Rosmarinic Acid from Perillae Herba Produces an Antidepressant-Like Effect in Mice through Cell Proliferation in the Hippocampus

Naoki Ito,[a] Takeshi Yabe,[a,b] Yuji Gamo,[a,c] Takayuki Nagai,[a,b] Tetsuro Oikawa,[a] Haruki Yamada,[a,b] and Toshihiko Hanawa*,[a,c]

[a] *Department of Clinical Research, Oriental Medicine Research Center, Kitasato University; Tokyo 108–8642, Japan:* [b] *Kitasato Institute for Life Sciences & Graduate School of Infection Control Sciences, Kitasato University; Tokyo 108–8641, Japan: and* [c] *Department of Oriental Medicine, Doctoral Program of Medical Science, Graduate School of Medical Science, Kitasato University; Kanagawa 228–8555, Japan.*

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Rosmarinic acid (RA) is one of major polyphenolic ingredients of Perillae Herba (a leaf of *Perilla frutescens*), and has an antidepressant-like property in animal models of depression. However, the mechanism(s) underlying this activity are unknown. Recent studies have reported that regulation of hippocampal neurogenesis is associated with the pathogenesis of depression. To elucidate the mode of action of RA-induced antidepressant-like activity, proliferative effect of RA on newborn cells in the dentate gyrus of mouse hippocampus was investigated using immunohistochemical analysis with bromodeoxyuridine (BrdU), a marker of proliferating cells. RA treatment for 7 or 14 d significantly increased in the number of BrdU-positive cells in inverse correlation with significant reductions in immobility in a forced swimming test, an animal model of depression, in a dose-dependent manner. However, locomotor activities were not affected. These results suggest that RA produces an antidepressant-like effect at least in part *via* the proliferation of newborn cells in the dentate gyrus of the hippocampus.

Key words rosmarinic acid; antidepressant-like effect; neurogenesis; bromodeoxyuridine; forced swimming test

Rosmarinic acid (α-*O*-caffeoyl-3,4-dihydroxyphenyl-lactic acid; RA) (Fig. 1) is one of major polyphenolic ingredients of Perillae Herba.[1] RA has multiple biological activities, including anti-oxidant, anti-allergic and anti-inflammatory effects.[2—5] Moreover, RA exhibits anti-apoptotic and anti-oxidant effects in astrocytes[6] as well as neuroprotective effects in the neurotoxicity of amyloid β.[7,8] These findings imply that RA may be useful for the treatment of dysfunction not only in the immune system but also in the nervous system.

Perillae Herba is a component herb of such Kampo medicines as Kososan (Xiang-Su-San in Chinese) and Hangekobokuto (Banxia-Houpo-Tang in Chinese), which are used clinically to improve depressive mood and which have exhibited antidepressant-like activities experimentally as well.[9,10] Moreover, RA and its major metabolite, caffeic acid (CA), have exhibited antidepressant-like activities in an animal model of depression.[11] The mode of action was determined not to be the inhibition of monoamine transporters or monoamine oxidases.[12] Instead, several studies have been reported that CA produces antidepressant-like effect *via* indirect modulation of the α1A-adrenoceptor system[13] and that CA attenuates the down-regulation of cortical brain-derived neurotrophic factor (BDNF) transcription that results from stressful conditions.[14] However, the mode of action for this antidepressant-like activity remains unknown. The clarification of the mechanisms underlying RA's and CA's actions may help to elucidate on the mechanisms underlying the antidepressant-like effects of Kososan and Hangekobokuto.

Disruption of neurogenesis in the dentate gyrus in the hippocampus plays a crucial role in the mechanism by which stress facilitates depression.[15,16] Furthermore, chronic antidepressant treatment increases neurogenesis in rat hippocampus,[17] and hippocampal neurogenesis requires the behavioral recovery effects of antidepressants.[18] Thus, the possibility has been thought that neurogenesis-induced compounds directly and/or indirectly produce antidepressant-like effects.

