Modificational Changes in Function and Morphology of Cultured Macrophages by Geraniin

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ABSTRACT—Treatment of peritoneal macrophages with geraniin, isolated from *Geranium funbergii*, markedly induced the phagocytosis of living yeasts. Marked increases in phagocytosis and acid phosphatase activity in macrophage lysates were observed 24 hr after the beginning of geraniin treatment. As observed by electron microscopy, macrophages that had been treated for 24 hr with geraniin had a markedly thickened surface layer which was positive to ruthenium red, compared to the control cells. In addition, geraniin treatment of macrophages appeared to induce remarkably large mitochondria, more coated pits and prominent lysosomal granules. In conclusion, the stimulation of phagocytosis and acid phosphatase activity of macrophages by geraniin treatment may involve alterations of the plasma membrane and cytoplasmic reorganization.

Geraniin, isolated from *Geranium funbergii*, belongs to a group of related tannins that have been reported to show many pharmacological activities such as inhibition of histamine release from mast cells (1), the ability to affect some platelet functions (2, 3), the lymphoproliferative effect (4) and the immunomodulatory effect (5), based upon binding tannins to membrane surface proteins of each cell (6). Recently, it has demonstrated that tannin mediates some functions of macrophages (7, 8). We have also reported that geraniin treatment in vitro induces significantly increased spreading of cultured macrophages and related changes in cytoskeletal organization (Y. Ushio et al., unpublished data). To further explore these issues, we examined the effects of geraniin on the phagocytosis, yeast killing activity and acid phosphatase activity in mouse peritoneal macrophages. We also reported that

the modification changes of functional activities by geraniin treatment might be caused by the altered plasma membrane and cytoplasmic reorganization.

MATERIALS AND METHODS

Animals

Macrophages were collected from male mice of the BALB/C strain (b.w. 25–30 g) obtained at the Shizuoka Experimental Animal Lab.

Cell culture

The mouse peritoneal macrophages were harvested by intraperitoneal injection to mice with Eagle's minimum essential medium (MEM: Nissui). The peritoneal cells were obtained by centrifugation at 1,200 rpm at 4°C for 10 min and were gently resuspended in MEM medium supplemented with 10% fetal

calf serum (FCS). The cells were plated on culture dishes (Falcon: 35×10 mm) at a concentration of 5×10^4 cells/dish and were incubated at 37°C in a 5% CO_2 atmosphere for 2 hr. Nonadherent cells were then gently removed by washing with Hank's balanced salt solution (HBSS), and the adherent cells were incubated in 10% FCS-MEM medium for 24 hr.

Addition of geraniin

After the macrophages were incubated for 24 hr, the cells were washed, and geraniin (Fig. 1) was added to the cell cultures with 2% FCS-MEM medium at the final concentrations of 2, 5, 10 and $20 \mu\text{g/ml}$. Geraniin used for the experiments was dissolved in ethanol (final concentrations of ethanol in the medium were 0.1%). The cells were further incubated for 1, 4, 8, 24 and 48 hr at 37°C in a 5% CO_2 atmosphere.

Phagocytosis of living yeasts

Phagocytosis was examined at 1, 4, 8, 24 and 48 hr after addition of geraniin. The living yeasts used for the experiments were from stock cultures of *Saccharomyces cerevisiae* (7–8 μm) maintained at 30°C on Sabouraud's dextrose agar slants. The yeasts were added with 10% FCS-MEM medium suspension into

the macrophage cultures at a concentration of 100 yeasts/single macrophage. The cells were incubated with yeasts at 37°C for 15 min in a 5% CO_2 atmosphere, washed thoroughly with phosphate-buffered saline (PBS) and fixed with 1% glutaraldehyde in PBS for 5 min. The number of yeasts ingested by a single macrophage was evaluated for 200 macrophages in random fields under phase contrast microscopy, and the values were expressed as the mean $\pm$ standard error. Furthermore, by electron microscopical observation, we confirmed the intracellular ingestion of yeasts by geraniin ($20 \mu\text{g/ml}$)-treated macrophages instead of simple attachment of yeasts to the cells (Fig. 2).

