lemon. Briefly, the three odorants were presented in random order to the participants. Participants smelled the odor and chose one of the four response alternatives (one correct response and three distracters) from the photographs corresponding to the odor stimulants. Each olfactory score was evaluated using a 3-point scale: 2 = completely identified, 1 = smelled but unable to distinguish, 0 = did not smell or distinguish. Individual olfactory score was calculated by adding the odor identification scores of all the items; totals ranged from 0 to 6.

Measurement of Upper Respiratory Protective Reflexes and Involuntary Swallowing Movement

The swallowing reflex and cough reflex sensitivity were assessed between 9:30 and 10:30 a.m. The swallowing reflex was provoked using a 1-mL bolus of distilled water injected into the pharynx through a nasal catheter. The subjects were unaware of the actual injection. Swallowing was identified according to submental electromyographic activity and visual observation of characteristic laryngeal movement. The swallowing reflex was quantified as latency of swallowing reflex (LTSR), timed from the injection to the onset of swallowing.[13] The individual cough reflex sensitivity to citric acid was evaluated using a tidal breathing nebulized solution delivered through an ultrasonic nebulizer (MU-32, Sharp Co. Ltd, Osaka, Japan).[14] Individual cough reflex threshold was defined as the concentration of citric acid (0.7–360.0 mg/mL in saline) at which the participant coughed more than five times during the 1-minute inhalation period. The number of involuntary swallowing movements was counted at rest in a supine position during inhalation of an odorant (BPO, LO, or distilled water) for 1 minute.

Measurement of Serum SP Concentration

Blood was collected before evaluation of cough-reflex sensitivity in a tube containing 0.5 U/mL aprotinin and 3 mM ethylenediaminetetraacetic acid before breakfast and immediately centrifuged to separate serum from the cell fraction. Serum SP was quantified as previously described.[15]

Regional Cerebral Blood Flow Study

Ten patients (4 men) with a history of aspiration pneumonia (mean age ± standard deviation 81.7 ± 8.5, mean MMSE score 13.5 ± 1.4, mean ADL score 15.5 ± 0.8) were randomly recruited from the BPO-treated group for SPECT-scanning examination with ^{123}I iodoamphetamine. Before the BPO intervention, magnetic resonance imaging revealed that, of the 10 patients, seven had multiple lacunar infarcts in the basal ganglia (n = 1), thalamus (n = 1), or deep white matter (n = 5) and that the other three had cortical infarcts in the bilateral frontal and right parietal regions (n = 1), right frontal regions (n = 2), left frontal regions (n = 1), and right parietal regions (n = 1). All 10 patients showed prolonged LTSR (>5.0 seconds) before the BPO intervention (mean 5.9 ± 1.1).

The SPECT-scanning examination was performed before the start of the study and at the end of the 1-month study with volatile BPO. All SPECT scans were performed with patients in stable condition with their eyes closed. Regional cerebral blood flow (rCBF) was measured by recording the distribution of radioactivity after the intravenous injection of ^{123}I-N-isopropyl-p-iodoamphetamine (IMP) with a triple-headed gamma camera (Multi-SPECT3, Siemens USA, Knoxville, TN), which has an axial field of the entire brain and cerebellum. Patients were placed in a supine position in a soft head restraint approximately 15 minutes after intravenous injection of 111 MBq of IMP into an antecubital vein.[16] Data were acquired by scanning in a three-dimensional mode for 30 minutes.

Statistics

The planned sample size for the study was based on power calculations related to the estimation of the confidence interval expected for the intervention group. This was based on the mean difference in LTSR between groups. The calculation yielded a sample size of 28 patients in each group, assuming a *t* test for two independent groups, with a two-sided alpha level of 0.05 and a statistical power of 80%.

Values were expressed as mean ± standard error. Data were transformed to logarithmic values of citric acid concentration for cough-reflex sensitivity. All data except for rCBF in SPECT scans were analyzed using statistical analysis software (Statview, version 5.0 for Windows, SAS Institute, Inc., Cary, NC). Comparisons of age, sex, ADL score, MMSE score, ability to identify odors, cough reflex sensitivity at baseline, and number of involuntary swallowing movements were performed between the BPO-treated group, the LO-treated group, and the odorless group according to the Kruskal–Wallis test. Comparisons of LTSR and serum SP of the three groups at baseline were performed using one-factor analysis of variance (ANOVA). Comparisons of LTSR, cough-reflex sensitivity, and serum SP between the pre- and poststudy periods in each group were performed using two-way repeated-measures ANOVA. The proportion of participants in the three groups with improved cough reflex sensitivity was compared using the chi-square and Fisher exact tests. Comparisons of the three groups with regard to LTSR and serum SP at Day 30 and cough reflex sensitivity at Day 30 were performed using one-factor ANOVA and the Kruskal–Wallis test, respectively.

