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FOOD & FUNCTION

Resveratrol derivative-rich melinjo (*Gnetum gnemon* L.) seed extract suppresses multiple angiogenesis-related endothelial cell functions and tumor angiogenesis

Kazuhiro Kunimasa[1], Toshiro Ohta[2], Hiroko Tani[3], Eishin Kato[4], Ryoji Eguchi[5], Kazuhiko Kaji[2,6], Katsumi Ikeda[7], Hideki Mori[1], Mari Mori[1], Tomoki Tatefuji[1,3] and Yukio Yamori[1]

[1] Institute for World Health Development, Mukogawa Women's University, Hyogo, Japan
[2] Graduate School of Nutritional and Environmental Sciences, University of Shizuoka, Shizuoka, Japan
[3] Institute for Bee Products and Health Science, Yamada Apiculture Center, Inc., Okayama, Japan
[4] Hosoda SHC Co., Ltd., Fukui, Japan
[5] Department of Thoracic Oncology, Hyogo College of Medicine, Hyogo, Japan
[6] The Open University of Japan, Chiba, Japan
[7] School of Pharmacy and Pharmaceutical Sciences, Mukogawa Women's University, Hyogo, Japan

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Angiogenesis is a promising target for cancer prevention and treatment. This study aimed to determine the antiangiogenic effects of melinjo (*Gnetum gnemon* L.) seed extract and its resveratrol derivative components, such as gnetin C (GC), gnetin L (GL), gnemonoside A (GMA), gnemonoside C (GMC), and gnemonoside D (GMD). An ethanol extract of melinjo seeds (EEMS) and the two gnetins markedly inhibited the proliferation and tube formation of human umbilical vein endothelial cells (HUVEC) stimulated with vascular endothelial growth factor and basic fibroblast growth factor. The inhibitory effects of GC and GL were much stronger than those of resveratrol. GMC and GMD inhibited only proliferation, whereas GMA had almost no effect on the two endothelial cell functions. The EEMS and GC also reduced the cell viability of tube-forming HUVEC, with accompanying ERK1/2 inactivation, and suppressed the migration of HUVEC. Furthermore, dietary intake of EEMS significantly inhibited tumor angiogenesis in a mouse dorsal air sac assay. In conclusion, we found that the EEMS and its resveratrol derivatives, particularly GC, suppress multiple angiogenesis-related endothelial cell functions and/or tumor angiogenesis, indicating that the melinjo seeds and the natural resveratrol derivatives may be useful for cancer prevention and treatment.

Keywords:
Angiogenesis / Endothelial cells / Melinjo seeds / Resveratrol derivatives / Tube formation

Angiogenesis is the formation of new blood vessels from the preexisting ones. Tumor-induced neovessels carry oxygen and nutrients into tumor tissue and functions as the primary path of metastasis [1]. In angiogenesis, quiescent endothelial cells (EC) are activated by angiogenic factors, such as vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF), which are produced by tumor cells and tumor-associated macrophages [2].

Correspondence: Dr. Kazuhiro Kunimasa, Institute for World Health Development, Mukogawa Women's University, 4-16 Edagawa-cho, Nishinomiya, Hyogo 663-8143, Japan
E-mail: kunimasa@mukogawa-u.ac.jp
Fax: +81-798-45-9981

Abbreviations: bFGF, basic fibroblast growth factor; **CCK-8,** cell counting kit-8; **EC,** endothelial cell; **EEMS,** ethanol extract of melinjo seeds; **ERK,** extracellular signal-regulated kinase; **GC,** gnetin C; **GL,** gnetin L; **GMA,** gnemonoside A; **GMC,** gnemonoside C; **GMD,** gnemonoside D; **HUVEC,** human umbilical vein endothelial cell; **MEK,** mitogen-activated protein kinase/extracellular signal-regulated kinase kinase; **VEGF,** vascular endothelial growth factor

The activated EC form new capillary vessels via extracellular matrix (ECM) degradation, migration, proliferation, and tube formation. Thus, these angiogenesis-related EC functions are promising targets for cancer prevention and treatment.