Intracellular killing of living yeasts

We examined intracellular killing activity of living yeasts by macrophages according to the method described by Grasso and Guay (9). After the phagocytosis assay made by 15 min-incubation of the control and geraniin-treated macrophages with yeasts, extracellular yeasts were thoroughly removed, and one series of control and geraniin-treated macrophages were immediately exposed for 30 min to distilled water at room temperature. Liberated yeasts were diluted in PBS, plated on Sabouraud's dextrose agar plates, and then the

Fig. 1. The chemical structure of geraniin.

Fig. 2. Transmission electron micrograph of macrophages treated with geraniin ($20 \mu\text{g/ml}$) for 24 hr and then incubated with yeasts for 15 min ($\times 20,000$).

agar plates were further incubated at 30°C for 48 hr. The other series of control and geraniin-treated macrophages were incubated with yeast-free fresh 10% FCS-MEM medium for an additional 165 min and 345 min and were then also lysed with distilled water. The yeasts liberated from the macrophages were plated. The colonies of yeasts on the agar plates were counted, and the total numbers of viable organisms per macrophage culture dishes were calculated.

Acid phosphatase activity

The macrophages (1×10^5 cells/dish) in cul-

ture dishes were solubilized by incubating for 30 min with 0.1% Triton X-100 in distilled water. Acid phosphatase activity in the lysates of the cells was determined by a using kit (Acid phosphatase kit, Wako). Protein concentration of the lysates was estimated by dye reagent (Bio-Rad) using bovine serum albumin as a standard.

Transmission electron microscopy

The cells incubated with 2% FCS-MEM medium containing geraniin for 24 hr were washed with HBSS and fixed with 1% glutaraldehyde at room temperature for 1 hr. After

a brief rinse with PBS, the cells were postfixed for 1 hr with 1% osmium tetroxide (OsO_4) in PBS, stained in 3% uranyl acetate for 30 min and washed with distilled water. The cells were dehydrated through a graded series of ethanol concentrations and embedded in Epon 812 at room temperature for 1 hr. The embedded block was removed from the culture dishes and trimmed. The ultra thin sections were made on an ultramicrotome, stained in 3% uranyl acetate, lead citrate and examined by a Hitachi HS-9 electron microscope.

Labelling with ruthenium red staining

The cells incubated with 2% FCS-MEM medium containing geraniin for 24 hr were fixed with 1% glutaraldehyde in 0.2 M veronal buffer containing 0.5 mg/ml of ruthenium red at room temperature for 1 hr and postfixed for 1 hr with 1% OsO_4 in the same buffer which also contained 0.5 mg/ml of ruthenium red. The following process was the same procedure as the above method of transmission electron microscopy.

Morphometry

The areas of cytoplasmic mitochondria within macrophages were determined using an electronic planimeter (Numonics) on electron micrographs of the cells fixed 24 hr after the addition of geraniin (20 $\mu\text{g}/\text{ml}$). The mean area of all mitochondria seen within 10 cells chosen randomly from the control and geraniin-treated groups was expressed as mean $\mu\text{m}^2 \pm$ standard error. For measurement of the mitochondrial areas, electron micrographs were taken at a magnification of 12,000.

The thickness of the ruthenium red-positive layer on the surface membrane of macrophages was also calculated using a micrometer on electron micrographs of the cells fixed 24 hr after addition of geraniin (20 $\mu\text{g}/\text{ml}$). The width of the layers at several points, about 1.7- μm apart, was calculated per single macrophage. The mean thickness of 10 cells randomly chosen from the control and geraniin-treated groups was expressed as the mean nm $\pm$ S.E. For measurements of the

thickness of the ruthenium red-positive layer, electron micrographs were taken at a magnification of 40,000.

Statistical analysis

All values represent the mean and standard error of three separate experiments. Statistical analysis was performed by Student's *t*-test for unpaired observations.

RESULTS

Effect of geraniin on phagocytosis of yeasts

The effect of geraniin on phagocytosis of yeasts was determined at 1, 4, 8, 24 and 48 hr after addition of geraniin to macrophages. The macrophages treated with geraniin showed increased phagocytosis of yeasts from 8 hr after addition. The increases in phagocytosis were 19%, 39% and 37%, respectively, at 8, 24 and 48 hr after the addition of geraniin (10 $\mu\text{g}/\text{ml}$), in comparison to the control cells (Table 1).

