

Carnosic Acid, a Component of Rosemary (*Rosmarinus officinalis* L.), Promotes Synthesis of Nerve Growth Factor in T98G Human Glioblastoma Cells

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Nerve growth factor (NGF) is a factor vital for the growth and functional maintenance of nerve tissue. The authors found that a rosemary (*Rosmarinus officinalis* L.) extract enhanced the production of NGF in T98G human glioblastoma cells. Furthermore, the results indicated that carnosic acid and carnosol, which are major components of the rosemary extract, were able to promote markedly enhanced synthesis of NGF.

Key words nerve growth factor; carnosic acid; carnosol; rosemary

Senile dementia has a tendency to increase with the shift to an aging society. This tendency has become a major large social problem. Dementia syndromes such as Alzheimer disease (AD) have their own pathologic characteristics including degeneration and loss of neurons in certain brain areas, such as the cholinergic neurons.

Nerve growth factor (NGF), a protein composed of 118 amino acid residues, is vital for the growth and functional maintenance of nerve tissue.^{1,2)} NGF functions as neurotrophic molecule for magnocellular cholinergic neurons in basal forebrain nuclei, which are specifically lost in AD. Considerable evidence from animal studies suggests that NGF may be useful in halting and slowing the progression of AD-related cholinergic basal forebrain atrophy.³⁾ Administration of NGF attenuated the degeneration of neurons and improved cognitive behavior in animals by stimulating central cholinergic neurons that are known to die during the development of AD.⁴⁾ A clinical trial using intracerebral infusion of NGF improved the patients' verbal episodic memory.⁵⁾ Accordingly, an elevation of the NGF level in the living body may be effective for treating disorders involving central functions such as AD.

However, exogenous NGF administration by intracerebral infusion is difficult and inconvenient. On the other hand, NGF administered by injection *via* peripheral routes is unable to reach the brain through the blood-brain barrier. Therefore low molecular-weight NGF inducers that pass through the blood-brain barrier would be good candidates for antidementia drugs.

The authors sought an herb extract that would promote the synthesis of NGF. As a result, it was found that rosemary extracts, the major constituents of which are carnosic acid and carnosol, has such an action. This paper reports that carnosic acid and carnosol were able to promote markedly enhanced synthesis of NGF in glioblastoma cells.

MATERIALS AND METHODS

Materials Eagle's minimum essential medium (MEM), Opti-MEM, fetal bovine serum (FBS), sodium pyruvate solution, and nonessential amino acids (NEAA) were obtained from Gibco BRL (U.S.A.). Anti-NGF antibody was from

Promega Co. (U.S.A.). Human beta-NGF was from PEPRO TECH EC (U.K.). β -Galactosidase-labeled anti-NGF antibody was purchased from Boehringer Mannheim (Germany). 4-Methylumbelliferyl-beta-D-galactoside, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), bovine serum albumin (BSA), and other chemicals used in this study were purchased from Sigma Chemical Co. (U.S.A.).

Rosemary Extract Dried leaves of rosemary (*Rosmarinus officinalis* L., 60 g) were soaked in water or aqueous 90% ethanol solution (300 ml), and extracted at 40 °C for 48 h. The water extract was freeze-dried (dry weight 7.2 g) and used in this study as the water-soluble part. The resultant 90% ethanol solution was concentrated to a volume of 100 ml. After concentration, the concentrate was filtered to remove insoluble materials. Water (200 ml) was added to the filtrate and the mixture was allowed to stand overnight at 4 °C. Subsequently, the mixture was again filtered to obtain the water-insoluble extract. The insoluble extract (dry weight 3.5 g) was used as the water-insoluble part in this study.