Antiangiogenic therapy must be administered as a long-term treatment to keep tumors in a dormant state. Edible plants with an acceptable safety profile have received increasing attention as practical tools to control tumor angiogenesis [3]. Furthermore, studies in this area have lead to the identification of various dietary components with both a favorable safety profile and antiangiogenic property, including resveratrol [4]. Edible plants can be attractive sources of useful angiogenesis inhibitors.

Melinjo (Indonesian name; *Gnetum gnemon* L.) is an arboreal dioecious plant widely cultivated in Southeast Asia, where its seeds and fruits are eaten as an ordinary vegetable. We recently found that an ethanol extract of melinjo seeds (EEMS) contains gnetin C (GC; resveratrol dimer), gnemonoside A (GMA; GC-diglucoside), and gnemonoside D (GMD; GC-monoglucoside) as major components and resveratrol, gnetin L (GL; resveratrol derivative), and gnemonoside C (GMC; GC-monoglucoside) as minor components (Fig. 1A and Supporting Information materials and methods) [5]. Diverse resveratrol derivatives, including resveratrol oligomers, have also been identified in several plants [6], some of which have stronger antitumor and antiangiogenic activities than resveratrol [7, 8]. These findings led us to speculate that resveratrol derivative-rich EEMS and its resveratrol derivative components may have potent antiangiogenic properties. In this study, we investigated their antiangiogenic activities using in vitro and in vivo angiogenesis models.

EC in tumor microenvironment have much higher proliferation rates than the quiescent EC in normal tissues [9], indicating that antiproliferative activity on EC is a useful indicator for identifying angiogenesis inhibitors. In a cell counting kit-8 (CCK-8) assay, EEMS (40 μg/mL), GC (5 μM), and GMD (5 μM) completely suppressed VEGF- and bFGF-induced human umbilical vein endothelial cell (HUVEC) proliferation (Fig. 1B), which were concentration-dependent (Supporting Information Fig. S1). Furthermore, GL and GMC at 5 μM markedly suppressed the proliferation, whereas GMA and resveratrol at 5 μM had very little inhibitory effect. Given the resveratrol derivatives in the EEMS, except for GMA, had stronger growth inhibitory effects than did resveratrol, the inhibitory effect of the EEMS may stem from such highly active derivatives, particularly GC and GMD. From the viewpoint of structure–activity relationships, we found that the methylation and glucosidation in A1 ring of GC negatively affected the growth inhibitory effect, whereas the glucosidation in B1 ring of GC had very little effect unless the A1 ring was additionally glucosidated. Further studies on these relationships may provide useful clue to develop novel resveratrol derivatives with more potent antiproliferative activity.

