Potential Ability of Hot Water Adzuki (Vigna angularis) Extracts to Inhibit the Adhesion, Invasion, and Metastasis of Murine B16 Melanoma Cells

Tomohiro ITOH,1,1 Hayato UMEKAWA,2 and Yukio FURUICHI2

1Imuraya Confectionary Co., Ltd., Takachaya 7-1-1, Tsu, Mie 514-8530, Japan
2Faculty of Bioresources, Mie University, Kamihama 1515, Tsu, Mie 514-8507, Japan

Received May 12, 2004; Accepted December 24, 2004

The 40% ethanol eluent of the fraction of hot-water extract from adzuki beans (EtEx.40) adsorbed onto DIAION HP-20 resin has many biological activities, for example, antioxidant, antitumorigenesis, and intestinal α-glucosidase suppressing activities. This study examined the inhibitory effect of EtEx.40 on experimental lung metastasis and the invasion of B16-BL6 melanoma cells. EtEx.40 was found significantly to reduce the number of tumor colonies. It also inhibited the adhesion and migration of B16-BL6 melanoma cells into extracellular matrix components and their invasion into reconstituted basement membrane (matrigel) without affecting cell proliferation in vitro. These in vivo data suggest that EtEx.40 possesses a strong antimetastatic ability, which might be a lead compound in functional food development.

Key words: adzuki beans (Vigna angularis); antimetastasis; B16-BL6 melanoma cells; matrix metalloproteinases

Metastasis, the major cause of cancer mortality, is a complex phenomenon in which tumor cells invade surrounding tissues, penetrate blood vessels, and exit vessels at distant sites to form secondary tumors.1) During the metastasis cascade, enzymic degradation of basement membranes, which is essential for cells to penetrate and exit blood vessels, is the target of antimetastatic drugs.2) Suppression of matrix metalloproteinases (MMPs), which is known to play an important role in invasion and metastasis in human cancer, might be connected to anti-tumor metastasis.

Adzuki beans (Vigna angularis) are a very important food material in the Far East. They have been used in traditional Chinese medicine for various purposes, e.g., as a diuretic, an antidote, and a remedy for dropsy and beriberi, but mainly for the production of traditional confectioneries (wagashi), e.g., youkan, manju, and amanatto, in Japan. When adzuki beans are used for confectioneries, they are boiled in a cooker and yield a hot water extract as a by-product, which is known to contain active ingredients but is wasted. It has been reported that the 40% ethanol fraction of hot-water extract from adzuki beans (EtEx.40) suppresses not only the proliferation of human stomach cancer cells in culture but also benzo(a)pyrene-induced tumorigenesis in mouse forestomachs.3,4) From these results, it was expected that EtEx.40 might inhibit experimental metastasis.

In this paper, the antimetastatic efficacy of EtEx.40 was studied on experimental lung metastasis of murine B16 melanoma (B16-BL6) in mice. The mechanisms of the antimetastatic effects of EtEx.40 were investigated using in vitro experiments relating to invasion, adhesion, and proliferation.

Materials and Methods

Preparation of test materials. A hot water extract of adzuki was obtained by boiling the beans of adzuki, harvested at Tokachi, Hokkaido, Japan. Concentrated in vacuo, the extract (about 15-liter) from about 2.5 kg of adzuki beans, gave about 30 g of a concentrate which was subjected to a open column chromatography on DIAION HP-20 (column size 5p × 300 mm) and eluted stepwise with distilled water and 40%, 60%, and 80% ethanol. The 40% ethanol fraction was evaporated to dryness and used for further analysis.

Animals. Four-week-old specific pathogen-free male C57BL/6J Jcl mice were purchased from Clea Japan, Tokyo. They were randomly assigned to group houses in the animal laboratory, which was maintained at a constant temperature (22 ± 2 °C) with a 12-h light and dark cycle (light from 08:00 to 20:00). All mice were fed commercial MF pellets (Oriental Yeast, Tokyo) and water ad libitum for one week to accustom them to their surroundings. This study was approved by the Mie University Animal Use Committee, and the animals were maintained according to the guidelines of Mie University for the care of laboratory animals.
**Cells and cell culture.** Murine melanoma cell lines (B16-BL6, highly metastatic) were provided by the Cell Resource Center for Biomedical Research of the Institute of Development, Aging and Cancer of Tohoku University. The B16-BL6 melanomas were maintained as a monolayer culture in Dulbecco’s Modified Eagle medium (DMEM) (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS), vitamin solution, 5% sodium pyruvate, nonessential amino acids and l-glutamine, 50 U/ml penicillin, and 50 μg/ml streptomycin at 37 °C in a humidified atmosphere of 5% CO₂/95% air.

