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# Involvement of Gicerin, a Cell Adhesion Molecule, in the Hematogenous Metastatic Activities of a Melanoma Cell Line

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**Abstract**

Gicerin is an immunoglobulin superfamily cell adhesion molecule that plays a role in development via its cell adhesive activities. After maturation, the gicerin expressionalmost disappears in most organs, except for the muscle and endothelial cells. Interestingly, various neoplastic cells strongly express gicerin in their cell membranes, indicating a potential function of gicerin in the development of malignancy. In the present study, we analyzed the potential role of gicerin in the metastasis of melanoma. Gicerin was found to be expressed in the cell membrane of the B16 implantable melanoma cell line. In addition, cell adhesion activity of B16was clearly promoted on gicerin proteins and HUVEC cells, an endothelial cell line. B16 cells were then implanted intravenously into nude mice in order to evaluate the metastatic activity of gicerin *in vivo*. Following implantation, metastatic lesions were frequently observed in the pulmonary tissues, whereas tumor emboli and extravasation were found in the pulmonary blood vessels. In contrast, no metastatic lesions were detected in the pulmonary tissues of the mice injected with B16 cells preincubated with anti-gicerin polyclonal antibodies. These findings suggest that gicerin enhances the pulmonary metastatic properties of melanoma cells by promoting endothelium-tumor interactions.

**Keywords:** melanoma, gicerin, cell adhesion molecule

## 1. Introduction

Gicerin is an immunoglobulin-superfamily cell adhesion molecule (CAM) purified from chicken smooth muscle as a neurite promotion factor (Kato *et al*., 1992; Hayashi & Miki, 1985; Taira*et al*., 1991).

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The genomic sequence of gicerin is highly homologous with that of B-CAM, BEN/DM-GRASP/SC1, HEMCAM and CD146/MelCAM (Campbell *et al*., 1994; Taira*et al*., 1994; Tanaka *et al*., 1991; Vainio*et al*., 1996). Gicerin contains five Ig-like domain structures in its extracellular region;each domain is rendered stable by a disulfide cross-link between two beta-pleated sheets. The cytoplasmic domain is relatively short and possesses potential recognition sites for protein kinases, although direct evidence of the phosphorylation of these sites is still lacking. Chicken gicerin exhibits a homophilic cell adhesion activity in addition to heterotypic adhesion to neurite outgrowth factor (NOF), a laminin-like molecule (Taira*et al*., 1994; Taniura*et al*., 1991; Tsukamoto *et al*., 1998). However, the heterophilic binding activity of gicerin has not been reported in other animals. Gicerin appears in embryonic tissues, including the nervous system, kidneys and respiratory system, and plays a role in cell migration, neurite extension, epithelialization and cell condensation via its cell adhesive activity (Hiroi*et al*., 2003; Tsukamoto *et al*., 1996, 1997a, 1997b, 1999a, 1999b, 2001, 2003). In mature tissues, gicerin is almost entirely absent in most tissues, restricted to only muscles and endothelial cells in blood vessels. Interestingly, it has been demonstrated that gicerin is overexpressed in various sporadic tumors in animals, including Wilm's tumors, oviductal adenocarcinomas, lymphomas, colorectal carcinomas and mammary gland adenocarcinomas (Adachi *et al*., 2010; Tsuchiya *et al*., 2003; Tsukamoto *et al*., 1997b, 1998, 1999b, 2001, 2003a, 2003b).In addition, a strong expression of gicerin has been detected in a metastatic melanoma cells in thelungs. Melanoma is one of the most aggressive tumor types, and various factors are thought to be involved in the pathogenesis of this malignancy (Tsao*et al*., 2004; Tiwary*et al*., 2014). However, the regulation of melanoma metastasis is currently poorly understood.

We previously speculated thatthe expression of gicerin may be involved in the metastasis of melanoma based on the following reasons: 1) malignant melanoma cells frequently metastasize to pulmonary tissues via the bloodstream (Tsao*et al*., 2004), 2)gicerin is enriched in melanoma cells, 3) vascular endothelial cells express gicerin on their surface, 4) gicerin promotes cell-cell interactions via its homophilic cell adhesive activity.