Effect of geraniin on acid phosphatase activity

Acid phosphatase activity of macrophages at 1, 4, 8, 24 and 48 hr after addition of geraniin was examined. The enzyme activity of control macrophages was maintained at the same level for the first 24 hr after the change of the medium to 2% FCS-MEM medium. However, the level of enzyme activity fell at 48 hr after changing the medium. The geraniin-treated macrophages showed the same level of enzyme activity as the control cells at 1, 4 and 8 hr after the addition of geraniin. The enzyme activity significantly increased in geraniin-treated cells at 24 hr after the addition. The activities of the cells treated with geraniin at concentrations of 10 and 20 $\mu\text{g}/\text{ml}$ were 26% and 43% higher than those of the control cells (Table 2).

Effect of geraniin on intracellular killing activity of yeasts within macrophages

The intracellular killing activity of living yeasts was determined at 24 hr after addition of geraniin. Viable yeasts within the macro-

Table 1. Effect of geraniin on phagocytosis of living yeasts

Treatment ^{a)}		Phagocytosis of yeasts (yeast cells/macrophage) ^{b)}				
	($\mu\text{g/ml}$)	1	4	8	24	48 (hr)
Control		5.8 $\pm$ 0.2	6.0 $\pm$ 0.3	6.3 $\pm$ 0.2	6.2 $\pm$ 0.2	4.3 $\pm$ 0.2
Geraniin	2	5.7 $\pm$ 0.2	6.1 $\pm$ 0.2	6.6 $\pm$ 0.2	7.0 $\pm$ 0.2 ^{c)}	5.5 $\pm$ 0.2 ^{d)}
	5	6.0 $\pm$ 0.2	6.4 $\pm$ 0.2	7.7 $\pm$ 0.3 ^{d)}	8.1 $\pm$ 0.2 ^{e)}	5.4 $\pm$ 0.2 ^{d)}
	10	6.3 $\pm$ 0.2	6.3 $\pm$ 0.2	7.5 $\pm$ 0.2 ^{d)}	8.6 $\pm$ 0.3 ^{c)}	5.9 $\pm$ 0.2 ^{e)}
	20	6.2 $\pm$ 0.2	7.5 $\pm$ 0.3 ^{d)}	7.3 $\pm$ 0.2 ^{d)}	7.8 $\pm$ 0.3 ^{d)}	5.7 $\pm$ 0.2 ^{d)}

^{a)}The macrophages were incubated with 2% FCS-MEM medium containing various concentrations of geraniin 24 hr after the cells were plated on culture dishes. ^{b)}The phagocytosis assay was performed at 1, 4, 8, 24 and 48 hr after addition of geraniin to the cells. ^{c)}P < 0.05. ^{d)}P < 0.01. ^{e)}P < 0.001.

Table 2. Effect of geraniin on acid phosphatase activity in the lysates of cultured macrophages

Treatment ^{a)}		Acid phosphatase activity (mU/mg protein) ^{b)}				
	($\mu\text{g/ml}$)	1	4	8	24	48 (hr)
Control		123.3 $\pm$ 8.6	119.2 $\pm$ 1.9	146.8 $\pm$ 10.9	138.7 $\pm$ 0.7	66.0 $\pm$ 2.9
Geraniin	2	119.6 $\pm$ 1.8	139.6 $\pm$ 1.3	158.9 $\pm$ 11.4	158.5 $\pm$ 2.5 ^{c)}	88.4 $\pm$ 1.0 ^{c)}
	5	128.7 $\pm$ 9.5	132.3 $\pm$ 5.3	161.4 $\pm$ 8.8	155.2 $\pm$ 5.1 ^{c)}	91.1 $\pm$ 4.4 ^{d)}
	10	131.6 $\pm$ 1.6	134.1 $\pm$ 9.3	145.0 $\pm$ 1.6	174.3 $\pm$ 1.0 ^{c)}	107.3 $\pm$ 7.0 ^{e)}
	20	124.7 $\pm$ 1.2	122.5 $\pm$ 2.6	172.9 $\pm$ 7.1	198.0 $\pm$ 6.2 ^{e)}	128.7 $\pm$ 3.8 ^{c)}