**Experimental lung metastasis of B16-BL6 melanomas.** The experimental pulmonary metastases were assessed by injecting tumor cells into the lateral tail veins of mice. The B16-BL6 melanoma cells (2 × 10⁴/200 μl/mouse) were implanted intravenously in the C57BL/6J mice.

During the first experiment, three groups of 6 C57BL/6J mice were fed experimental water containing 1% hot water extract from adzuki beans (the HWEA group), 1% EtEx.40 (the EtEx.40 group), or water as a control 2 weeks before tumor inoculation. During the second experiment, three groups of 6 C57BL/6J mice were fed experimental water containing 1% hot water extract from adzuki beans (the HWEA group), 1% EtEx.40 (the EtEx.40 group), or water as a control from the day after tumor inoculation. All mice were sacrificed on day 21 after tumor inoculation. After recording the weight of the lungs, the lungs were fixed in Bouin’s solution and the lung tumor colonies were counted under a dissection microscope.

**Cell proliferation.** B16-BL6 melanoma cells (1 × 10⁴ cells/ml) were seeded into 12-well plates (Nunclon™, Nalge Nunc International, Denmark). After 24 h pre-incubation the cells were cultured with various concentrations of EtEx.40 in DMEM for a further 48 h. After 48 h incubation, the medium-sample mixture was aspirated and changed to fresh 10% FBS-DMEM, added to various concentrations of EtEx.40, and incubated for 24 h. Alamar blue solution (100 μl/ml medium, TREK™ Diagnostic System) was added to each well for 4 h before the end of the incubation period. Alamar blue is an oxidation/reduction indicator that fluoresces red when it accepts electrons generated during cellular metabolism. The absorbance of the culture solution was determined from a standard curve. The standard curve was created as follows: B16-BL6 melanoma cells (1 × 10⁵–1 × 10⁶ cells/ml) were seeded into 12-well plates. Alamar blue dye was then added each well, and the plate was incubated at 37 °C for 6 h. After incubation, the plate was measured with an immunoplate reader.

**Assay for tumor invasion and migration.** Tumor cell invasion was assayed in a Transwell cell culture chamber with polyvinylpyrrolidone (PVP)-free polycarbonate filters with 8-μm pores (Kurabo, Osaka, Japan) according to the method of Saiki et al.⁵ PVP-free polycarbonate filters were precoated with 5 μg of fibronectin on the lower surfaces and dried at room temperature. Reconstituted basement membranes (matrigel) (Collaborative Research, Bedford, MA) were applied to the upper surfaces of the filters (5 μg/50 μl/filter) and dried at room temperature. The filters were designated matrigel/fibronectin-coated filters and washed extensively in phosphate-buffered saline (PBS) immediately before use. For the migration assay, the filters were precoated with fibronectin on the lower surfaces without coating them with matrigel.

Tumor cells in the log phase culture were harvested, washed with serum-free DMEM, and resuspended in DMEM containing 0.1% bovine serum albumin (BSA). Cell suspensions (1 × 10⁵ cells/100 μl) were added to the upper compartment of the Transwell cell culture chamber. Next, 10% FBS-DMEM (600 μl) was added to the lower compartment and incubated at 37 °C in a humidified atmosphere with 5% CO₂/95% air for 6 and 24 h for the migration and invasion assays respectively. Quantification of the cells that had invaded or migrated onto the lower surfaces was measured colorimetrically using the crystal violet staining method, and each assay was performed in triplicate.