Isolation of Carnosic Acid and Carnosol Rosemary (5 kg) was soaked in ethanol (20 l), and extracted at 40 °C. The resultant solution was concentrated to a volume of 1 l. The concentrate was filtered to remove insoluble materials. Water (2 l) was added to the filtrate and the precipitate (105 g) deposited at this time was filtered. The precipitate was dissolved in ethyl acetate, separated, and purified through silica gel column chromatography (developing solvent, ethyl acetate:hexane=1:4 v/v) to afford two substances. These were recrystallized from hexane to leave pale yellow crystals **1** (carnosic acid 1.5 g) and colorless crystals **2** (carnosol 0.8 g), respectively. The structures of compounds **1** and **2** were identified by the comparison of these spectral properties (¹³C- and ¹H-NMR) with those reported in the literature.^{6,7)}

Cell Culture Human glioblastoma cells (cell line T98G) were seeded in Eagle's MEM containing FBS (10%), sodium pyruvate (1×) and NEAA (1×) in wells of flat-bottomed 96-well plates (Corning, U.S.A.), at a cell density of 2×10⁴/well, and cultivated in a humidified atmosphere containing 5% CO₂/air for 3 d. The medium was then replaced by Opti-MEM medium containing 5 mg/ml of BSA. The cultivation was continued for a further 6 d with the medium re-

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placed at intervals of 3 d. After removing the medium, fresh medium supplemented with the above extract, carnosol, or carnosic acid 50 μl /well was added and cultivation was continued for a further 4 d. After cultivation, the supernatants were used as sample solution. Viability was examined using the MTT method.⁸⁾

NGF Measurement NGF content was measured by enzyme-linked immunosorbent assay (ELISA) with anti-NGF antibody as follows. Anti-NGF antibody 1 $\mu\text{g}/\text{ml}$ solution (50 μl) was added to each well of a 96-well microplate (Nunc, Denmark), and the plate was allowed to stand for 18 h at 4 °C. After washing the plate with phosphate-buffered saline containing 0.05% Tween 20 (PBS-T), 1% BSA solution (100 μl) was added to each well and the plate was incubated at 4 °C overnight. Subsequently, the plate was washed with PBS-T and above sample solution (50 μl) was added to each well. After the reaction at room temperature for 1 h, the plate was washed with PBS-T. Beta-galactosidase-labeled anti-NGF antibody (0.4 unit/ml) solution (50 μl) was added to each well, and incubated at room temperature for 1 h. After washing the plate with PBS-T, 0.5 mg/ml of 4-methylumbelliferyl-beta-D-galactoside solution (200 μl) was added and incubated at room temperature for 4 h in the dark. The fluorescence intensity of 4-methylumbelliferone produced was measured on a fluorescence reader (PerSeptive Biosystems, CytoFluor Series 4000), and the content of NGF contained in the sample solution was determined using a standard curve obtained from a human beta-NGF standard solution.

HPLC Carnosic acid and carnosol in water-insoluble rosemary extract dissolved in ethanol were measured using reverse-phase HPLC. The HPLC system consisted of a Shimadzu Model CLASS-LC10 liquid chromatograph equipped with a spectrophotometric detector SPD-M10AVP diode array detector and a C18 column ($\mu\text{Bondasphere}$: 50 \times 3.9 mm i.d., C18, 5 μm , 100A, Waters, U.S.A.). The column was eluted with 2 (v/v)% acetic acid and acetonitrile (45:55 v/v) at a flow rate of 1.0 ml/min, and the elute was monitored at 230 nm.

Reverse-Transcriptase PCR T98G cells were treated with carnosic acid for 48 h in Opti-MEM medium containing 5 mg/ml of BSA. Then total RNA was prepared using TRI reagent (Sigma Chemical Co., U.S.A.) according to the manufacturer's protocol. The total RNA (1 μg) from the cultured T98G cells was reverse-transcribed at 48 °C for 40 min into cDNA using a TAKARA reverse-transcriptase (RT)-PCR kit (TAKARA BIO Inc., Japan) in a 20 μl volume. PCR was performed using oligonucleotides complementary to the 5' and 3' ends of the coding sequence of NGF (purchased from Promega Co.) and beta-actin (5'-GAT CAT TGC TCC TCC TGA GC-3', 5'-CAC CTT CAC CGT TCC AGT TT-3'). Amplification was performed for 30 cycles of 94 °C for 30 s, 60 °C for 30 s, 72 °C for 2 min (NGF); 25 cycles of 94 °C for 30 s, 58 °C for 30 s, 72 °C for 2 min (beta-actin) in a DNA thermal cycler heat block (ASTEC, MODEL PC-801). The PCR products were separated by 2% agarose gel electrophoresis and visualized with ethidium bromide staining.

