Inhibitory effects of rosmarinic acid on the proliferation of cultured murine mesangial cells

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Abstract
Background. Rosmarinic acid is a phenolic compound widely distributed in Labiatae herbs such as rosemary, sweet basil, and perilla, which are frequently used with meat and fish dishes in Western and Asian countries. In the present study we investigated the effects of rosmarinic acid on cultured murine mesangial cell proliferation.

Methods. Cultured murine mesangial cells were stimulated by growth factors with or without rosmarinic acid, and [³H]thymidine incorporation was measured in regard both to signal transduction and to cell cycle dependency. In other experiments, mRNA extracted from the cells was analysed by Northern blotting.

Results. Rosmarinic acid inhibited the cell proliferation induced by platelet-derived growth factor (PDGF) (P<0.01; IC₅₀ values, 1.4 µg/ml) or tumour necrosis factor-α (P<0.01; IC₅₀ values, 3.8 µg/ml), and these effects involved both the G₀/G₁ and G₁/S phases of the cell cycle. Rosmarinic acid also suppressed the mRNA expressions of PDGF and c-myc in PDGF-stimulated mesangial cells.

Conclusions. Rosmarinic acid inhibits cytokine-induced mesangial cell proliferation and suppresses PDGF and c-myc mRNA expression in PDGF-stimulated mesangial cells. Rosmarinic acid in Labiatae herbs might be a promising agent to prevent mesangial cell proliferation.

Keywords: glomerulonephritis; Labiatae herbs; mesangial cell proliferation; rosmarinic acid

Introduction

Rosmarinic acid (Figure 1) is a widely distributed phenolic compound in various Labiatae herbs [1,2] such as Ocimum basilicum (sweet basil), Melissia officinalis (lemon balm), Rosmarinus officinalis (rosemary), Mentha spicata (spearmint), and Perilla frutescens (perilla). Previous studies revealed that rosmarinic acid has several anti-inflammatory effects such as inhibitory effects on a complement-dependent inflammatory process [3], and 5-lipoxygenase [4] and histamine releases from mast cells [5]. Rosmarinic acid also has anti-oxidative activity [6].

Recent studies have shown that glomerular mesangial cell proliferation, one of the major histological findings in various renal diseases, is mediated by various humoral factors such as inflammatory cytokines, eicosanoids, angiotensin II, and reactive oxygen species in the development of glomerulonephritis [7]. In these inflammatory cytokines, platelet-derived growth factor (PDGF), interleukin (IL)-1, tumour necrosis factor (TNF)-α, and IL-6 have been demonstrated as mesangial proliferative cytokines, which are secreted not only by infiltrating macrophages or monocytes but also by mesangial cells as autocrine factors [8]. These cytokines are also involved in the pathophysiology of chronic renal failure via the induction of mesangial cell proliferation [9]. Therefore, agents intervening in mesangial cell proliferation may be promising for the treatment of various types of mesangioproliferative glomerulonephritis.

In the present study, we evaluated the effects of rosmarinic acid on PDGF- or TNF-α-induced proliferation and PDGF-induced mRNA expression of PDGF and c-myc in cultured murine mesangial cell. We found in the present study that rosmarinic acid is
capable of inhibiting mesangial cell proliferation and suppressing mRNA expression of PDGF and c-myc.

**Subjects and methods**

**Materials**

Rosmarinic acid was purchased from Extrasyntèse (Genay, France). Recombinant human PDGF-BB homodimer, TNF-α, and insulin were purchased from Gibco BRL (Gaithersburg, MD, USA), Genzyme (Cambridge, MA), and Eli Lilly (Indianapolis, IN) respectively; 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) was purchased from Nacalai Tesque (Kyoto, Japan).