**Cell adhesion assay.** B16-BL6 melanoma cells (1 × 10⁵/ml) were cultured in T25 culture flasks (Nunclon™, Nalge Nunc) with serum-free DMEM for 24 h. After incubation, equal numbers of B16-BL6 melanoma cells (1 × 10⁵ cells/100 μl) were seeded into type IV collagen-coated 96-well plate (Bicocat, Becton Dickinson, Bedford, MA). Various concentrations of EtEx.40 were then added prior to incubation at 37 °C in a humidified atmosphere with 5% CO₂/95% air for 60 min. The wells were washed twice with warm PBS to remove any unattached cells, then the attached cells were stained with 0.5% crystal violet in 20% methanol for 30 min. After thorough washing with distilled water, the residual stained cells were lysed with 50 μl of 30% acetic acid, and the absorbance of the lysates was measured at 590 nm in an immunoreader.

**Gelatin zymography.** B16-BL6 melanoma cells (1 × 10⁵ cells/ml) were cultured in T25 culture flasks (Nunclon™, Nalge Nunc) with serum-free DMEM containing varying concentrations of EtEx.40 for 24 h. After 24 h incubation, the supernatant was collected and concentrated to × 100 with a Vivaspin 6 VS060 (Sartorius, Tokyo). The conditioned medium was used for detecting gelatinase activity, which was examined by gelatin zymography. Briefly, supernatant samples of B16-BL6 melanoma cells cultured in FBS-free medium were loaded with non-reducing sample buffer onto a
10% sodium dodecyl sulfate (SDS)–polyacrylamide gel (7.5% w/v) containing 1 mg/ml gelatin, and electrophoresed. After removal of the SDS from the gels by washing them twice with 2.5% Triton X-100 solution for 30 min and once with 10 mM Tris–HCl buffer (pH 8.0) for 30 min with shaking, the gels were incubated in 50 mM Tris–HCl buffer (pH 8.0) containing 5 mM CaCl₂, 0.1 μM ZnCl₂, and 0.05% NaN₃ at 37°C for 40 h to activate the MMPs. The activated gels were stained with 1% Coomassie Blue R-250 in 10% methanol and 5% acetone with shaking and subsequently destained with 10% methanol and 5% acetone solution. The gelatinolytic activity of each gelatinase was detected as a clear band against a blue background.

Measurement of DPPH radical-scavenging activity. To measure antioxidant activity, a 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical-scavenging assay was carried out according to the previous method with a slight modification. Briefly, the DPPH radical-scavenging activity was measured in a reaction mixture containing 1 mM DPPH radical solution, 0.1 mL, 99% ethanol 0.8 mL, and 0.1 mL of sample solution. The solution was rapidly mixed and scavenging capacity was measured spectrophotometrically by monitoring the decrease in absorbance at 517 nm. The antioxidant activity of EtEx.40 was expressed as IC₅₀, which was defined as the concentration of EtEx.40 required for inhibition of the formation of DPPH radicals by 50%. Vitamin C (L-ascorbic acid) and Vitamin E (α-tocopherol) were used as positive controls.

Statistical analysis. All data were analyzed first by one-way ANOVA, and subsequently by Duncan’s multiple-range test. The differences among the means were considered significant at p < 0.05.

Results

Experimental lung metastasis of B16-BL6 melanomas
The effect of EtEx.40 on experimental lung metastasis produced by i.v. injections of B16-BL6 melanoma cells was investigated. As Table 1 shows, the number of lung tumor colonies was significantly reduced in the mice fed EtEx.40 compared with the control group. However, effectiveness by giving EtEx.40 beforehand was not seen.

Cell proliferation
When B16-BL6 melanoma cells were incubated with EtEx.40 for 72 h, EtEx.40 did not affect cell proliferation at concentrations ranging from 0.01 to 1 μg/mL (Fig. 1A).

Assay for tumor invasion and migration
Tumor cell invasion into the extracellular matrix and basement membranes is crucial in the complex multi-stage process of metastasis. Therefore the effect of EtEx.40 on tumor cell invasion into the matrigel was examined. B16-BL6 melanoma cells were added to the upper compartment of the chamber with or without EtEx.40 for 24 h. Invasion of the tumor cells through the matrigel/fibronectin-coated filters tended to decrease in a dose-dependent manner, with EtEx.40 concentrations ranging from 0.01 to 10 μg/mL (Fig. 1B). Similarly, the haptotactic migration of B16-BL6 melanoma cells through the fibronectin-coated filters was also significantly inhibited in a dose-dependent manner by the addition of EtEx.40 ranging from 0.1 to 10 μg/mL (Fig. 1C).