Statistical Analysis Results are expressed as mean \pm S.D. The means for each point were generated from triplicate determinations. The effects of various treatments were compared using Student's *t*-test for unpaired samples.

RESULTS AND DISCUSSION

It is known that memory is improved with the use of rosemary (*R. officinalis* L.).⁹⁾ However, the effective component has not been elucidated. In addition, NGF is a vital factor for the growth and functional maintenance of nerve tissue, and a clinical trial using NGF was reported to improve the patients' verbal episodic memory.⁵⁾ Consequently, the authors examined whether a rosemary extract influences NGF production.

T98G human glioblastoma cells were used in this evaluation.¹⁰⁾ First, a highly sensitive sandwich ELISA for human NGF using a fluorescence substrate was developed to determine NGF in T98G culture supernatants. The standard curve for human NGF ELISA is shown in Fig. 1, and the minimal detection limit of this ELISA was estimated to be 5 pg/ml. In this system, the promoting action of epinephrine, as a positive control, with respect to the production of NGF was exhibited at concentrations ranging from 0.05 to 1 mM, and NGF content in the medium increased by 15 fold with the administration of 1 mM of epinephrine (Fig. 3).

When T98G cells were cultured in the medium supplemented with 5 or 10 $\mu\text{g}/\text{ml}$ of water-insoluble rosemary extract, the NGF content almost doubled (Table 1). However, the effects of the water-soluble rosemary extract were much lower than those of the water-insoluble extract. These results indicate that the water-insoluble fraction of rosemary extract contains an enhancer of NGF production. Next, the two major components of the fraction were isolated. The structures of the compounds were identified by the comparison of

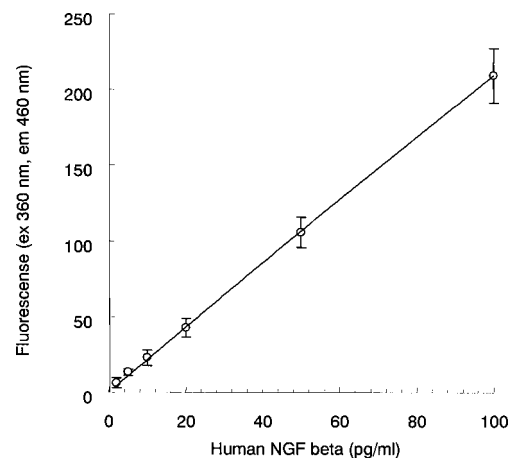


Fig. 1. Standard Curve of the Sandwich ELISA for Human NGF
Points represent mean value \pm S.D. of three determinations.

Table 1. Effect of Rosemary Extract on NGF Secretion in T98G Cells

	Rosemary extract ($\mu\text{g}/\text{ml}$)	NGF (pg/ml)	NGF content (%)
Control	0	6.2 \pm 0.5	100 \pm 8
Water-insoluble	5	12.9 \pm 1.7*	208 \pm 27
	10	15.6 \pm 3.3*	252 \pm 53
Water-soluble	5	6.8 \pm 2.4	110 \pm 39
	10	7.4 \pm 1.3	119 \pm 21

T98G glioblastoma cells were incubated with water-soluble or -insoluble rosemary extract for 4 d. Then NGF content in culture supernatant was determined with ELISA using anti-NGF antibody. Results are reported as the mean of triplicate measurements \pm S.D. Significantly different from control values: **p* < 0.03.

