly (800 μg/ml).

[2] Each value represents the mean ± S.E.M., n=5. *Significantly different from Matrigel/aFGF/heparin (Control), $p<0.05$.

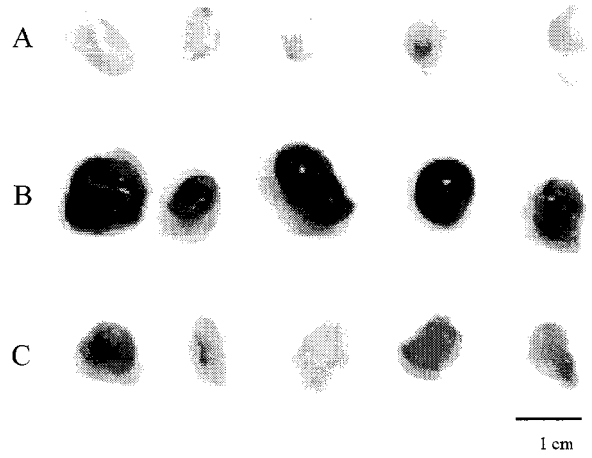

Fig. 4 Photographs of Matrigel 5 days after subcutaneous injection of mice with 0.5 ml of Matrigel without aFGF and heparin (A), Matrigel supplemented with 1 ng/ml aFGF and 64 U/ml heparin (B) or the Matrigel/aFGF/heparin mixture plus royal jelly (800 μg/ml) (C)

distinguished from surrounding tissue and produced only a minor or local angiogenic response (Fig. 4A). However, Matrigel supplemented with 1 ng aFGF and 64 U heparin per ml produced gels that induced an angiogenic reaction (Fig. 4B). The Matrigel/aFGF/heparin mixture caused a significant increase of the weight of the gel and the hemoglobin content in the gels at 6 days after implantation compared with the Matrigel without aFGF and heparin (Table IV). Royal jelly (800 μg/ml) inhibited the increases in the weight and hemoglobin content of the gels (Fig. 4C and Table IV).

Discussion

The removal of certain cancers, for example, breast carcinoma, colon carcinoma and osteogenic sarcoma, may be followed by the rapid growth of distant metastases to lung, liver, *etc.* Therefore, new anticancer agents with antitumor and antimetastatic activities are now being sought. In previous reports, we showed that stilbenes from the heartwood of *Cassia* and *Polygonum* species inhibited tumor metastases to the lung through the inhibition of tumor-induced neovascularization.[7-10] As part of our series of studies of the antitumor and antimetastatic activities of natural products, in the present study we examined the inhibitory effects of royal jelly on tumor growth and tumor metastasis to the liver in mice implanted intrasplenically with LLC. It has been reported that subcutaneous LLC-implantation in the foot pad or back in C57BL/6 mice resulted in lung metastasis in addition to tumor growth.[11-14] In the present study, we found that the intrasplenic implantation of LLC cells resulted in tumor metastasis to the liver. The present study showed that tumor growth in the spleen and liver metastasis were inhibited by the oral administration of royal jelly for 20 consecutive days at a dose of 300 or 600 mg/kg in control mice. Antitumor and antimetastatic actions by royal jelly did not show a significant difference at doses between 300 and 600 mg/kg. Thus, it was indicated that these effects of royal jelly were dose-independent. This reason is unknown. On the other hand, royal jelly (1 to 1000 μg/ml) had no cytotoxic effect against LLC cells *in vitro* (data not shown).

Angiogenesis is the growth of new capillary blood vessels from preexisting capillaries and postcapillary venules. Solid tumors cause neovascularization and the resultant angiogenesis from solid tumors stimulates tumor growth and metastasis.[15-18] Therefore, to clarify whether antiangiogenesis might be involved in the inhibition of the growth of primary tumors and metastasis to the liver, we examined the effects of royal jelly on Matrigel-induced neovascularization using *exo-vivo* model. Female C57BL/6 mice were injected subcutaneously with Matrigel containing aFGF and heparin with or without royal jelly. Royal jelly inhibited the Matrigel-induced neovascularization at a concentration of 800

μg/ml. Experiments are now in progress to isolate the active substance from various fractions of royal jelly using this *exo-vivo* model. This finding indicates that the antitumor and antimetastatic activities of royal jelly may be partly due to direct inhibition of angiogenesis induced by solid tumors. This is the first report showing that royal jelly has an antiangiogenic effect.