Statistical analysis for voxel-by-voxel comparison of rCBF in SPECT scans was performed using Statistical Parametric Mapping 99 software (London, UK) implementation of a general linear model. After spatial normalization, stereotactically normalized images were smoothed using a Gaussian kernel with a full width of 12 mm at half maximum.[16] Multiple comparison in the global brain was performed. The regional-to-cerebellar IMP uptake ratio (cerebral blood flow ratio) was used as a measure of the relative perfusion rate in the insular region and orbitofrontal region. Comparison of rCBF distributions in the insular region and the orbitofrontal cortices between pre- and post-BPO intervention for 1 month was performed using the paired Student *t* test.

Significance levels were defined as $P < .05$.

RESULTS

In this randomized, prospective trial, there were no significant differences in multiple parameter baseline factors (age; sex distribution; ADL status; cognitive function; the

Table 1. Characteristics of Participants and Outcomes of Each Treatment in Nursing Home Patients

Characteristic	Odorless n = 35	Black Pepper Oil n = 35	Lavender Oil n = 35	*P*-value*	*P*-value†
Age, mean ± SD	84.5 ± 4.2	84.3 ± 7.1	86.2 ± 4.9		
Sex: male:female	8:27	9:26	7:28		
Activities of daily living, mean ± SD	8.4 ± 6.6	10.8 ± 6.3	9.5 ± 6.5		
Mini-Mental State Examination score, mean ± SD	12.4 ± 7.3	11.2 ± 7.7	11.2 ± 7.7		
Olfactory identification, mean ± SD	2.1 ± 2.4	2.2 ± 2.0	1.7 ± 2.3		
Latency of swallowing reflex, seconds, mean ± SD					
Baseline	15.8 ± 19.6	17.6 ± 21.5	14.8 ± 15.1		
1 minute later	15.2 ± 17.4	6.4 ± 7.8*	13.2 ± 12.5	.03	
Day 30 (dropouts)	14.4 ± 17.3 (2)	4.4 ± 2.6* (1)	13.6 ± 15.4 (2)	.005	<.001
Log concentration of citric acid for cough threshold, mean ± SD, mg/mL					
Baseline	1.3 ± 0.5	1.2 ± 0.6	1.1 ± 0.5		
Day 30 (dropouts)	1.3 ± 0.5 (2)	1.2 ± 0.5 (1)	1.1 ± 0.9 (2)		
Serum substance P, mean ± SD, pg/mL					
Baseline	34.3 ± 8.1	35.3 ± 9.0	32.9 ± 10.1		
Day 30 (dropouts)	30.9 ± 8.7 (2)	40.8 ± 10.6‡ (1)	34.9 ± 8.4 (2)	.03	.04
Number of swallows for 1 minute, mean ± SD					
Baseline	0.5 ± 0.3	0.4 ± 0.3	0.4 ± 0.5		
During smell	0.5 ± 0.5	3.7 ± 2.5+	0.3 ± 0.3	<.001	<.001

* Representative of the comparison between that at baseline and that 1 minute later or at Day 30.
† Representative of the overall group comparison.
‡ Significance at $P < .05$.
SD = standard deviation.

ability to identify odors; and medications such as angiotensin-converting enzyme inhibitors, neuroleptics, and amantadine) between participants in the odorless group, the BPO-treated group, and the LO-treated group (Table 1). In addition, LTSR, cough-reflex sensitivity, and serum SP concentration at baseline were not significantly different in the three groups ($P > .05$).

The nasal inhalation of BPO caused a significant shortening of LTSR at 1 minute soon after its first nasal inhalation by the naive participants ($P = .03$) (Table 1). The LTSR in the BPO-treated group at 30 days was significantly less than the baseline value, whereas those of the other groups were not ($P = .005$). The LTSR of the BPO-treated group at 30 days was also significantly shorter than those of the LO-treated and the odorless groups at 30 days ($P < .001$) (Table 1).