We hypothesized that RA may induce an antidepressant-like effect by modulating hippocampal neurogenesis. The present study investigated whether RA treatment for 7 or 14 d would modulate cell proliferation in the dentate gyrus of the hippocampus, using bromodeoxyuridine (BrdU) immunohistochemical analysis.

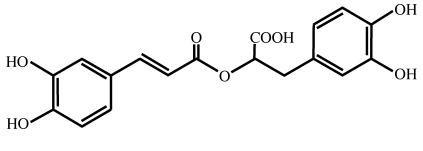

Fig. 1. Chemical Structure of Rosmarinic Acid

MATERIALS AND METHODS

Animals Adult (7-week-old) male ddY mice (Japan SLC, Hamamatsu, Japan), weighing 35—40 g, were used in the experiments. The mice were housed individually under conditions of constant temperature (23±2 °C) and humidity (55±10%) with food and water available *ad libitum* unless otherwise specified, and a 12/12 h light–dark cycle (8:00—20:00). All animal experiments were performed according to the Guidelines for the Care and Use of Laboratory Animals at the Kitasato Institute and Kitasato University.

Drug Treatment Rosmarinic acid (RA) (Sigma, St. Louis, MO, U.S.A.) was dissolved in saline. RA (1.0, 2.0, 4.0 mg/kg/bw, i.p.) were administered for 7 or 14 d (Figs. 2A, B). To label dividing cells, bromodeoxyuridine (BrdU) (Roche Diagnostics, Indianapolis, IN, U.S.A.) was dissolved in saline with 0.007 N NaOH. BrdU (100 mg/kg/bw, i.p.) was administered twice, once at 24 h and once at 3 h before fixation for BrdU immunohistochemistry (Fig. 2B).

Forced Swimming Test This paradigm (Fig. 2A) was performed as the forced swimming test (FST) with some modifications as described previously.[19] Briefly, mice were

* To whom correspondence should be addressed. e-mail: hanawa-t@kitasato.or.jp

placed individually into 5 l beakers (height 27 cm, diameter 18 cm) filled with 4 l of water (23±1 °C) for 15 min (FS). After 15 min, the mice were removed and allowed to dry before being returned to their home cages. They were then separated into groups according to the duration of immobility during the first 5 min of the FS, in order to minimize the variation in immobility among the groups. A mouse was judged to be immobile when it ceased struggling and remained floating motionless in the water, making only those movements necessary to keep its head above water. At 60 min after the last administration of drugs, the mice were placed again into 5 l beakers with 4 l of water, and the total duration of immobility during the 5 min FST was measured. The behavioral experiments were carried out between 13:00 and 16:00.

Open Field Test Spontaneous locomotor activities of mice were measured in an open field test (OFT) (Fig. 2A). Briefly, at 1 d before the FST, the mice were placed individually in an opaque open field box (40×40×40), in which they were allowed to move freely. The total distance and duration of movement, using a video tracking system (EthoVision; Noldus), was measured for the 5 min OFT. The behavioral experiments were carried out between 13:00 and 16:00.

Immunohistochemistry Three hours after the final administration of BrdU, the mice were anesthetized with ether and perfused transcardially with cold phosphate-buffered saline (PBS) and subsequently with cold 4% paraformaldehyde solution at twice the volume of body weight. The brains were collected from the skull and postfixed in 4% paraformaldehyde solution at 4 °C overnight. Serial coronal brain sections of 50 μm thickness were cut with a vibratome, and the sections were stored in PBS/NaN_3 at 4 °C until ready to use.