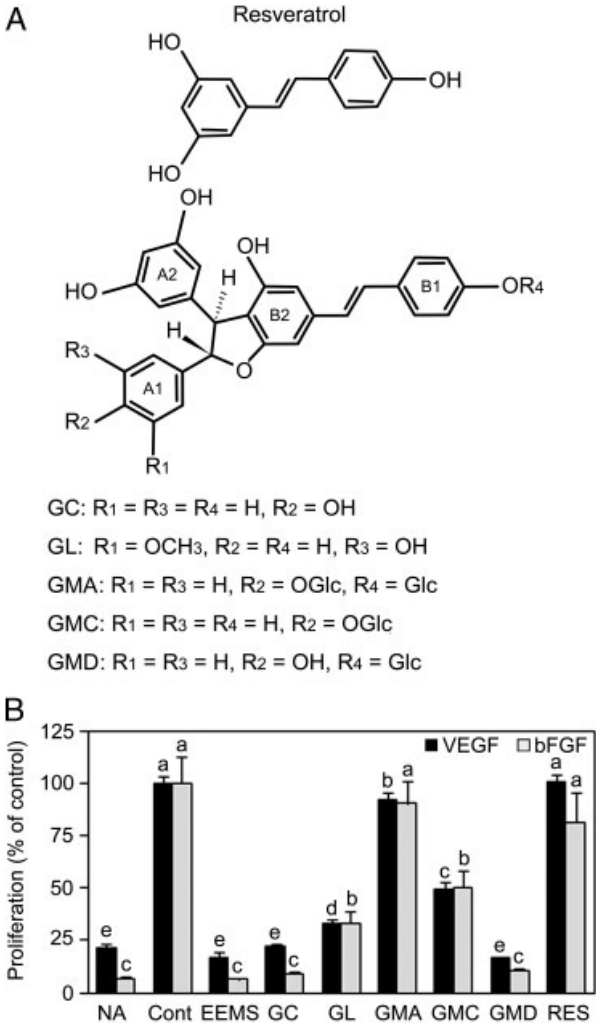

Figure 1. (A) The chemical structures of resveratrol and its derivatives in the EEMS. (B) The effect of the EEMS and its resveratrol derivatives on the VEGF- or bFGF-induced proliferation of HUVEC. HUVEC were treated with vehicle, EEMS (40 μg/mL), each resveratrol derivative (5 μM), or resveratrol (RES; 5 μM) in the presence of 10 ng/mL VEGF or 10 ng/mL bFGF for 72 h. NA indicates no angiogenic stimulus (VEGF- or bFGF-free condition). Cell proliferation was determined with a CCK-8 assay. Data are shown as mean ± SD, $n = 4$. Means without a common letter differ significantly ($p < 0.05$).

We next investigated the effects of the EEMS and the resveratrol derivatives on the secretion and activation of matrix metalloproteinase-2, an ECM-degrading enzyme essential for tumor angiogenesis [10]. Gelatin zymographic analysis revealed that the EEMS and its components had very little effect on pro-MMP-2 secretion and its activation (Supporting Information Fig. S2). In the future studies, we should focus on the effects of the EEMS and its components on plasminogen activator, another angiogenesis-promoting proteinase [10].

We further investigated the effects of the EEMS and its components on tube formation in an in vitro angiogenesis

model using collagen matrices (Fig. 2A and B). The EEMS (80 μg/mL), GC (10 μM), and GL (10 μM) completely inhibited VEGF- and bFGF-induced tube formation of HUVEC, which were concentration-dependent with the IC_{50} values of 33.9 μg/mL for the EEMS, 2.6 μM for GC, and 3.2 μM for GL (Supporting Information Fig. S3). The values of GC and GL were much lower than that of resveratrol (28.9 μM). In contrast, the three gnemonosides at 10 μM had very little effect (Fig. 2A and B), indicating that the glucosidation of GC negatively affect its inhibitory effect. Considering the relative amount of GC (27.0 mg/g of EEMS) and GL (a minor component) in the EEMS, the inhibitory effect of the EEMS on tube formation might be attributed to GC. Lee et al. reported that the greater apoptosis efficacy of heyneanol A, a natural resveratrol tetramer, than that of resveratrol may be attributed to its increased lipophilicity [8]. A hydrophobic benzofuran skeleton in the EEMS components contributes to increase their lipophilicity. Thus, the