The nasal inhalation of any odorants (BPO, LO, and distilled water) during the 30-day period did not significantly affect cough-reflex sensitivity (Table 1). The number of involuntary swallowing movements for 1 minute in the BPO-treated group was significantly greater than in the odorless group and the LO-treated group ($P < .001$) (Table 1). Serum SP at 30 days in the BPO-treated group was significantly greater than at baseline ($P = .03$), whereas it did not change significantly in the LO-treated and odorless groups ($P = .53$). Serum SP in the BPO-treated group at 30 days was also significantly greater than in the LO-treated and odorless groups ($P = .04$) (Table 1).

The LTSR in the group of BPO-treated patients was significantly longer than the initial LTSR, according to the SPECT scan (5.9 ± 1.1 vs 2.7 ± 1.1 seconds). Voxels within the insular cortex in the brains of these patients were significantly larger ($P < .001$) (Figure 2). Comparison of rCBF before the nasal inhalation of volatile BPO with that after the study showed that rCBF in the right medial orbitofrontal cortex (anterior cingulate cortex) and the left insular cortex was significantly greater (x,y,z = −10, 54, −10, z = 4.29; k = 330 voxels, x,y,z = 46, 32, −4, z = 4.00; k = 139 voxels, respectively) ($P < .001$) (Figure 2).

DISCUSSION

It was found that olfactory stimulation using BPO significantly improved the sensory and reflexive motor movement of swallowing, presumably via activation of the right insular cortex, the function of which is reported to be impaired in patients with dysphagia. It was previously reported that patients with depressed swallowing reflex over 5 seconds were at high risk for the development of pneumonia.[17] This function was impaired in the present patients, suggesting dysphagia and a high risk of pneumonia. Therefore, olfactory stimulation using BPO is a possible new remedy for treatment of elderly patients at high risk of pneumonia.

Olfactory stimulation, so called aromatherapy, is not limited to any particular subjects, because nasal inhalation of odorants is simple and easy for older people regardless of their level of consciousness or physical and mental status. Unlike the case of medication, there is no need to worry about any side effects or about a participant's ability to adhere to oral instructions. Some subjects with severe

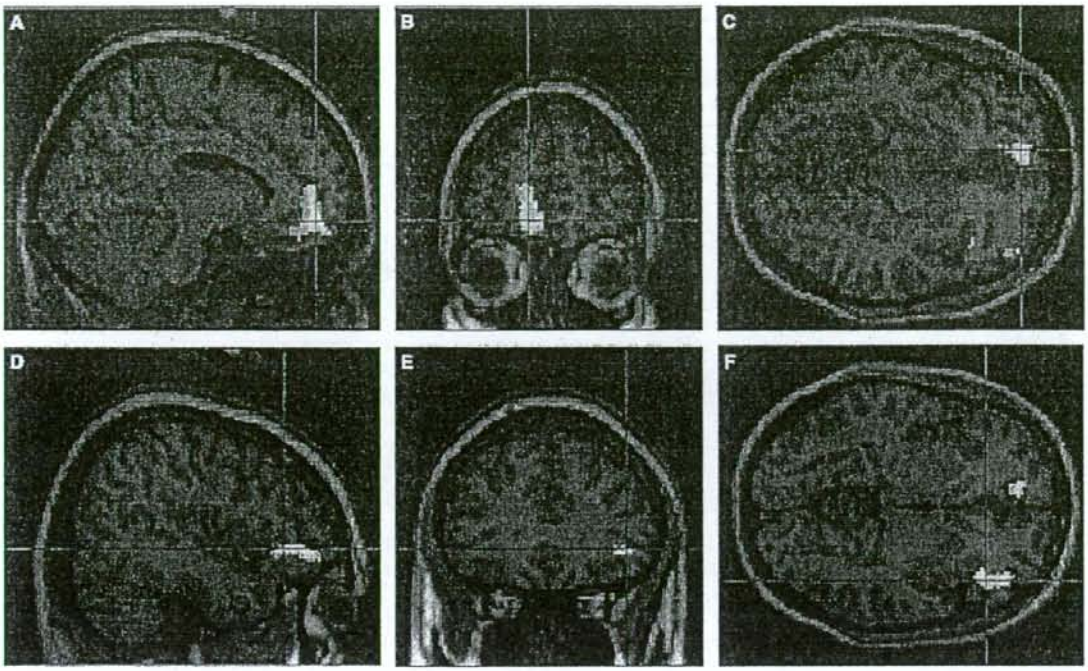

Figure 2. Brain single photon emission computed tomography scans obtained 30 days after nasal stimulation with black pepper oil. Enhanced intensity in the right medial orbitofrontal cortex (anterior cingulate cortex) (A, B, and C) and left insular cortex (D, E, and F) shows greater regional cerebral blood flow. (A and D, sagittal; B and E, coronal; C and F, horizontal slice).

dysphagia, such as those who had undergone tracheostomy or who required a nasogastric tube for feeding, were able to accept this remedy.