In the present study, the potential role of the gicerin expression in melanoma pulmonary metastasis was examined using an implantable melanoma cell line and specific neutralizing antibody against gicerin.

## 2. Materials & Methods

### 2.1 Expression of Gicerin in a the Melanoma Cell Line,B16

B16 cells were fixed in Zamboni's solution and incubated with a rabbit polyclonal antibody against gicerin protein at 4°C overnight after washing in PBS. The cells were then reacted with an FITC-conjugated swine antibodyagainst rabbit Ig at 37°C forone hour following sufficient washing with PBS andfinally mounted with glycerol,after which the specific signals in the gicerin proteins were examined under a fluorescent microscope with a B-filter (Nikon). For the Western blot analysis, B16 cells and mouse lung and cerebrum tissue were homogenized in PBS and centrifuged at 15,000 rpm for 15 minutes. The pelletswere solubilized by incubating in 10 mM of Tris/acetate (pH 8.0), 1 mM of EDTA and 0.5% NP-40 (octylphenoxypolyethoxyethanol; Sigma, St Louis, Mo., USA) at room temperature for 1.5 hours on a rotating shaker and then centrifuged at 15,000 rpm for 30 minutes. The resultant supernatants were separated via 7.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and electrotransferred to a Bio-Rad polyvinyl difluoride membrane. The blots were blocked with 2% skim milk in PBS containing 0.05% Tween 20 (PBST) for one hour at 37°C and then incubated with a rabbit polyclonal antibody against gicerin diluted in PBST with 2% skim milk for one hour at 37°C. After washing with PBST three times and incubating with a horseradishperoxidase-conjugated secondary antibody to rabbit IgG (DAKO) in PBST with 2% skim milk for one hour at 37°C, the membranes were washed three times in PBST and PBS and the blots were visualized in DAB solution (Tsukamoto *et al*., 1998).

### 2.2 Injection of Melanoma Cells into the Tail Vein in the Nude Mice

B16cells were preincubated with anti-gicerin rabbit polyclonal antibodies or preimmune IgG for one hour at 37°C and then suspended at $4.3 \times 10^7$ cells/mL in DMEM. The cells (0.1mL) were injected into the tail vein in adult male nude mice (five animals for each cell with/without anti-gicerin antibodies).

Fourteen days after the injection, the mice were sacrificed under deep anesthesia with pentobarbital solution, and the lungs were removed and fixed withbuffered formalin for histopathology.

All animal experiments were performed in accordance with the guidelines for studies using laboratory animals issued by the Kyoto Prefectural University Experimental Animal Committee (No. KPU240401).

### 2.3 Histopathology

The samples were embedded in paraffin and cut into 3-µm sections using a microtome. The sections were then stained with hematoxylin and eosin (H&E) as a routine procedure and observed under a light microscope.

### 2.4 In Vitro Cell Adhesion of the Melanoma Cells to Thegicerin Proteins

The B16 cells were seeded onto culture dishes coated with gicerin proteins or normal mouse immunoglobulins (*as a negative control*) and then incubated in DMEM for one hour at 37°C. Following a gentle and brief washing with PBS, the cells adhering to the dish were observed under a microscope, and the cell number per area was determined. The examination was performed in five areas for each protein coating, and the average score and standard deviation (SD) were calculated.

### 2.5 In Vitrocell Adhesion of Melanoma Cells to Vascular Endothelial Cells

Endogenous gicerin-positive HUVEC cells, a vascular endothelial cell line,were cultured on disheswith DMEM containing 10% FCS at 37°C and used as feederlayers. B16 cell suspensions were preincubated with anti-gicerin polyclonal antibodies or preimmune IgG for one hourat 37°C. After washing in PBS and centrifugation, the B16 cells were resuspended in DMEM and seeded onto the HUVEC monolayers. Following 30minutesof incubation at 37°C, the cultures were gently washed with DMEM and removed, and the B16 cells adhering to the feederlayers were observedunder a light microscope.The cell number per area was determined;the examination was performed in five areas, and the average score and SD were calculated.