**Mesangial cell culture**

Murine mesangial cells were prepared according to the method of MacKay et al. [10]. Briefly, glomeruli were isolated from 10–20-week-old female Balb/c mice (Shimizu Laboratory Materials, Kyoto) by sieving, and were then cultured in 100-mm culture dishes (Iwaki Glass, Funabashi, Japan) which were coated with human fibronectin (Koken, Tokyo, Japan). The culture medium used was a 3:1 mixture of Dulbecco’s modified Eagle’s medium and F12 (Gibco BRL) supplemented with 20% heat-inactivated fetal calf serum (FCS, Biowhitaker, Walkersville, MD), 100 U/ml penicillin, and 100 μg/ml streptomycin (Gibco BRL). Mesangial cells surrounding the glomeruli were removed using cloning rings and passed to 24-well plates (Iwaki). The identification of mesangial cells was based on the following characteristics: appearance of spindle shape; positive staining with phalloidin (Molecular Probes, Junction City, OR); negative staining with an antibody to cytokeratin (Triton Biosciences Inc, Alameda, CA) or to von Willebrand factor (Dakopatts, Glostrup, Denmark), and survival in medium containing t-valine as a substitute for L-valine (Gibco BRL). The cells at passages 5–15 were used for the experiments.

**Cell proliferation assay**

After mesangial cells had been seeded into fibronectin-coated 24-well plates (2 × 10^4 cells/well) and incubated with 20% FCS medium for 96 h, the cultures were synchronized by 48-h FCS depletion. Quiescent cells were stimulated for 24 h with 10 ng/ml PDGF or 100 U/ml TNF-α in 0.5% FCS medium, together with one of several different concentrations of rosmarinic acid. Twenty hours after stimulation was begun, a pulse of 1 μCi/well of ^[3]H)thymidine (NEN Products, Boston, MA) was added to the culture. To measure the amount of radioactivity incorporated into the nuclei of cells, the cultures were washed with phosphate-buffered saline (PBS, 0.01 M, pH 7.2), followed by 5% trichloroacetic acid in PBS. The precipitates were then lysed with 1 N NaOH in 0.02% sodium dodecyl sulphate (SDS). Radioactivity was measured with a liquid scintillation counter (Beckman, Palo Alto, CA). TriPLICATE cultures were used in all assays, and the results are shown as radioactivity (c.p.m.) or the ratio of the radioactivity in the rosmarinic acid-treated group to the untreated group. The experiments were repeated at least three times, and similar results were obtained each time.

**Evaluation of cell viability**

Cell viability was evaluated measuring the extent of MTT reduction by the cells, and by the amount of lactate dehydrogenase (LDH) released from the cells. In the assay using MTT, mesangial cells grown in 96-well plates (1 × 10^4 cells/well) were incubated in the medium described above containing rosmarinic acid for 24 h. The cells were subsequently incubated in the medium containing 0.5 mg/ml MTT for 4 h. An equal volume of 0.04 N HCl in isopropanol solution was then added to each well to dissolve formazan crystals; optical density at 570 nm was measured. In another experiment, mesangial cells grown in 96-well plates (3 × 10^4 cells/well) were incubated for 48 h in the medium containing rosmarinic acid. The medium was then collected and the cells were lysed with 1% Triton X-100 in PBS. LDH activity in the medium or cell lysate was measured with an LDH assay reagent (Wako Pure Chemical Industries, Osaka, Japan). The percentage of released LDH was calculated from the ratio of LDH activity in the medium related to the sum of the LDH activity in the medium and cell lysate.

**Evaluation of cell-cycle dependency**

In order to evaluate the cell-cycle dependency, quiescent mesangial cells were incubated with 10 ng/ml PDGF for 24 h. Rosmarinic acid (final concentration, 5 μg/ml) was added to the medium at 0, 2, 4, 8, or 16 h post-PDGF administration. ^[3]H)Thymidine (1 μCi/well) was added at 20 h and the cells were further incubated for 4 h. ^[3]H)Thymidine incorporation into the cell nucleus was measured as mentioned above. Control cells were treated with PDGF without rosmarinic acid. Another experiment was carried out according to the method of Shirotani et al. [11], using PDGF as a competence factor, which transfers cells from the G₀ to the G₁ (G₀/G₁) phase, and insulin as a progression factor, which transfers cells from the G₁ to the S (G₁/S) phase. Quiescent mesangial cells were stimulated with 10 ng/ml PDGF for 2 h and the cells were then washed with serum-free culture medium, followed by stimulation with 30 μg/ml insulin for 22 h. At each stimulation, 1 or 5 μg/ml rosmarinic acid was added. ^[3]H)Thymidine was applied for the last 4 h, and the radioactivity was measured as above.