BrdU labeling was conducted in 24-well plates for free-floating immunohistochemistry. Briefly, DNA denaturation was performed by incubation in 50% formamide/2×SSC for 2 h at 65 °C, followed by a 2×SSC rinse. Sections were then incubated in 2 N HCl for 30 min at 37 °C to separate double-stranded DNA, rinsed in 0.1 M borate buffer (pH 8.5) for 15 min, and rinsed in PBS for 15 min×6. After blocking for 2 h with 1% BSA in PBS containing 0.3% Triton X-100 (PBS-T), the sections were incubated overnight at 4 °C with mouse anti-BrdU IgG (1 : 1000; Chemicon, CA, U.S.A.) in PBS-T containing 1% BSA. The following day, the sections were rinsed in PBS-T for 10 min×3, incubated for 2 h at room temperature (RT) with biotinylated horse anti-mouse IgG (1 : 200; Vector Laboratories, Burlingame, CA, U.S.A.), rinsed in PBS-T for 10 min×3, and incubated for 2 h at RT with avidin–biotin complex using the Vectastain Elite ABC kit (Vector Laboratories) in PBS-T. The sections were then rinsed in PBS-T and reacted with PBS containing 0.05% diaminobenzidine (Sigma) and 0.02% H_2O_2. After rinsing in PBS, the sections were counterstained with 0.05% toluidine blue (Sigma), dehydrated in a series of ethanol, cleared in xylene, and coverslipped with Permount (Fisher Scientific International, Fair Lawn, NJ, U.S.A.). All counts of BrdU-labeled cells were performed at 200× and 400× under a light microscope (Olympus BX-41), omitting cells in the outermost focal plane. The total number of BrdU-labeled cells in the dentate gyrus per mouse was determined as the sum of 6 sections in every three sections.

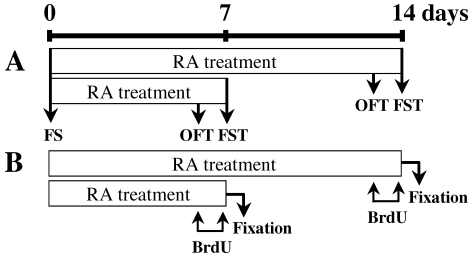

Fig. 2. Schedules of the Drug Treatment for (A) Behavioral Strategies (FST and OFT) and (B) BrdU Immunohistochemistry

FS; forced swimming.

Immunofluorescence Double BrdU/Nestin staining was conducted in 24-well plates for free-floating immunofluorescence. Briefly, the pretreatment for BrdU labeling was conducted as mentioned above. After blocking for 2 h with 1% BSA in PBS-T, the sections were incubated overnight at 4 °C with rat anti-BrdU IgG (1 : 50; Oxford Biotechnology, Kidlington, U.K.) and mouse anti-Nestin IgG (1 : 500; Chemicon) in PBS-T containing 1% BSA. After rinsing in PBS-T for 10 min×3, the sections were incubated for 2 h at RT with biotinylated chicken anti-rat IgG (1 : 200; Vector Laboratories), rinsed in PBS-T for 10 min×3, and incubated for 2 h at 4 °C with AlexaFluor 488-conjugated streptavidin (1 : 1500; Molecular Probes, Eugene, OR, U.S.A.) and AlexaFluor 594-conjugated goat anti-mouse IgG (1 : 1500; Molecular Probes). The sections were then rinsed at 4 °C in PBS-T and PBS for 10 min×3, and coverslipped with VECTASHIELD (Vector Laboratories). Double stained cells in the dentate gyrus were observed under a fluorescent microscope (BZ-8000, KEYENCE, Woodcliff Lake, NJ, U.S.A.).

Statistical Analysis Results are presented as means±S.E. Statistical analysis was performed by one-way analysis of variance (ANOVA) followed by Dunnett's test with StatView 5.0 software (SAS Institute, Cary, NC, U.S.A.). Differences were considered significant at $p<0.05$.