## 3. Results

### 3.1 Expression of Gicerin Proteinsin the B16 Cells

Immunohistochemically, the expression of gicerin in the B16 cells was examined using an anti-gicerin polyclonal antibody. Consequently, gicerin proteins were expressed strongly on the B16 cell membrane (Fig. 1).In addition, a Western blot analysis showed that gicerin proteins were expressed in the cell membrane fractions in the nude mouse lungs and B16 cells at around 120 kDa (Fig. 2). In contrast, the normal mouse cerebrum did not exhibit a gicerin expression.

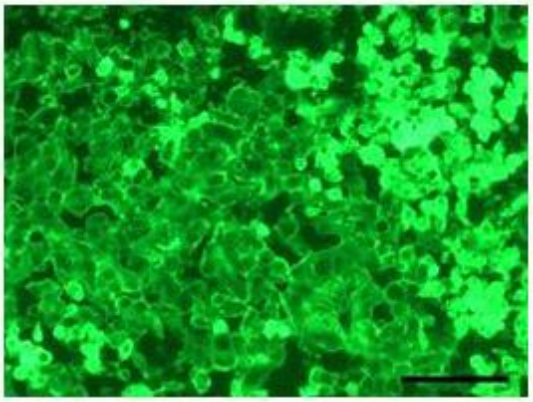

**Fig. 1: Immunocytochemistry of the gicerin expression in the melanoma cell line, B16 cells Gicerin was enriched in the B16 cells, especially on the cell surface. Bar= 200 µm**

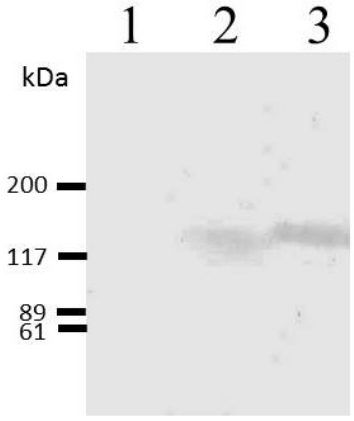


**Fig. 2: Western blot analysis of gicerin proteins in the melanoma B16 cells. Membrane fractions in the cerebrum (lane 1), lungs (lane 2) and B16 cells (lane 3) were used. Each lane is loaded with 20 µg under reducing conditions. Note that gicerin is expressed as an approximately 120-kDa protein in the lungs and melanoma cells, but is not found in the cerebrum. The bars in the left panel indicate the molecular weight marker in kDa.**

### 3.2 Adhesion of Melanoma Cells to Gicerin Proteins

A B16 cell attachment assay was carried out on the gicerin-coated dishes. On the control proteins, only asmall number of B16 cells adhered to the dish. In contrast, cell adhesion was strongly promoted on the gicerin-coated dishes (Fig. 3). These findings indicate that the cell adhesive activities of the B16 cells were promoted by the gicerin proteins.