**RNA purification and northern blot analysis**

Mesangial cells were grown in 100-mm dishes until subconfluent and then grown for 48 h in FCS-free medium. After stimulation with 10 ng/ml PDGF together with 5 or 25 μg/ml rosmarinic acid for 24 h, total RNA was extracted by the acid guanidinium–phenol–chloroform method [12]. After the separation of 10 μg of RNA by formaldehyde/1% agarose gel electrophoresis, the RNA was transferred to a nylon membrane (Biodyne, Pall Bio Support, East Hills, NY). A ^32P-labelled cDNA probe was prepared with the Megaprime DNA labelling system (Amersham Life Sciences, Buckinghamshire, UK). Hybridization for glyceroldehyde-3-phosphate dehydrogenase (GAPDH) was used to normalize for any differences in RNA loading. The following cDNA probes in plasmid vectors were used: murine PDGF-B cDNA, from Dr Daniel F. Bowen-Pope (University of Washington School of Medicine, WA); c-myc and GAPDH were obtained from American Type Culture Collection (Rockville, MD). Hybridization was performed at 65°C for 12 h and the membrane was then autoradiographed. The radioactivity of the corresponding bands was quantitatively...
measured by a Fuji BAS 2000 Bio-Image Analyser (Fuji Film Co., Tokyo). After the radioactive probes were stripped off the membranes, rehybridization for the GAPDH probe was performed. The experiments were repeated three times with different batches of cell preparation, and similar results emerged.

**Statistical analysis**

A one-way analysis of variance (ANOVA) and Student’s t-test were applied. Differences with \( P < 0.05 \) were considered significant. IC\(_{50}\) values were obtained by fitting of the data to the general dose-response equation for a single binding component.

**Results**

**Effects of rosmarinic acid on mesangial cell proliferation and cell viability**

Rosmarinic acid significantly reduced the basal DNA synthesis, measured as the \([^{3}H]\)thymidine incorporation in murine mesangial cells under serum-free conditions (basal, 1360.4 ± 81.5; 1 µg/ml, 993.9 ± 49.4, \( P < 0.01 \); 5 µg/ml, 697.4 ± 45.8, \( P < 0.001 \) c.p.m.). PDGF (10 ng/ml) and TNF-\(\alpha\) (100 U/ml) each increased the DNA synthesis by mesangial cells, by four- and sixfold (3683.0 ± 360.2 vs 896.6 ± 154.5 c.p.m.; 5000.1 ± 669.9 vs 822.5 ± 81.3 c.p.m. respectively). These increases were significantly inhibited by rosmarinic acid, in a dose-dependent manner (0.2, 1, and 5 µg/ml of rosmarinic acid under PDGF stimulation, 3615.8 ± 154.6 (85.9%), 1730.4 ± 324.6 (53.0%, \( P < 0.01 \)), and 1277.7 ± 287.6 c.p.m. (34.7%, \( P < 0.01 \)) respectively. Under TNF-\(\alpha\) stimulation, 0.2, 1, and 5 µg/ml of rosmarinic acid resulted in 4618.8 ± 333.2 (92.4%), 3551.6 ± 137.5 (71.0%, \( P < 0.05 \)), and 2263.7 ± 398.9 c.p.m. (45.3%, \( P < 0.01 \)) respectively (Figure 2). The concentrations of rosmarinic acid that caused 50% inhibition (IC\(_{50}\)) of the PDGF- and TNF-\(\alpha\)-induced proliferations were 1.4 µg/ml and 3.8 µg/ml respectively. In other experiments to confirm cell viability, we measured the reducing activity of the cells on MTT and the LDH activity released from the cells to the medium. As shown in Table 1, rosmarinic acid did not significantly alter these parameters up to a dose of 25 µg/ml.

**Evaluation of cell-cycle dependency**

To investigate the phase(s) of the cell cycle at which rosmarinic acid inhibits mesangial cell proliferation, we administered rosmarinic acid after PDGF treatment. Rosmarinic acid was effective in inhibiting mesangial cell DNA synthesis when added at 0, 2, 4, and 8 h but not 16 h after the addition of PDGF (Figure 3). Another experiment using PDGF as a competence factor and insulin as a progression factor showed that rosmarinic acid inhibited \([^{3}H]\) thymidine incorporation in both the G\(_{0}/G\_1\) and G\(_{1}/S\) phases in a dose-dependent manner. The inhibitory effect of rosmarinic acid was slightly higher in the G\(_{1}/S\) phase than in the G\(_{0}/G\_1\) phase (Figure 4).