RESULTS

Effects of RA Treatment on the Duration of Immobility In the FST, RA treatment (2.0, 4.0 mg/kg) for 7 d significantly reduced the duration of immobility in a dose-dependent manner (138.7±22.8 and 133.1±19.9, respectively) compared with the saline-administered control (217.0±21.6) (Fig. 3A). Moreover, RA treatment (1.0, 2.0, 4.0 mg/kg) for 14 d significantly reduced the duration of immobility in a dose-dependent manner (130.6±15.4, 123.8±10.5, 107.2±15.7, respectively) compared with the saline-administered

Table 1. Effects of RA Treatment (1.0, 2.0, 4.0 mg/kg, i.p.) for 7 and 14 d on the Total Distance and Duration of Movement in the Open Field Test

	Total distance (cm/5 min)		Total duration (sec/5 min)	
	7 d	14 d	7 d	14 d
Saline	1892.7±62.0	1965.2±181.3	230.2±6.1	233.7±10.9
RA 1.0 mg/kg	2178.2±154.9	1960.2±143.4	247.2±5.6	230.3±12.5
RA 2.0 mg/kg	2208.5±219.5	2042.4±149.9	239.3±10.4	237.3±9.7
RA 4.0 mg/kg	2310.2±124.4	1819.2±129.6	253.9±6.3	222.4±9.9

The total distance and duration of movement were measured during a 5 min OFT on a day before FST. Each value represents the mean±S.E. (n=11—12).

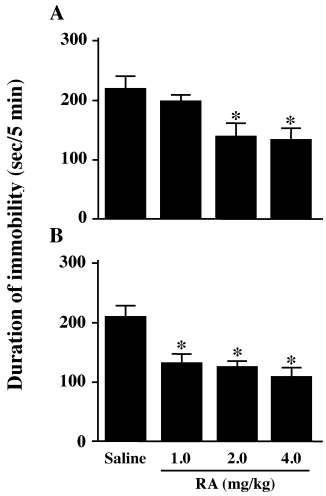

Fig. 3. Effects of RA Treatment (1.0, 2.0, 4.0 mg/kg, i.p.) for (A) 7 and (B) 14 d on the Duration of Immobility in the FST

The duration of immobility was measured during a 5 min FST at 60 min after the last treatment of RA. Each value represents the mean±S.E. (n=11—12). $*p<0.05$ *vs.* saline-administered control with Dunnett's test.

control (208.1±19.3) (Fig. 3B). However, neither total distance nor total duration of movement in the OFT was affected by RA treatment (1.0, 2.0, 4.0 mg/kg) for 7 or 14 d (Table 1).

Effects of RA Treatment on the Number of BrdU-Positive Cells in the Dentate Gyrus Using normal mice, independent of behavioral experiments, we used BrdU immunohistochemistry to examine the proliferative effects of RA treatment for 7 or 14 d on dividing cells, such as neural progenitor cells, in the dentate gyrus. RA treatment (2.0, 4.0 mg/kg) for 7 d significantly increased the number of BrdU-positive cells in a dose-dependent manner (430±23.1, 475.7±18.1, respectively) compared with the saline-administered control (328.7±21.4) (Figs. 4A, B).

The BrdU-positive cells in the dentate gyrus mostly expressed Nestin (Fig. 4C). In addition, RA treatment (1.0, 2.0, 4.0 mg/kg) for 14 d significantly increased the number of BrdU-positive cells in a dose-dependent manner (325.2±8.87, 339.2±20.2, 409.8±21.2, respectively) compared with the saline-administered control (227.8±22.2) (Fig. 5).