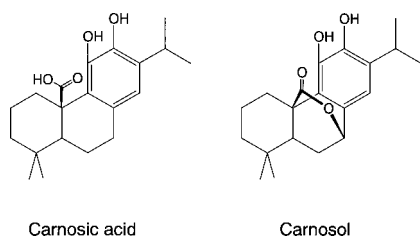


Fig. 2. Chemical Structures of Carnosic Acid and Carnosol

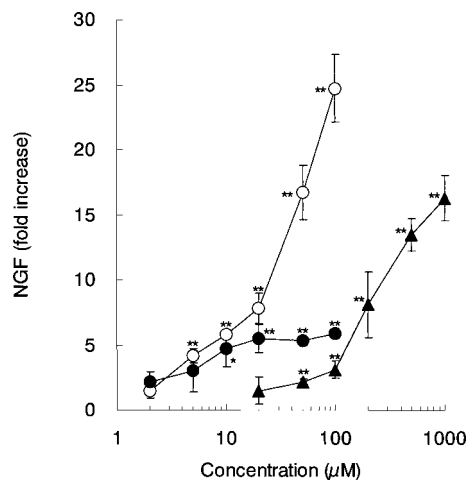


Fig. 3. Effect of Carnosic Acid and Carnosol on NGF Secretion in T98G Cells

The experimental conditions were the same as those described in Table 1. ○, carnosic acid; ●, carnosol; ▲, epinephrine. Results are expressed as the mean of triplicate measurements ± S.D. Significantly different from control values: * $p < 0.05$, ** $p < 0.01$.

spectral properties, and these compounds were determined to be carnosic acid and carnosol (Fig. 2).

As shown in Fig. 3, carnosic acid and carnosol increased NGF synthesis in a concentration-dependent manner. This effect of carnosic acid was exhibited at concentrations ranging from 5 to 100 μM , and NGF content in the medium increased by about 25 fold. Furthermore, it was confirmed that the level of NGF mRNA also increased with the administration of carnosic acid (Fig. 4). However, cytotoxicity was induced with the administration of carnosic acid doses higher than 200 μM .

Carnosic acid 1 $\mu\text{g}/\text{ml}$ (3.2 μM) and carnosol 0.08 $\mu\text{g}/\text{ml}$ (0.25 μM) were included in 10 $\mu\text{g}/\text{ml}$ of the water-insoluble rosemary extract solution as a result of the measurement with HPLC. Although carnosol 0.25 μM was ineffective, NGF production almost doubled with the administration of carnosic acid 3.2 μM (data not shown). This result almost equalled that of the rosemary extract 10 $\mu\text{g}/\text{ml}$ (Table 1). These results suggest that the promoting action of rosemary extract on NGF production is attributed to carnosic acid.

Furukawa *et al.* reported that 3,4-dihydroxyphenyl derivatives with two saturated side chains were effective in stimu-

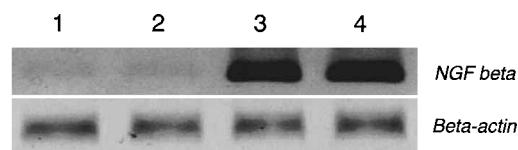


Fig. 4. Gel Analysis of RT-PCR Products of T98G Cells Treated with Carnosic Acid

T98G cells were treated for 48 h with vehicle (lanes 1, 2) or carnosic acid 50 μM (lanes 3, 4). RT-PCR was performed as described under Materials and Methods.

lating NGF synthesis and that the catechol portion is essential for this stimulation.¹¹⁾ The structures of carnosic acid and carnosol also meet the conditions they established. Therefore the catechol portions of carnosic acid and carnosol maybe play an important role in the stimulating NGF synthesis.

Although carnosol is a derivative of carnosic acid, the promoting action of carnosic acid on NGF synthesis was much stronger than that of carnosol. It has been reported that carnosic acid and carnosol exhibit antioxidative and antimutagenic actions.^{12,13)} An interesting point of note is that carnosic acid has more effective antioxidative and antimutagenic actions than carnosol. Therefore the mechanisms of these effects of carnosic acid are possibly associated with the stimulation of NGF synthesis. However, further investigation is required for elucidation of the precise mechanisms.

To the best of our knowledge, the data presented here are the first to demonstrate the promoting action of components in rosemary on NGF synthesis. These components may be used as a lead compound for anti-AD agents. However, further studies are required to determine the mechanism by which carnosic acid induces NGF synthesis. The promoting action of carnosic acid on NGF synthesis *in vivo* is currently under investigation.

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