Cell adhesion assay
Since the adhesion to and motility of tumor cells in the extracellular matrix are considered to be important steps in the invasive processes of metastatic tumor cells, the effects of EtEx.40 on adhesion were examined. Incubation of B16-BL6 melanoma cells with 0.1 to 1 μg/mL of EtEx.40 for 60 min significantly inhibited cell adhesion to the type IV collagen-coated substrate in a concentration-dependent manner (Fig. 1D).

Gelatin zymography
Gelatinases/type IV collagenase enzymes play a major role in the facilitation of cancer metastasis.

Table 1. Inhibitory Effect of the 40% Ethanol Eluent of the Partial Fraction Adsorbed onto DIAION HP-20 Resin of Hot-Water Extracts from Adzuki Beans (EtEx.40) on Lung Metastasis by i.v. Injection of B16-BL6 Melanoma Cells in C57BL/6 Mice

<table>
<thead>
<tr>
<th>Group</th>
<th>No. of colonies in lung</th>
<th>Range</th>
<th>Weight of lung (mg)</th>
<th>Body weight gain (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Vehile (control)</td>
<td>152 ± 10⁷</td>
<td>129–181</td>
<td>610 ± 91</td>
</tr>
<tr>
<td>2</td>
<td>Hot-water extract of adzuki beans</td>
<td>10 mg/ml</td>
<td>110 ± 12²</td>
<td>75–142</td>
</tr>
<tr>
<td>3</td>
<td>EtEx.40</td>
<td>10 mg/ml</td>
<td>80 ± 9⁹</td>
<td>52–108</td>
</tr>
</tbody>
</table>

Those not sharing a common superscript letter are significantly different at p < 0.05 by Duncan’s multiple range test.

Twenty thousand cells of highly metastatic B16-BL6 were injected i.v. into C57BL/6 mice. Lungs were excised on day 21, and metastasis foci in them were counted. In this experiment, three groups of 6 C57BL/6 Jel mice each were caused to drink an experimental water containing 1% hot water extract from adzuki beans (HWEA group) or 1% EtEx.40 (EtEx.40 group) or water as the control from the 2 weeks before tumor inoculation or the after tumor inoculation. Value are the means ± SD (n = 6).
MMP-2 and -9 are a family of zinc-dependent proteolytic enzymes known to degrade type IV collagen in the basal membrane, and their activities are regulated at various levels, such as during secretion and the activation of proMMP to enzymatically active MMP. Since the production of MMPs, including MMP-2 and -9, by tumor cells is closely correlated with the metastatic and invasive potentials in vivo, we investigated the effect of EtEx.40 of MMPs by B16-BL6 melanoma cells as detected by gelatin zymography. As shown in Fig. 2A, incubation of tumor cells with EtEx.40 resulted in inhibited MMP expression in a concentration-dependent manner. And suppression of MMP-2 and -9 expression by EtEx.40 was stronger than that of vitamin C or E (Fig. 2B).

Measurement of DPPH radical-scavenging activity
Recently, it was reported that peroxide stimulated cancer cell motility and metastasis.7,8) Thus measurement of radical-scavenging activity of this extract might
be connected with anti-metastatic activity. The free radical-scavenging activity of EtEx.40 was tested by this ability to bleach the stable radical DPPH. This assay provided information on the reactivity of the compounds with a stable free radical. Because of the odd electron, DPPH shows a strong absorption band at 517 nm in visible spectroscopy (deep violet color). As this electron becomes paired off in the presence of a free radical scavenger, the absorption vanishes, and the resulting decolorization is stoichiometric with respect to the number of electrons taken up. Vitamins C and E, reference drugs, exhibited scavenging activity with IC_{50} values of 8.1 and 9.9 µg/ml respectively. EtEx.40 also exhibited a DPPH radical-scavenging activity with a IC_{50} value of 2.1 µg/ml. (Fig. 3) The phenolic compounds epicatechin and quercetin also revealed scavenging activity on DPPH radicals with IC_{50} values of 12.8 and 8.2 µM respectively (data not shown).