Black pepper is generally recognized as a fairly simple spice whose flavor comes from the molecule piperine, a transient receptor potential (TRP) vanilloid 1 agonist similar to capsaicin.[18] The benefit of stimulation of the TRP channel family using capsaicin,[19,20] as well as using hot or cold temperature, has been previously reported.[21] In light of these findings, it is conceivable that volatile BPO improves the swallowing movement by stimulating the brain via the olfactory sensory system. Videofluoroscopy examination indicated that the nasal inhalation of volatile BPO significantly reduced the pooling of isotopes on the recessus piriformis in the three elderly subjects who were examined (data not shown). Olfactory treatment using volatile BPO for 30 days also brought about a significant shortening of LTSR from baseline. Daily stimulation might result in cortical reorganization, making these reflexes easy to provoke.[22]

Meanwhile, the nasal inhalation of LO did not have any effect on coughing or on the reflexive swallowing movement. The effect of LO is known to counter insomnia and promote restful sleep. As predicted, unlike with BPO, nasal inhalation of LO did not stimulate the level of consciousness of participants.

Silent aspiration of oropharyngeal secretion, which is a cause of aspiration pneumonia, is often the consequence of insufficient SP release due to cerebrovascular disease.[1] Several types of pharmacological and mechanical stimulation increase the local SP concentration in human sputum or saliva and improve the swallowing reflex and cough-reflex sensitivity. The increase in serum SP with volatile BPO in this study might be closely related to improvement of the swallowing reflex. Capsaicin has been reported to release SP not only from sensory neurons but also from human lymphocytes.[23] Intervention with an ACE inhibitor also resulted in an increase in serum SP.[24] Nasal inhalation of volatile BPO might affect the whole body, resulting in an increase in serum SP by some unknown process involving the dopaminergic nerve in the brain.

Previous research has shown that the swallowing movement, including drinking water, activates portions of the insular, operculum, inferior precentral gyrus, and cerebellum and that frequent tongue movement produces substantial increases in insular activity, as shown using positron emission tomography imaging.[4,25] Using SPECT imaging, the current study revealed a dysfunction in the bilateral insular region in patients with a history of aspiration pneumonia.[5] The signal from olfactory information is projected to the primary olfactory cortex, such as the pyriform cortex and a portion of the right amygdala, right orbitofrontal cortex, anterior cingulate cortex, and left insular cortex (adjacent to the gustatory cortex).[26] Taken together, these findings indicate that olfactory stimulation using volatile BPO might alleviate swallowing dysfunction by enhancing activation mainly of the left insular cortex via the olfactory system.

Olfactory impairment or depression of the ability to identify odors has been reported in 80% of participants

aged 80 and older[27] and patients with neurogenerative diseases such as Alzheimer's disease or Parkinson's disease.[28] In this study, the ability to identify odors was evaluated using olfactory thresholds, smell recognition, and naming of smell. It is difficult to evaluate the olfactory threshold only. In the odor identification test, photographs were employed to minimize the effect of cognitive deficits on the naming of smells, but scores on this test were low, and no difference in identifying odors was found between the three groups because they were much older (mean age 85.8 ± 2.2) with much lower cognitive function (scoring approximately 10 points on the MMSE).

The anterior cingulate cortex, which was activated by volatile BPO in this study, has been recognized as a key region for central processing of initiation, motivation, and goal-directed behavior.[29] Further study of whether volatile BPO can enhance nutritional status in older people with malnutrition due to deteriorated motivation of feeding behavior is needed.

This study indicates a potential ability for older people to reduce the risk of aspiration bronchitis or pneumonia. Nasal inhalation of volatile BPO might be useful for patients with nasofeeding tubes, tracheotomization, or mechanical ventilation who constitute a high-risk group for aspiration pneumonia.

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Effects of menthol on the triggering of the swallowing reflex in elderly patients with dysphagia

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Aims
To investigate the effect of menthol on swallowing reflex sensitivity in elderly patients with dysphagia.