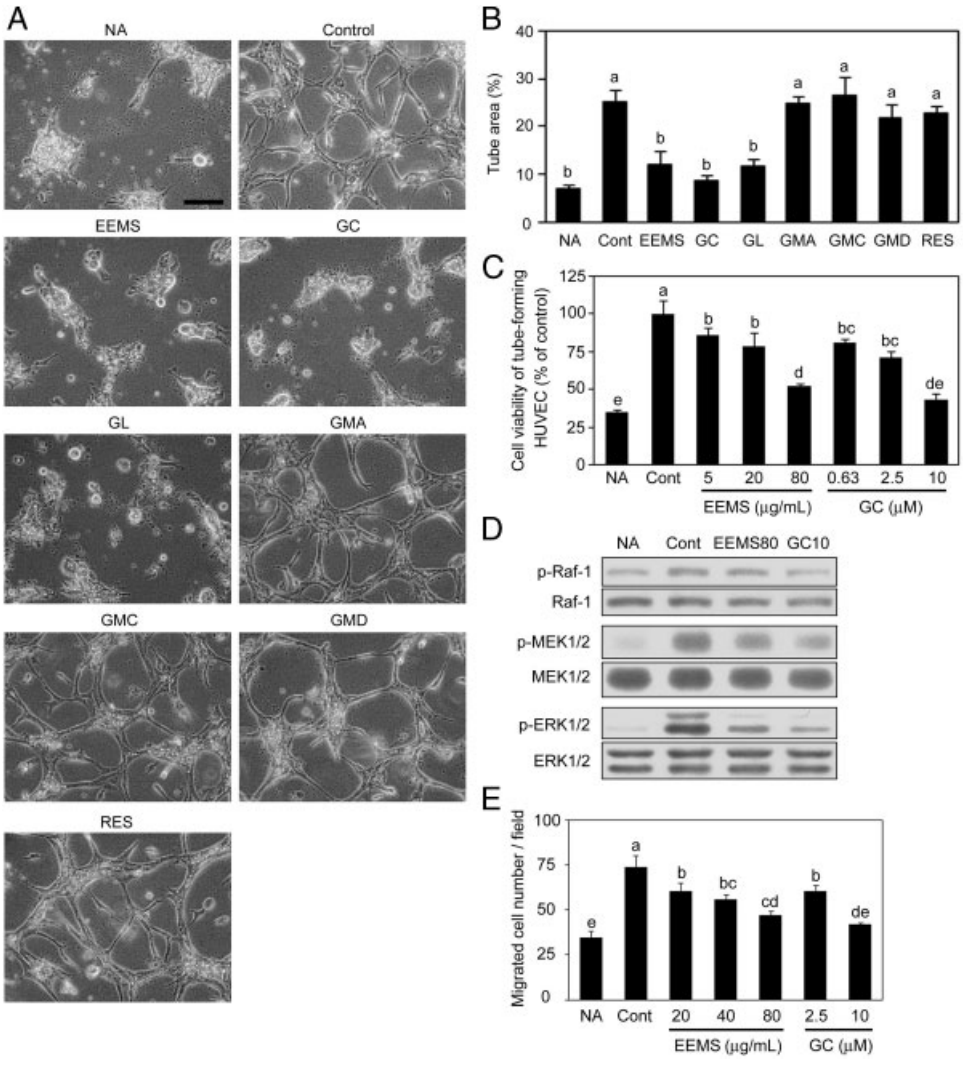

Figure 2. The effect of the EEMS and its resveratrol derivatives on tube formation, cell viability, survival signaling, and migration of the VEGF- and bFGF-stimulated HUVEC. (A) HUVEC were induced between two layers of type I collagen matrices to form capillary tube-like structures in the presence of 10 ng/mL VEGF and 10 ng/mL bFGF with vehicle, EEMS (80 μg/mL), each resveratrol derivative (10 μM), or resveratrol (RES; 10 μM) for 24 h. NA indicates no angiogenic stimuli. The bar indicates 100 μm. (B) The formed tube area in (A) was determined by calculating the ratio of the formed tube area to the total area of the picture field. Data are shown as mean ± SD, $n = 3$. (C) HUVEC were induced to form three-dimensional tube-like structures in the presence of 30 ng/mL VEGF and 30 ng/mL bFGF with vehicle, EEMS, or GC for 24 h. The cell viability of tube-forming HUVEC was determined with a CCK-8 assay. Data are shown as mean ± SD, $n = 4$. (D) After a 0.5-h treatment with vehicle, EEMS, or GC, cellular proteins were collected from tube-forming HUVEC. Changes in Raf-1, MEK1/2, and ERK1/2 were analyzed by Western blotting. (E) HUVEC were allowed to migrate to the acellular area in the presence of 10 ng/mL VEGF and 10 ng/mL bFGF with vehicle, EEMS, or GC for 6 h. The migrated cell number per field was counted. Data are shown as mean ± SD, $n = 3$. Means without a common letter differ significantly ($p < 0.05$).