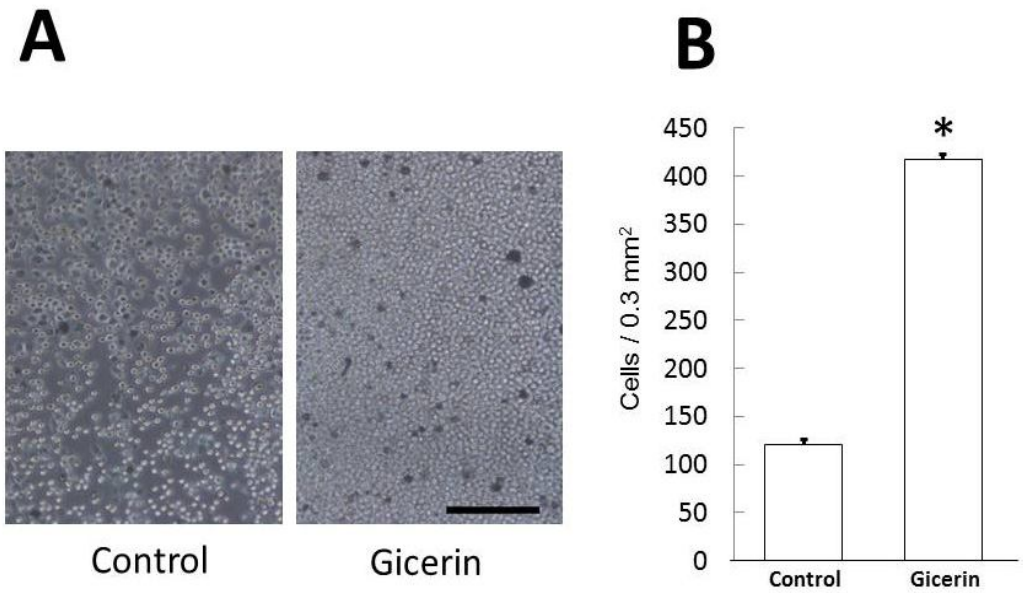


**Fig. 3: Cell Adhesion Activity of the B16 cells with Gicerin Proteins. B16 cells were seeded onto control- or gicerin protein-coated dishes. A large number of cells adhered to the gicerin coating compared with the control proteins (A). The number of adhesive cells in each dish was counted in an area of 0.3 mm2 using light microscopy. Each bar represents the mean±SD of five areas in each protein-coated dish (B). *P<0.01 compared with the control protein-coated dish (Student's t-test).**

### 3.3 Attachment of Melanoma Cells to Vascular Endothelial Cells

The B16 cells were seeded into the human endothelial cell line, HUVEC. Numerous numbers ofB16 cells treated with preimmune IgG adhered to the feeder layers. In contrast, the number of B16 cells treated with anti-gicerin antibodies adhering to the feeder layers was clearly decreased (Fig. 4).

These findings indicate that the adhesive activity of gicerin promotes the interaction of melanoma cells with vascular endothelial cells.

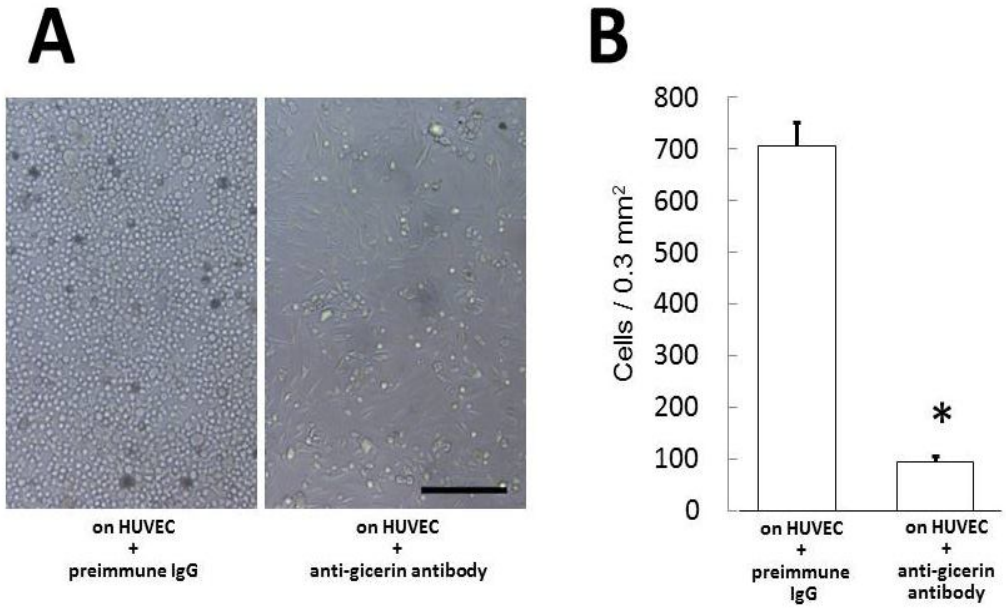


**Fig. 4: Cell adhesion activity of the B16 cells with HUVEC cells. B16 cells were incubated with preimmune IgG or anti-gicerin antibodies and seeded onto the feeder layers of HUVEC cells. A large number of preimmune IgG-treated cells adhered on HUVEC cells compared with those treated with the anti-gicerin antibodies (A). The number of adhesive cells on the HUVEC cells was counted in an area of 0.3 mm² using light microscopy. Each bar represents the mean±SD of five areas in each cell culture (B). **P<0.01* compared with the preimmune IgG-treated cells (Student's *t*-test).**

3.4 Pulmonary Metastasis of Melanoma Cells in the Nude Mice

Based on above mentioned in vitroresults, we next focused on the potential functions of gicerin with respect to the hematogenousmetastasis of melanoma cells. Followingi.v.implantation with B16 cells in the nude mice, macroscopic metastasis was detected in the lungs in four of five micetreated with preimmune IgG (Table 1). In contrast, no metastatic lesions were found in any of the mice implanted with B16 cells pre-reacted with anti-gicerin antibodies. A histopathological analysis showed tumor emboliin the pulmonary vein and extravasation into the pulmonary parenchymain many of the B16-injected mice (Fig. 5, Table 1).