Methods and results
The swallowing reflex sensitivity of institutionalized elderly patients was evaluated as a latent time of swallowing reflex (LTSR), induced by the injection of 1 ml solution into the pharynx. LTSR was significantly shortened in a concentration-dependent manner, from 13.8 s [95% confidence interval (CI) 11.1, 16.5] by distilled water to 9.4 s (95% CI 7.1, 11.8) by 10^{-3} M menthol.

Conclusion
Using menthol with elderly patients with dysphagia may improve the sensitivity of their swallowing reflex, resulting in prevention of aspiration pneumonia.

Introduction

Morbidity and mortality from aspiration pneumonia continues to be a major health problem in the elderly [1]. Dysphagia, such as delayed triggering of the swallowing reflex, an important respiratory defence mechanism, predisposes to aspiration pneumonia. Triggering of the swallowing reflex could be accelerated if swallowed material was cooled down, even in dysphagic patients [2]. Moreover, it has been clinically accepted that there are dysphagic patients who benefit from a therapeutic procedure known as thermal stimulation, a technique consisting of a brief, light touch with a cooled laryngeal mirror to the anterior faucial pillars followed by the application of small amounts of iced fluid [3]. This suggests that repeated cold stimulation restores sensitivity to trigger the swallowing reflex in dysphagic patients. In 2002, two groups independently cloned and characterized the cold receptor as a member of a transient receptor potential (TRP) superfamily, named TRPM8 [4, 5]. TRPM8 could be activated not only by

cooling with an activation temperature <25–28 °C and but also by menthol, a chemical agent found in mint. Therefore, we examined the effect of menthol in elderly patients with dysphagia.

Patients and methods

The elderly patients were recruited from institutionalized patients located near Sendai, Japan. This long-term care facility is for older patients with a physical handicap or mental deterioration. Residents are highly dependent on their caregivers for assistance with the activities of daily life. From residents in the nursing home, we selected patients who fed themselves or needed help eating and had had a stable condition for a minimum of the last 2 months. We excluded patients who had either a feeding tube or percutaneus endoscopic gastrostomy. We assessed the latent time of swallowing reflex (LTSR) in 42 patients who met the criterion and had given informed consent. Written informed consent was obtained from all study patients. A detailed explanation of the study was given to each patient. When patients lacked the ability to understand the study, or the capacity to consent, their family was asked on their behalf. This protocol was approved by the Institutional Review Board of the Tohoku University Ethics Committee (no. 2004-379).

The swallowing reflex was induced by a bolus injection of 1 ml distilled water into the pharynx through a nasal catheter (8 Fr). The subjects were unaware of the actual injection. Swallowing was identified by submental electromyographic (EMG) activity and visual observation of characteristic laryngeal movement. EMG activity was recorded from surface electrodes on the chin. The swallowing reflex was evaluated by the latency of response, timed from the injection to the onset of swallowing [6]. The mean LTSR was 10.1 s [95% confidence interval (CI) 7.1, 13.2] for the 42 patients. The ultimate goal of the study was to develop a medicine for dysphagia. We focused on patients with mild to moderate dysphagia due to the potential risk of aspiration in patients with very severe dysphagia. We excluded patients with a LTSR of >20 s and <5 s, resulting in the selection of 14 patients.

Menthol (L-menthol; Sigma-Aldrich, St Louis, MO, USA) was dissolved in distilled water using a sonicator. Various concentrations (10^{-4}–10^{-2} M) of menthol and distilled water at room temperature were injected in a double-blind, randomized manner at 2-min intervals. After completing all the dosages of the menthol prepared, we changed the catheter to a two-lumen indwelling catheter (7 Fr). One lumen was used to inject cold water and the other to measure the temperature of the injected water at the larynx by a miniature thermocouple (MT-29/2; Physitemp, Clifton, NJ, USA). The cold distilled water was then placed on a bed of ice until just before the application and then injected. Statistical analysis was conducted using SPSS version 9.0J (SPSS Inc., Chicago, IL, USA). The comparisons among groups were done by one-way ANOVA with posthoc application of Fisher's least-significant-difference test. $P < 0.05$ was taken as significant.