^{a)}The macrophages were incubated with 2% FCS-MEM medium containing various concentrations of geraniin 24 hr after the cells were plated on culture dishes. ^{b)}The enzyme activity was determined at 1, 4, 8, 24 and 48 hr after addition of geraniin to the cells. ^{c)}P < 0.05. ^{d)}P < 0.01. ^{e)}P < 0.001.

phage culture gradually declined with the time of incubation. The killing activity of yeasts by geraniin-treated macrophages was at the same level as that by control cells after incubation of the cells for an additional 165 min or 345 min (Table 3).

Transmission electron microscopy

Transmission electron microscopy showed that the control macrophages had a large number of small vacuoles localized mainly at the periphery of the cells and some dense granules in the cytoplasm. The cells treated with geraniin (10 $\mu\text{g/ml}$) for 24 hr showed enlarged mitochondria. The cells treated with geraniin (20 $\mu\text{g/ml}$) for 24 hr showed remarkably large

mitochondria, more coated pits and prominent lysosomal granules within the enlarged cells, compared to the control cells (Fig. 3). In addition, quantitative examinations using an electronic planimeter showed that the mean area of the mitochondria within geraniin-treated macrophages was 71% larger than that within the control cells (Table 4).

Labelling with ruthenium red staining

An electron-dense surface coat was seen in the control macrophages based upon the negative surface charge of the cells. In the cells treated with geraniin (20 $\mu\text{g/ml}$) for 24 hr, the surface layer positive to ruthenium red was found to be significantly thicker than in the

Table 3. Effect of geraniin on intracellular killing activity of yeasts by macrophages

Treatment ^{a)}		Percentage of ingested yeasts after 15 min incubation	
	($\mu\text{g/ml}$)	3	6 (hr) ^{b)}
Control		36 $\pm$ 4	23 $\pm$ 2
Geraniin	10	39 $\pm$ 5	25 $\pm$ 3
	20	38 $\pm$ 2	21 $\pm$ 5

^{a)}The macrophages were further incubated with fresh 2% FCS-MEM medium containing various concentrations of geraniin 24 hr after the cells were plated on culture dishes. ^{b)}After incubating the control and geraniin-treated macrophages for a further 165 or 345 min, the cells were incubated with living yeasts for 15 min and then the killing activities determined.

Table 4. Effect of geraniin on area of mitochondria and the thickness of ruthenium red-positive layer of macrophages

Treatment ^{a)}		The mean area of mitochondria (μm^2)	The thickness of ruthenium red-positive layer (nm) ^{b)}
	($\mu\text{g/ml}$)		
Control		0.97 $\pm$ 0.09	28.5 $\pm$ 2.7
Geraniin	20	1.67 $\pm$ 0.14 ^{c)}	42.6 $\pm$ 4.2 ^{c)}

^{a)}The macrophages were incubated with 2% FCS-MEM medium containing geraniin 24 hr after the cells were plated on culture dishes. ^{b)}The analysis of morphometric parameters was performed on electron micrographs of macrophages fixed 24 hr after the addition of geraniin. ^{c)}P < 0.01.

control cells (Fig. 4). In addition, quantitative examination using a micrometer showed that the mean thickness of the ruthenium red-positive layer on the membrane surface of geraniin-treated cells was 50% thicker, compared to that of the control cells (Table 4).

DISCUSSION

Geraniin remarkably increased the intracellular acid phosphatase activity of macrophages 24 hr after the beginning of geraniin treatment. Acid phosphatase is mainly located in the lysosomal granules (10, 11). The lysosomal granules in the cytoplasm of geraniin-treated macrophages were conspicuous 24 hr after geraniin treatment by electron microscopy. In addition, geraniin-treated cells

appeared to show more coated pits along the surface membrane. Coated pits may be involved in the fluid-phase pinocytosis and the membrane recycling (12). Unlike phagocytosis, pinocytosis is ongoing and constitutive and not generally susceptible to stimulation. Pinocytosis can internalize solutes either in the fluid phase or by adsorption of the substrate to the cell membrane (13). So, there is a possibility that the conspicuous lysosomal granules and increased number of coated pits, which could be seen within geraniin-treated macrophages, might be caused by the activated pinocytic process comprising internalization of soluble small vesicles including geraniin, followed by invagination of the surface membrane of macrophages.