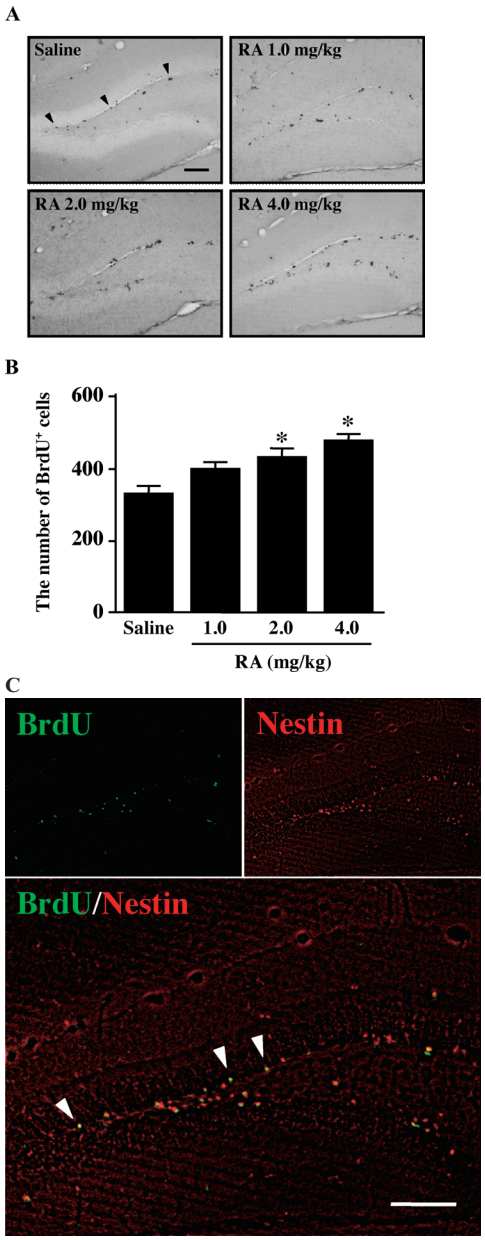

Fig. 4. Effect of RA Treatment (1.0, 2.0, 4.0 mg/kg, i.p.) for 7 d on the Number of BrdU-positive Cells in the Dentate Gyrus of Mice

(A) Photomicrographs of the BrdU-positive cells (arrowhead) in the dentate gyrus. Scale bar=100 μm. (B) The number of BrdU-positive cells in the dentate gyrus. (C) Immunofluorescence representative section of the dentate gyrus showing BrdU (green) and Nestin (red)-positive cells. Arrowheads indicate double-stained cells (yellow). Scale bar=100 μm. Each value represents the mean±S.E. (n=11). $*p<0.05$ *vs.* saline-administered control with Dunnett's test.

DISCUSSION

The present study demonstrates that RA treatment for 7 or 14 d produces an antidepressant-like effect while simultaneously increasing cell proliferation in the dentate gyrus in dose- and time-dependent manners.

RA significantly reduced immobility in a dose-dependent manner (Fig. 3) and did not change spontaneous locomotor activities (Table 1), indicating that RA has antidepressant-like activity. Our results were inconsistent with those in a previous report[11,12] which found that RA (2.0 mg/kg) in mice produces an antidepressant-like effect in a U-shaped manner. However, the discrepancy is not meaningful, since RA in the previous report was administered once to mice, whereas in our study it was administered repeatedly (7 or 14 d) (Fig. 2). A single administration of caffeic acid (CA), which is a metabolite of RA, produces an antidepressant-like effect in a dose-dependent manner not a U-shaped manner.[12] Therefore, the antidepressant-like effect induced by repeated administration of RA may be attributable in part to the effect of CA.

RA treatment produced an increase in BrdU-positive cells in the dentate gyrus (Figs. 4, 5). BrdU, an analogue of thymidine, is incorporated into dividing and proliferating cells. In the present study, Nestin-positive cells as a marker for progenitor cells were found predominantly in the dentate gyrus, and most BrdU-positive cells expressed Nestin (Fig. 4C). Thus, our results indicate that RA treatment increases the proliferating cells such as neural progenitor cells. All three doses (1.0, 2.0, 4.0 mg/kg) were increased the number of BrdU-positive cells to 20.5, 30.8, and 44.7% of saline-ad-