Results

For the 14 patients who completed the study, the mean age was 88 ± 3 (SD) years (range 81–95). The mean LTSR induced by distilled water at room temperature was 13.8 s (95% CI 11.1, 16.5). The LTSR was significantly shortened in a concentration-dependent manner by the menthol (Figure 1). The mean LTSRs induced by 10^{-4} M, 10^{-3} M and 10^{-2} M menthol were 9.4 s (95% CI 7.1, 11.8), 6.1 s (95% CI 4.7, 7.6) and 3.9 s (95% CI 2.8, 5.0), respectively. The cold water also significantly shortened the LTSR and the effect was comparable to that of 10^{-4} M menthol. The mean LTSR induced by distilled water at room temperature was 8.1 s (95% CI 6.4, 9.7). The temperature of the injected cold water at the outlet of the catheter was 23.5 ± 1.2 °C (mean ± SD). There were no harmful effects or unpleasant feelings exhibited by patients during or after the study.

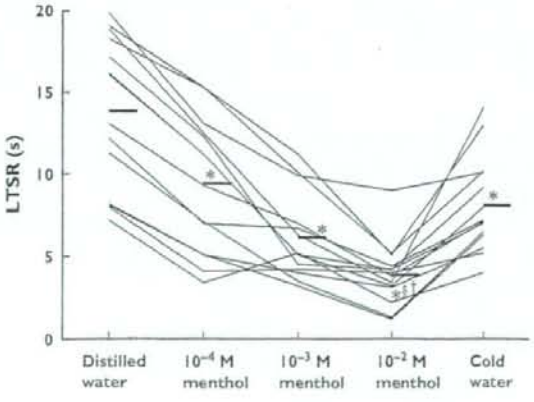

Figure 1

Latent time of swallowing reflex (LTSR) induced by distilled water at room temperature and ice cold water, and 10^{-4} M, 10^{-3} M and 10^{-2} M menthol in each subject ($N = 14$). *$P < 0.0005$ *vs.* distilled water at room temperature (distilled water); §$P = 0.0013$ *vs.* 10^{-2} menthol; †$P = 0.0001$ *vs.* distilled ice cold water.

Discussion

The study showed that menthol has an effect similar to that of cold temperature on triggering the swallowing reflex, suggesting the involvement of TRPM8 in the neural afferent of the swallowing reflex. The menthol dosage used was lower than that used to induce cold hyperalgesia by applying to the surface of the skin [7], but higher than that used to activate *in vitro* TRPM8 channel [4, 5]. Very recently, another cold receptor, TRPA1, was cloned and characterized [8]. TRPA1 is activated by noxious cold temperature (<18 °C) and by pungent compounds such as cinnamaldehyde and mustard oil, but not by menthol. Further studies are needed to elucidate the possible involvement of TRPA1 in the swallowing reflex.

Our results suggest that menthol stimulation as well as cold stimulation restores sensitivity to the triggering of the swallowing reflex in dysphagic patients. The addition of menthol to liquids or food may stimulate the swallowing reflex and help to prevent aspiration pneumonia in the elderly with dysphagia. The physiotherapy for dysphagia, known as thermal stimulation of reflex, requires considerable effort by physiotherapists or caregivers. Therefore, an alternative pharmacotherapy is needed. We have previously shown that the lozenge containing capsacin, an agonist of receptor for hot temperature (TRPV1), could improve the swallowing reflex in the dysphagic elderly [9]. Hence, letting a lozenge containing menthol dissolve in the mouth before meals in the dysphagic elderly may improve the sensitivity of the swallowing reflex. Since applying the lozenge is much easier than the procedure of thermal stimulation, evaluation of the efficacy of the menthol lozenge is warranted to prevent aspiration pneumonia in elderly dysphagic patients.

Another important defence reflex from aspiration pneumonia is cough reflex. Interestingly, menthol is known to lower cough reflex sensitivity [10]. The swallowing and cough reflexes are not always impaired simultaneously [11]. There are patients whose swallowing reflex is impaired but whose cough reflex is hypersensitive rather than impaired. Menthol may be a remedy for such patients.

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Dietary Intakes and Plasma 8-Iso-Prostaglandin $F_{2\alpha}$ Concentrations in Community-Dwelling Elderly Japanese: The Tsurugaya Project

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Abstract: We examined the association between dietary intakes and oxidative stress status in elderly Japanese. We analyzed cross-sectional data from a community-based Comprehensive Geriatric Assessment conducted in 2002. The subjects included 961 Japanese subjects aged 70 years or older who were non-daily antioxidant supplements users. We measured plasma total 8-iso-prostaglandin (PG)$F_{2\alpha}$ concentrations, a measurable lipid peroxidation biomarker, using a specific enzyme immunoassay kit. Dietary intakes were assessed through a food frequency questionnaire. Subjects were divided into three groups according to their dietary intake frequencies. Logistic regression was applied to calculate the odds ratios (ORs) for being in the highest tertile of plasma 8-iso-$PGF_{2\alpha}$ concentration.