Discussion

This study indicates that intakes of EtEx.40 attenuates experimental lung metastasis produced by i.v. inoculation of B16-BL6 melanoma cells, possibly through inhibition of the adhesion to, invasion and migration through, and enzymatic degradation of the extracellular matrix. Tumor migration towards interstitial stroma is an important step during tumor invasion of basement membranes. Cell motility is regulated by autocrine and paracrine mechanisms through tumor- or host-derived cytokines.\(^9\) Although the stimulatory mechanism of cell motility by many kinds of cytokines is clearly characterized, little is known about the inhibitors of cell motility or the signaling mechanism. This study indicates that EtEx.40 inhibits the haptotactic migration of B16-BL6 melanoma cells to fibronectin. Invasion of B16-BL6 melanoma cells into matrigel/fibronectin and B16-BL6 melanoma cell adhesion to type IV collagen was observed using an in vitro assay without affecting cell viability. Degradation of the extracellular matrix by
tumor cells follows tumor adhesion to the basement membranes during the invasive process.\textsuperscript{10} Of the several known MMPs, MMP-2 and -9 are secreted by a variety of tumor cells, and levels of these enzymes correlate with the metastatic potential of tumor cells.\textsuperscript{11} As shown in Fig. 2, EtEx.40 suppressed MMP-2 and -9 expression in B16-BL6 melanoma cells.

The physiological functions of the Rho family, including Rho (e.g., RhoA, B, and C), Rac, and Cdc42 proteins have been widely investigated.\textsuperscript{12} They regulate a wide variety of cell functions, especially cytoskeletal reorganization during cell motility.\textsuperscript{13} RhoC regulates actin cytoskeletal organization, which is believed to provide the driving force for cell migration.\textsuperscript{14} Recently, it was reported that RhoC apparently up-regulates both expression levels and activities of MMPs, leading to enhancement of invasive activity.\textsuperscript{15} Since the rise of Rho-dependently phospholipase D activity by insulin is controlled by protein kinase C (PKC), it is known that there is PKC as an upstream signal of Rho.\textsuperscript{16, 17}

Moreover, Klann \textit{et al.} have reported the mechanism of the activation of PKC by reactive oxygen species (ROS).\textsuperscript{18} ROS raises the activity of PKC, and since PKC carries out activity of the translocation to the cell membrane of Rho, it turns out that the signal of ROS-PKC-Rho motility exists. Perhaps it led to reducing ROS controlling cancer metastasis. So, in this experiment, when the anti-oxidative activity of EtEx.40 was measured using DPPH, activity stronger than vitamin C or E was found. And MMP-2 and -9 expression by treatment with vitamin C or E was stronger than EtEx.40. Since vitamin C was itself a very unstable hydrophilic substance and it promptly inactivated by cancer cells, it was reported that EtEx.40 is about 50% polyphenols (data not shown). Inoue \textit{et al.} indicated that EtEx.40 is about 50% polyphenols (data not shown). Inoue \textit{et al.} indicated that these compounds have the scavenging activity of superoxide radicals.\textsuperscript{28, 29} Recently, it appeared that the scavenging activity of polyphenols such as (−)-epigallocatechin gallate might be involved in the antimetastatic process.\textsuperscript{30} The results of the Folin-Denis method indicated that EtEx.40 is about 50% polyphenols (data not shown).

Rutin, epicatechin, naringin, and naringenin are also known to inhibit lung tumor nodule formation,\textsuperscript{26} and quercetin inhibits the invasion of B16-BL6 melanoma cells by decreasing pro-MMP-9.\textsuperscript{27} It has been reported that these compounds have the scavenging activity of superoxide radicals.\textsuperscript{28, 29} Therefore, further examination of these effects is required.

Most of the MMP inhibitors that act as antimetastasis drugs are pseudopeptides with a catalytic zinc-chelating group, and thus they competitively inhibit MMPs. But recently, several antimetastatic drugs or anti-tumor compounds have been found in natural products such as trees, vegetables, and herbs.\textsuperscript{21–23} It has been reported, for example, that crucumin and catechin strongly inhibit lung metastasis induced by melanoma cells in mice.\textsuperscript{24, 25}

References