和文抄録

ローヤルゼリーは働き蜂の咽頭腺から分泌され，幼虫の餌となり，それを食した蜂は女王蜂となる。著者らは，脾臓内に高転移能のルイス肺癌細胞移植したマウスへのローヤルゼリー300mg/kgもしくは600mg/kgの投与は，腫瘍重量および肝臓への癌転移を有意に減少させたことを見出した。ローヤルゼリーの抗腫瘍および抗転移効果を明らかにする目的で，著者らはマトリゲルによる血管新生に対する影響を*exo-vivo*モデル，すなわち，ローヤルゼリーを添加あるいは無添加の酸性線維芽細胞成長因子とヘパリン含有マトリゲルをマウスの皮下に移植して検討した。ローヤルゼリーはマトリゲルによって誘導される血管新生を抑制した。これらの結果から，ローヤルゼリーの抗腫瘍および抗転移効果の作用の一部は，血管新生の阻害によるものと推測される。

*〒791-0295 愛媛県温泉郡重信町志津川
愛媛大学医学部医化学第二教室 木村善行

References

1) Willson, R.B. : Royal jelly. A review. *American Bee Journal* **97**, 356-359, 396-399, 1957.
2) Okuda, H., Kameda, K., Morimoto, C., Matsuura, Y., Chikaki, M. and Jiang, M.: Studies on insulin-like substances and inhibitory substances toward angiotensin-converting enzyme in royal jelly. *Honeybee Science* **19**, 9-14, 1998. (In Japanese)
3) Townsend, G.F., Morgan, J.F. and Hazlett, B.: Activity of 10-hydroxydecenoic acid from royal jelly against experimental leukaemia and ascetic tumors. *Nature* **183**, 1270-1271, 1959.
4) Townsend, G.F., Morgan, J.F., Tolnai, S., Hazlett, B. Morton, H.J. and Shuel, R.W.: Studies on the in vitro antitumor activity of fatty acids I. 10-hydroxy-2-decenoic acid from royal jelly. *Cancer Res.* **20**, 503-510, 1960.
5) Tamura, T., Fujii, A. and Kuboyama, N.: Antitumor effects of royal jelly. *Folia Pharmacol. Jpn.* **89**, 73-80, 1987. (In Japanese)
6) Passaniti, A., Taylor, R.T., Pili, R., Guo, Y., Long, P.V., Haney, J.A., Pauly, R.R., Grant, D.S. and Martin, G.R.: Methods in laboratory investigation. A simple, quantitative method for assessing angiogenesis and antianigiogenic agents using reconstituted basement membrane, heparin, and fibroblast growth factor. *Laboratory Invest.* **67**, 519-528, 1992.
7) Kimura, Y., Baba, K. and Okuda, H.: Inhibitory effects of active substances isolated from *Cassia garrettiana* heartwood on tumor growth and lung metastasis in Lewis lung carcinoma-bearing mice (Part 1). *Anticancer Res.* **20**, 2899-2906, 2000.
8) Kimura, Y., Baba, K. and Okuda, H.: Inhibitory effects of active substances isolated from *Cassia garrettiana* heartwood on tumor growth and lung metastasis in Lewis lung carcinoma-bearing mice (Part 2). *Anticancer Res.* **20**, 2923-2930, 2000.
9) Kimura, Y. and Okuda, H.: Effects of naturally occurring stilbene glycosides from medicinal plants and wine, on tumor growth and lung metastasis in Lewis lung carcinoma-bearing mice. *J. Pharm. Pharmacol.* **52**, 1287-1295, 2000.
10) Kimura, Y. and Okuda, H.: Resveratrol isolated from *Polygonum cuspidatum* root prevents tumor growth and metastasis to lung and tumor-induced neovascularization in Lewis lung carcinoma-bearing mice. *J. Nutr.* **131**, 1844-1849, 2001.
11) DeWys, W.D.: Studies correlation the growth rate of a tumor and its metastases and providing evidence for tumor-related systemic growth-retarding factor. *Cancer Res.* **32**, 374-379, 1972.
12) Gorelik, E., Segal, S. and Feldman, M.: Growth of local tumor exerts a specific inhibitory effect on progression of lung metastases. *Inter. J. Cancer* **21**, 617-625, 1978.
13) Gorelik, E., Segal, S. and Feldman, M.: Control of lung metastasis progression in mice: Role of growth kinetics of 3LL Lewis lung carcinoma and host immune activity. *J. Nat. Cancer Inst.* **65**, 1257-1264, 1980.
14) O'Reilly, M.S., Homgren, L., Shing, Y., Chen, C., Rosenthal, R.A., Moses, M., Lane, W.S., Cao, Y., Sage, E.H. and Folkman, J.: Angiostatin: A novel angiogenesis inhibitor that mediates the suppression of metastases by a Lewis lung carcinoma. *Cell* **79**, 315-328, 1994.
15) Koch, A.E., Halloran, M.M., Hassle, C.J., Shah, M.R. and Polverini, P.J.: Angiogenesis mediated by soluble forms of E-selectin and vascular cell adhesion molecule-1. *Nature* **376**, 517-519, 1995.
16) Bischoff, J.: Cell adhesion in vascular biology: Cell adhesion and angiogenesis. *J. Clin. Invest.* (Suppl) **100**, s37-s39, 1997.
17) O'Reilly, M.S., Boem, T., Shing, Y., Fukai, N., Vasios, G., Lane, W.S., Flynn, E., Birkhead, J.R., Olsen, B.R. and Folkman, J.: Endostatin: An endogenous inhibitor of angiogenesis and tumor growth. *Cell* **88**, 277-285, 1997.
18) Boem, T., Folkman, J. Browder, T. and O'Reilly, M.S.: Antiangiogenic therapy of experimental cancer does not induce acquired drug resistance. *Nature* **390**, 404-407, 1997.