greater lipophilicity of GC and GL without hydrophilic glucose moiety may be responsible for their stronger inhibitory effects than that of resveratrol. Furthermore, Roberts et al. reported that the ratio of $S+G_2/M$ phase in tube-forming EC within collagen matrices is much lower than that of proliferating EC on gelatin-coated dishes (2 versus 22%) [11], indicating that most tube-forming EC are under cell cycle arrest. Thus, GMC and GMD that markedly inhibited the proliferation of HUVEC but not the tube formation may mainly target cell cycle regulators that are active in the proliferating EC, but not in tube-forming ones. Further studies on these points provide the novel insights into angiogenesis inhibitors with the specificity for cell cycle phase.

To further investigate the effects of the EEMS and GC, the most effective component among the resveratrol derivatives tested, on the cell viability of tube-forming HUVEC, we performed a CCK-8 assay in a three-dimensional tube formation model. The cell viabilities of tube-forming HUVEC treated with the EEMS (80 μg/mL) and GC (10 μM) were decreased to 26.2 and 12.3% of the control (vehicle-treated) group, respectively (Fig. 2C). Furthermore, western blot analyses of Raf-1-MEK1/2-ERK1/2 signaling pathway, a representative survival pathway that is activated by VEGF and bFGF [2], revealed that the EEMS and GC significantly attenuated VEGF- and bFGF-induced ERK1/2 phosphorylation in tube-forming HUVEC (Fig. 2D and Supporting Information Fig. S4). GC also suppressed the phosphorylation of Raf-1 and MEK1/2. Consistent with these findings, ERK1/2 inactivation by U0126, a specific MEK inhibitor, caused a reduction in tube formation and the cell viability of tube-forming HUVEC (Supporting Information Fig. S5). Furthermore, we previously revealed that dietary factors inhibit tube formation and reduce the EC viability partly via ERK1/2 inactivation [12, 13]. Thus, it seems reasonable to assume that the EEMS and GC exerted their antiangiogenic effects partly via ERK1/2 inactivation. In contrast, the EEMS and GC had very little on the phosphorylation of VEGF receptor-2 and signal transducer and activator of transcription 3, a key transcriptional factor in VEGF-induced angiogenic signaling [14] (Supporting Information Fig. S6).

EC migration is an essential component in the neovessel formation. Because the EEMS and GC markedly inhibited such tube formation, we examined the effects of the EEMS and GC on EC migration using a wound-healing migration assay (Fig. 2E and Supporting Information Fig. S7). The migration of VEGF- and bFGF-stimulated HUVEC was significantly diminished by the EEMS and GC treatment.