**Table 1. Effects of rosmarinic acid on the viability of murine mesangial cells**

<table>
<thead>
<tr>
<th>Rosmarinic acid (µg/ml)</th>
<th>Reducing activity on MTT (OD(_{570}))</th>
<th>Release of LDH (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.618 ± 0.016</td>
<td>10.9 ± 5.0</td>
</tr>
<tr>
<td>1</td>
<td>0.625 ± 0.011</td>
<td>Not tested</td>
</tr>
<tr>
<td>5</td>
<td>0.622 ± 0.011</td>
<td>18.1 ± 3.9</td>
</tr>
<tr>
<td>25</td>
<td>0.598 ± 0.029</td>
<td>13.6 ± 5.6</td>
</tr>
</tbody>
</table>

Mesangial cells were incubated with the indicated concentration of rosmarinic acid for 24 h, and the cell viability was evaluated by measuring cellular reduction of MTT and the amount of LDH released from the cells. The assay using MTT was expressed as mean ± SD (\( n = 5 \)) of OD\(_{570}\). The assay measuring LDH released from the cells was expressed as mean ± SD (\( n = 3 \)) of the ratio of LDH released from the cells (%). No significant difference was observed compared with the control group.

![Fig. 2. Effects of rosmarinic acid on (A) PDGF-and (B) TNF-\(\alpha\) induced murine mesangial cell proliferation. Quiescent mesangial cells were incubated with 10 ng/ml PDGF or 100 U/ml TNF-\(\alpha\) and the indicated concentration of rosmarinic acid for 24 h; \([^{3}H]\) thymidine was added for the last 4 h. The radioactivity (c.p.m.) incorporated into the cells is shown. Each column represents the mean ± SD (\( n = 3 \)). *\( P < 0.05 \) and **\( P < 0.01 \) compared with control.](image-url)
Rosmarinic acid inhibits cultured mesangial cell proliferation

**Fig. 3.** Time course study of the inhibitory effect of rosmarinic acid on PDGF-induced mesangial cell proliferation. Quiescent mesangial cells were incubated with 10 ng/ml PDGF for 24 h, and 5 μg/ml rosmarinic acid was added 0, 2, 4, 8 or 16 h after PDGF administration. Control cells were treated with PDGF and without rosmarinic acid. [³H]Thymidine was added for the last 4 h, and the radioactivity (c.p.m.) incorporated into the cells is shown. Each column represents the mean ± SD (n = 3). **P < 0.01 and ***P < 0.001 compared with control.

**Fig. 4.** Effect of rosmarinic acid added at the time of (A) PDGF (G₀/G₁) or (B) insulin (G₁/S) stimulation on mesangial cell proliferation. Quiescent mesangial cells were incubated with 10 ng/ml PDGF for 2 h. After washing with basal medium, mesangial cells were further incubated with 30 μg/ml insulin for 22 h; 5 μg/ml rosmarinic acid was added at each stimulation. [³H]Thymidine was added for the last 4 h. The radioactivity (c.p.m.) incorporated into the cells is shown. Each column represents the mean ± SD (n = 3). **P < 0.01 and ***P < 0.001 compared with control.

**Effect of rosmarinic acid on the expression of PDGF and c-myc mRNA**

PDGF is known as an autocrine mediator of mesangial cells after exogenous PDGF stimulation [8]. The expression of PDGF mRNA induced by exogenous 10 ng/ml PDGF for 20 h was significantly decreased by the addition of 5 or 25 μg/ml rosmarinic acid by 40 and 29% respectively, as determined by the densitometric analysis of autoradiographs from Northern blots. The expression of c-myc mRNA was also significantly decreased by 5 and 25 μg/ml rosmarinic acid, by about 62 and 40% respectively, after a 20-h incubation with PDGF (Figure 5).