Frequent intake of orange or other citrus fruits, or persimmon, strawberry, or kiwi fruit was associated with lower plasma 8-iso-$PGF_{2\alpha}$ concentrations, respectively. After adjustment for potential confounders, the ORs and 95% confidence intervals (CIs) for orange or other citrus fruits were 1.00 (reference), 0.66 (0.47, 0.92), and 0.58 (0.39, 0.87) (p for trend, 0.009). Intake of persimmon, strawberry, or kiwi fruit showed similar results. These associations were partly explained by vitamin C intake. Other dietary intakes had no association. Intake of fruits may have a beneficial effect against oxidative stress in elderly Japanese.

Key words: Diet, oxidative stress, 8-iso-prostaglandin $F_{2\alpha}$, elderly people, Japan

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Introduction

Oxidative stress is caused by an imbalance between free radical generation and antioxidant defenses. Oxidative stress has been implicated in the pathogenesis of many chronic disorders, such as atherosclerosis, cancer, neurodegeneration, and aging itself [1–4]. Therefore, obtaining information about the oxidative stress status of human populations is an essential task.

An analytic approach for quantifying the oxidative stress status *in vivo* is provided by measurements of a lipid peroxidation biomarker, 8-iso-prostaglandin (PG)$F_{2\alpha}$ in plasma and urine. 8-iso-$PGF_{2\alpha}$ is one of the four known classes of F_2-isoprostanes, which are formed nonenzymatically through free radical-catalyzed attack on esterified arachidonic acid [5, 6].

Determining the factors associated with oxidative stress is an important task. Dietary factors have attracted increasing interest due to their relatively potent radical-scavenging activities or potential contribution to oxidative damage. Consumption of antioxidant-rich foods may be beneficial by counteracting the formation of highly reactive oxygen species [7, 8]. In contrast, consumption of foods high in polyunsaturated fatty acids (PUFAs) could increase lipid peroxidation [9, 10]. Previous studies have addressed the association of oxidative damage and plasma antioxidant concentrations [11–13]. However, only two reasonably large-scale data are available from the US and Spain, on the distribution of oxidative damage due to dietary intakes using a reasonably reliable biomarker in general human populations [13, 14]. In addition, to our knowledge, no studies have examined the association among Asian populations, whose dietary habits and genetic background are substantially different from Western populations.

We therefore designed this cross-sectional analysis to investigate the association between dietary intakes and plasma lipid peroxidation using 8-iso-$PGF_{2\alpha}$ as a biomarker among community-dwelling elderly Japanese aged 70 years or older. To increase the likelihood of detecting an impact of dietary intakes on plasma oxidative status, studies focusing on elderly subjects are needed, because in younger subjects it has been suggested that the endogenous antioxidant defense system, including antioxidant enzymes, is adequate to minimize the levels of *in vivo* oxidant stress damage [15].

Subjects and Methods

Study population

The Tsurugaya Project was a community-based Comprehensive Geriatric Assessment (CGA) conducted among elderly Japanese subjects living in Tsurugaya district, a suburban area of Sendai City in northern Japan, between July and October 2002 [16, 17]. CGA is a structured approach to measuring the physical, mental, and social functioning of elderly people in order to promote healthy aging, and to assess early deterioration that may result in the need for long-term care [18, 19].

At the time of the study, there were 2730 people aged 70 years or older living in the Tsurugaya district. We sent invitation letters to all of these people asking them to participate in the health survey. Of those invited, 1198 participated in the survey and 1178 (43.2%) gave written informed consent to be included in the analysis. The study protocol was approved by the institutional review board of Tohoku University Graduate School of Medicine.

Among the 1178 subjects who gave written informed consent, plasma 8-iso-$PGF_{2\alpha}$ data were obtained from 1149 subjects. We excluded eight subjects from the analysis because they had provided insufficient information about their dietary intakes to calculate total energy intake. We excluded another 180 subjects who had taken daily antioxidant supplements. A qualified pharmacist inspected all medicines, including non-steroidal anti-inflammatory drugs (NSAIDs), and supplements that each study subject showed him, and which each subject reported taking daily, then classified them according to whether they contained vitamin A, vitamin C, or vitamin E. Data from 961 subjects contributed to the final analyses.