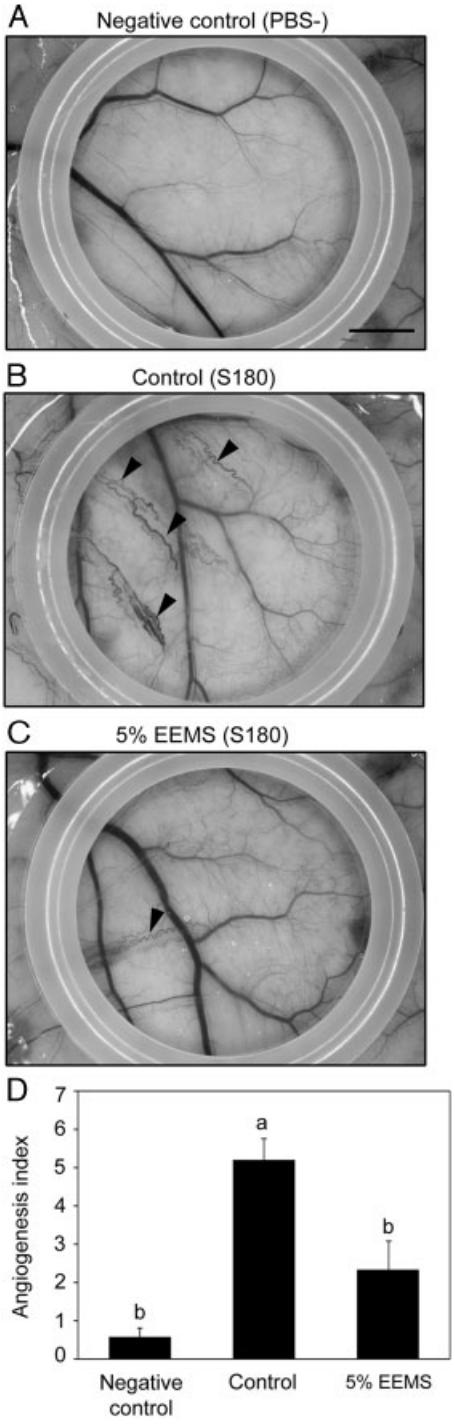

Figure 3. The suppressive effect of the EEMS on tumor-induced angiogenesis in a mouse dorsal air sac assay. (A-C) Female ICR mice were fed a standard (A, B) or 5% EEMS (C) diet from day 1 to day 9. On day 4, a chamber containing PBS (A) or S180 sarcoma cells (B, C) was implanted under the skin of the back. On day 9, the skin was excised and photographed. The arrowheads represent tumor-induced vessels with a zigzagging character. The bar indicates 3 mm. (D) The angiogenesis index was defined as the number of tumor-induced neovessels longer than 3 mm. Data are shown as mean ± SEM ($n = 6$ for negative control and EEMS-treated groups and 5 for control group). Means without a common letter differ significantly ($p < 0.05$).

We also confirmed that ERK1/2 inactivation by U0126 had almost no effect on the migration of VEGF- and bFGF-stimulated HUVEC (Supporting Information Fig. S5). Thus, the inhibitory effects of the EEMS and GC on tubular morphogenesis are attributed not only to reductions in cell viability via ERK1/2 inactivation, but also to inhibition of migration via ERK1/2-independent actions. Further studies are needed to clarify the molecular mechanisms underlying the inhibitory effects of the EEMS and GC on EC migration.

To evaluate the effects of the EEMS on tumor-induced angiogenesis in vivo, we performed a mouse dorsal air sac assay. In mice implanted with the chamber containing PBS and fed a standard diet (the negative control group), there was little or no formation of neovessels (Fig. 3A). In contrast, in mice implanted with the chamber containing S180 sarcoma cells and fed a standard diet (the control group), there was a strong induction of tumor-induced neovessels characterized by zigzag lines (Fig. 3B). Such tumor angiogenesis was substantially reduced when the mouse were fed a 5% EEMS diet (Fig. 3C). The angiogenesis indices (the number of newly formed blood vessels) were 0.6 ± 0.2 (mean $\pm$ SEM, $n = 6$), 5.2 ± 0.6 ($n = 5$), and 2.3 ± 0.8 ($n = 6$) for the negative control, control, and EEMS-treated groups, respectively (Fig. 3D). No sign of toxicity, such as decreases in body weight or food intake, were observed in any groups. These results indicate that the EEMS is orally active in suppressing tumor angiogenesis without unacceptable side effects. In the present study, we did not determine the plasma levels of the resveratrol derivatives in the EEMS and the effects of the EEMS and its components on VEGF and bFGF expression in tumor cells and tumor-associated macrophages. Further studies on these points are needed.

Taken together, our data demonstrated that the EEMS and its resveratrol derivative components suppress multiple angiogenesis-related EC functions and/or tumor angiogenesis. Although epidemiological studies are needed to determine whether melinjo seeds contribute to cancer prevention in humans, a daily intake of melinjo seeds may be useful for antiangiogenic therapies. We also found that GC has much stronger antiangiogenic activities compared with those of resveratrol and the other derivatives tested. Thus, GC may be a promising lead compound for developing an effective chemotherapeutic agent with antiangiogenic properties.

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