On the other hand, no metastatic lesionswere not detected in the lungs of any of the nude mice implantedwith B16 cells reacted with the anti-gicerin antibodies. Accordingly, it is clear that gicerin proteinsare involved in the hematogenousmetastasis of melanoma cells to the lungs.

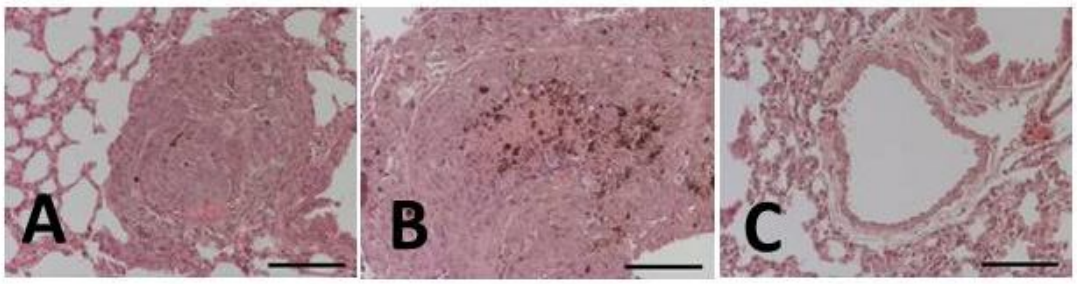


**Fig. 5: Histopathology of the nude Mouse Lungs Following i.v. Injection of B16 Cells**

The lungs were examined histopathologically at eight days after cell injection. Severe tumor embolism in the blood vessels and extravasation to the pulmonary parenchyma were observed in the mouse lungs implanted with the preimmune IgG-treated B16 cells (A, B). In contrast, no metastatic lesions were detectable in the mouse lungs implanted with B16 cells treated with anti-gicerin antibodies (C). Bar= 200 µm.

## 4. Conclusion

In the present study, endogenouspositive B16 cells clearly adhered to gicerin proteinsvia gicerin homophilic(gicerin-gicerin)adhesion. It is well known that *in situ* metastasis involves a multistep process characterized by a changing relationship between tumor cells and normal tissues, in which the tumor cells must detach from the primary site, intravasate through neighboring cells and/or ECMnetwork and bind to blood vessels and extravasateto sites of metastasis (Albelda, 1993; Cunningham *et al*., 1995; Takeichi, 1993; Tiwary*et al*., 2014).Endothelial cells express gicerin proteins continuously. Therefore, we speculated tumor cell interactions with endothelial cells are enhanced by gicerin adhesive activities, resulting in tumor intravasation, embolism and extravasation. The present findings demonstrated that gicerinpromoted *in vitro* cell interactionsbetween melanoma and endothelial cells.

In addition, the implantation model in nude mice showed that the melanoma cellshighly metastasizedto the pulmonary vein, where theyembolizedand invaded the surrounding pulmonary tissue.Thesefindings suggestthat melanoma cells attach to the surface of endothelial cells via gicerin-gicerin binding and embolize vessels, after which the promotion of cell invasion by gicerin-gicerin and/or gicerin-ECM binding leads to extravasation through the vessel wall.In our other recent studies, gicerin was found to beexpressed in the microvilli of L-929 transfectants and firmly bound to actin filamentsvia moesin, a member of the ERM family (Okumura *et al*., 2001). Therefore, the interactionsbetween gicerin and actin filamentsmay increase the invasive activity of tumor cells.

In conclusion, the present study provides evidence that gicerin promotes the pulmonary metastasis of B16 melanoma cells via its adhesive activity.

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