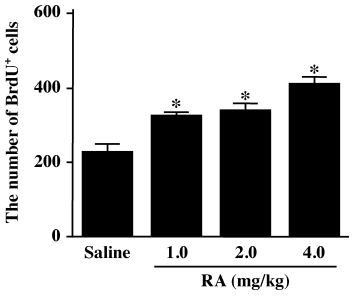

Fig. 5. Effect of RA Treatment (1.0, 2.0, 4.0 mg/kg, i.p.) for 14 d on the Number of BrdU-positive Cells in the Dentate Gyrus of Mice

Each value represents the mean±S.E. (n=10—11). $*p<0.05$ *vs.* saline-administered control with Dunnett's test.

ministered control after 7 d of treatment (Fig. 4), and to 42.8, 48.9, and 79.9% after 14 d of treatment (Fig. 5). Nevertheless, the number of BrdU-positive cells after 7 d of treatment was larger than that of BrdU-positive cells after 14 d of treatment. The difference may be due to the difference in the lot of anti-BrdU antibody used between 7 and 14 d. When BrdU immunohistochemistry was conducted using the same lot antibody, the number of BrdU-positive cells were not different between 7 and 14 d (data not shown). The present study suggests that RA, at least, increases the proliferating cells in both a time-dependent manner and a dose-dependent manner.

Antioxidant and neuroprotective effects of RA lead to the improvement of memory impairment induced by amyloid β, which is a protein related to Alzheimer's disease.[7] It is well known that depression is a frequent complication of Alzheimer's disease, and that the loss of neurons in this disease is associated with depression.[20—22] Thus, our results may raise the possibility that RA is useful for patients with Alzheimer's disease who suffer from a depressive mood. Protective effect of RA in astrocytes results in part from its antiapoptotic property.[6] Moreover, several reports suggest that antidepressants promote cell proliferation in the dentate gyrus by antiapoptotic and neuroprotective activities.[23,24] Therefore, these effects of RA may result in the increase of cell proliferation in the dentate gyrus. However, it remains to be unclear why increase in BrdU-positive cells in the dentate gyrus shows an antidepressant-like effect, and further studies are needed to address the issue. In several reports, X-irradiation of a restricted region of mouse brain containing the hippocampus prevents the neurogenic and antidepressant-like effects of antidepressants,[18] and the number of BrdU-positive cells and the duration of immobility in the FST are correlated in a negative fashion.[25] These findings indicate that increase in cell proliferation plays an essential role in an antidepressant-like effect. Although we demonstrated the immunohistochemical and behavioral studies using discrete mice for simple interpretation, both studies using the same mice can show more direct interaction between increases in cell proliferation in the dentate gyrus and behavioral expressions of antidepressant-like effect of RA, and warrant examination. It is still unclear whether RA modulates the increase in cell proliferation directly or indirectly, although RA is translocated to the brain.[26] Our preliminary data demonstrated that RA itself did not directly exhibit a proliferative effect on rat fetal brain-derived neural stem/progenitor cells *in vitro* (data not shown). Thus, RA may indirectly rather than directly increase cell proliferation in the dentate gyrus. Further studies are needed to clarify the effect of RA on cell proliferation in detail.

In the present study, RA-induced cell proliferation was found in the hippocampus of normal mice. To further clarify the effect of RA on the pathology of depression, it is necessary to investigate the effect of RA on cell proliferation using depression-like model mice, in which cell proliferation is impaired in the dentate gyrus. It is still not known whether the modulation of cell proliferation by RA from Perillae Herba contributes to the antidepressant-like property of Kampo medicines such as Kososan and Hangekobokuto, which contain Perillae Herba as a component herb. Further study of the antidepressant-like action of oral administration of RA may help to elucidate in detail the association between RA and the antidepressant-like properties of Kososan and Hangekobokuto.

In conclusion, the present study is the first report that the up-regulatory action of RA-induced cell proliferation may be one of the mechanism(s) of the antidepressant-like effect of RA. Further studies on neurogenesis of RA are needed to elucidate the mechanisms underlying RA's antidepressant-like effect.

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