**Discussion**

In the present study, we found that rosmarinic acid, which is widely distributed in Labiatae herbs, inhibited both PDGF-and TNF-α-induced mesangial cell proliferation. Since rosmarinic acid inhibited mesangial cell proliferation induced by different mitogens, the inhibitory effects may involve mechanisms independent of the mitogens and their trans-receptor signalling modes. Cell proliferation is a complex phenomenon involving the interaction of growth factors with cell membranes, the successive phosphorylation of various proteins in the cytoplasm, and the regulation of the cell cycle at the nuclear level [13]. To investigate the site(s) at which rosmarinic acid acts in signal transduction, we conducted a time-course study in which rosmarinic acid was applied to mesangial cells several hours after PDGF stimulation, and we found that rosmarinic acid was effective when added up to 8 h after the stimulus. Early signal transduction pathways usually occur within minutes to 1–2 h after the addition of a stimulus in vitro, as has been shown for the induction of phospholipid hydrolysis, phosphorylation pathways,
mesangial cell proliferation in both the G₀/G₁ and G₁/S phases, the effect being slightly more pronounced in the G₁/S phase. These results suggest that in the process of cell proliferation, rosmarinic acid might regulate DNA synthesis both in early and in late signal transduction.

In mesangial cells, exogenous PDGF can induce autocrine PDGF mRNA expression, which is considered to stimulate further proliferation in mesangial cells. The proto-oncogene, c-myc is a central regulator of cell proliferation closely linked to the cell cycle machinery [16], and is rapidly induced by mitogenic growth factors such as PDGF [17]. The present Northern blot analysis at 20 h after exogenous PDGF stimulation indicated that rosmarinic acid suppressed the expression of PDGF and c-myc mRNA in a dose-dependent manner. Recent studies revealed that reactive oxygen species act as cellular signals in PDGF-induced mitogenesis, and these include the expression of the proto-oncogenes c-fos and c-myc [18]. Since rosmarinic acid has potent anti-oxidative activity [6], this compound might suppress c-myc mRNA expression by scavenging reactive oxygen species induced by exogenous PDGF stimulation. PDGF is produced in PDGF-stimulated mesangial cells as an autocrine factor, which is considered to further stimulate mesangial cell proliferation. The suppressive effects of rosmarinic acid on PDGF and c-myc mRNA expression would contribute to its anti-proliferative activity on mesangial cell.

The leaves of perilla contain at least 0.3% weight of rosmarinic acid [2]. For example, if 5 g of fresh leaves are consumed, more than 15 mg of rosmarinic acid would be ingested, absorbed through the gastrointestinal tract, and circulated in the serum in an unmetabolized chemical structure [19]. This dose per body weight nearly corresponds to the concentrations used in our in vitro study. Our recent study revealed that oral administration of rosmarinic acid suppressed glomerular cell proliferation and infiltration in rat thy-1 nephritis [20]. Therefore, the use of Labiatae herbs may be a promising way for protection against chronic aggravation of renal diseases.

In conclusion, we found that rosmarinic acid exhibited antiproliferative effects in cultured murine mesangial cells and suppressive effects on PDGF and c-myc mRNA expression. It is tempting to speculate that rosmarinic acid in Labiatae herbs is a promising agent to prevent mesangioproliferative glomerular diseases.

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References


and oncogene expression [14]. Our present results suggest that rosmarinic acid did not inhibit DNA synthesis at the S phase but rather suppressed the entry of mesangial cells into the S phase.

The next experiment was conducted to determine whether rosmarinic acid acted in the G₀ to G₁ phase or during the G₁ to S phase. In the process of cell proliferation, there are two critical steps: competent factors such as PDGF are involved in the transition from the G₀ phase to the G₁ phase in the first step, and progression factors such as insulin are involved in the onset of DNA synthesis promoting the progression of the competent cells from the G₁ to the S phase [15]. Rosmarinic acid inhibited the PDGF-induced

Fig. 5. Effects of rosmarinic acid on PDGF-induced mRNA expression of PDGF and c-myc of mesangial cells. Quiescent mesangial cells were incubated with 10 ng/ml PDGF in the absence or presence of 5 or 25 μg/ml rosmarinic acid for 20 h, and RNA was subsequently extracted and analysed by northern blot. (A) mRNA expression of PDGF, c-myc, and GAPDH. Lane 1, control; lane 2, rosmarinic acid (5 μg/ml); lane 3, rosmarinic acid (25 μg/ml). The signal intensities of mRNA expression were quantified, and changes in the signal intensities of PDGF (B) and c-myc (C) relative to GAPDH are shown.


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