Macrophages play a major role in host re-

Fig. 3. Transmission electron micrographs of cultured macrophages ($\times 12,000$) a): Control cells possessed a large number of small vacuoles localized in the periphery of the cells. b): The cells treated with geraniin ($20 \mu\text{g/ml}$) for 24 hr showed large mitochondria (white arrows) and more coated pits (black arrow) within the cells.

Fig. 4. Electron micrographs of macrophages stained by ruthenium red. ($\times 40,000$) a): Control cells possessed the electron dense layer on the cell surface b): In the cells treated with geraniin ($20 \mu\text{g}/\text{ml}$) for 24 hr, the surface layer positive to ruthenium red was found to be significantly thicker than that in the control cells.

sistance by ingesting and killing microorganisms. Geraniin had no effect on the intracellular killing activity of living yeasts after ingestion. The number of yeasts killed by geraniin-treated macrophages might seem to be actually much more than that by the control cells, because the geraniin-treated macrophages ingested much more yeasts than control cells, even if the percent of yeasts killed intracellularly in geraniin-treated cells was the almost same as that in control cells. It is unclear whether the significant increase in acid phosphatase activity in geraniin-treated cells really affected the killing activity of yeasts and was caused by the alteration of the lysosomal mechanism. Furthermore, we must examine the activation mechanism of lysosomal enzyme activity to clarify whether the activation was caused by simple adsorption of geraniin to the surface membrane of macrophages or by delivery of geraniin to the lysosomes by fluid-phase pinocytosis.

Staining with ruthenium red provides a strong contrast of the cell surface and the invaginating tubules beneath it against the cytoplasm (14). The ruthenium red staining method is generally believed to allow detection of minute amounts of sulfate and carboxyl groups of acidic polysaccharides. As observed by electron microscopy, geraniin treatment seemed to induce remarkably thick sections on the ruthenium red-positive cell surface of macrophages, compared to control cells. We suggested that these thick layers were caused by geraniin binding to the some glycoproteins of the cell surface.

We have reported that geraniin treatment to mouse resident peritoneal macrophages in culture induces phagocytosis of yeasts in a dose-dependent manner. The significant enhancement of phagocytosis was recognized 24 hr after the beginning of geraniin treatment. The significant increases in both phagocytosis and enzyme activity were exhibited 24 hr after geraniin treatment. Although they are not directly related to each other, modification of the cell surface of macrophages by geraniin participated in not only increased the attach-

ment and phagocytic process of yeasts, but also affected the transmembrane communication and coordinated cytoplasmic control. It is apparent that the plasma membrane is not an autonomous cell organelle. It is coupled to various cytoplasmic structures and other cell organelles (15). The morphological changes including changes in some ultrastructural features are accompanied by important changes in the physiology and function of macrophages (16, 17). We had already obtained the results that geraniin enhanced induced spreading, redistribution of cytoplasmic actin and extension of microtubules into the peripheral cytoplasm of cultured macrophages *in vitro*. The results suggested that the highly induced spreading activity in macrophages caused by geraniin appeared to be closely accompanied by stabilization of the microfilaments and microtubule network by geraniin binding to contractile proteins related to the cytoskeleton or membrane-associated proteins within the lipid bilayer. Taking this into consideration, we concluded that development of mitochondria, increase in phagocytosis of yeasts and enhancement of intracellular acid phosphatase activity in geraniin-treated macrophages were also induced by surface modification of macrophages by binding geraniin and were closely related to the increase in induced spreading and a highly organized cytoskeleton of macrophages caused by geraniin. We must further investigate what kind of cellular process such as cell division, development, differentiation towards death is concerned with functional modification and alteration of the surface membrane of macrophages by geraniin.

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