Assessment of dietary intakes

The dietary intakes were assessed by a food frequency questionnaire (FFQ), which was a short-version of a previously published FFQ [20]. In the present questionnaire, for each of 69 food items listed, participants were asked how often, on average, over the past month, they had consumed that food. Participants chose from among seven or nine possible frequencies, which ranged from "never" to "2 or more times/day" for almost all foods or "8 or more cups/day" for rice and miso-soup. Foods consumed in large quantities or high in nutrients of interest in our study objectives were selected, tested, and reported among the food items. We grouped the subjects into three categories according to their food consumption: < 1 time/week, 1–6 times/week, or ≥ 1 time/day for almost all foods; ≤ 1 cup/day, 2 cups/day, or ≥ 3 cups/day for rice and miso-soup.

We computed nutrient intakes by multiplying the consumption frequency of each unit of food by the nutrient amount of the specified portions, using composition values from Standard Tables of Food Composition published by the Science and Technology Agency of Japan [21]. In a validity study of the present FFQ (short-version), the FFQ was compared with three-day dietary records. Correlation coefficients of individual foods between the average intakes assessed from the dietary records and the FFQ were distributed from 0.24 for sugars to 0.66 for fruits. Corresponding correlation coefficients of individual nutrients were 0.56 for total energy, 0.53 for carbohydrate, 0.46 for protein, 0.43 for fat, 0.50 for vitamin A, 0.60 for vitamin C, and 0.56 for vitamin E, respectively.

Plasma 8-iso-prostaglandin F2α measurements

Total (esterified plus free) 8-iso-$PGF_{2\alpha}$ concentrations were assayed in plasma by a specific enzyme immunoassay (EIA) kit (Cayman Chemical: Ann Arbor, MI) [22–25].

For total 8-iso-$PGF_{2\alpha}$ measurement, peripheral venous blood samples were collected in Na_2EDTA (disodium ethylenediaminetetraacetic acid)- and Na_4EDTA-coated cold polyethylene tubes containing indomethacin, an inhibitor of cyclooxygenase, and aprotinin, an inhibitor of kallikreins, to prevent any *in vitro* formation of 8-iso-$PGF_{2\alpha}$. After collection, blood samples were immediately cooled at 4 °C and transferred to the laboratory within 4 hours. In the laboratory, the samples were centrifuged at 3000 × g at 4 °C for 10 minutes. The plasma fraction was removed and stored at –80 °C for later 8-isoprostane assay. The antiserum used in this assay has 100% cross-reactivity with 8-isoprostane, 0.2% with $PGF_{2\alpha}$, $PGF_{3\alpha}$, PGFI, and PGF_2 and 0.1% with 6-Keto $PGF_{2\alpha}1$. The intra-assay and interassay variabilities were within 6% for both. The detection limit of the assay was 4 pg/mL.

Other variables

The questionnaire in the CGA included: (1) demographic characteristics: sex, age; (2) physical health: (a) The physical functioning status assessed using the six-item physical functioning status measure of the Medical Outcomes Study (MOS) Short-form General Health Survey [26] those able to perform vigorous activity (MOS scores of 5 or 6), moderate activity but not vigorous activity (MOS scores of 2–4), and limited activity (MOS scores of 0 or 1)); (b) history of diagnosis of chronic disease; (3) lifestyle habits: smoking, alcohol drinking.

Body mass index (BMI; weight in kilograms divided by height in meters squared) was calculated from participants' height and weight measurements. Subjects were classified by World Health Organization (WHO) guidelines for Asians into three BMI categories: underweight (< 18.5 kg/m²), normal weight (18.5–22.9 kg/m²), overweight or obesity (≥ 23.0 kg/m²) [27].

Statistical analysis

Study participants were classified into tertiles of plasma 8-iso-$PGF_{2\alpha}$ concentrations. We used multivariate logistic regression analysis to calculate odds ratios (ORs) for being in the highest tertile of plasma 8-iso-$PGF_{2\alpha}$ concentrations relative to the food consumption frequencies, with the lowest frequency category treated as the reference group. Trend tests were performed by including the ordinal variable in a linear regression analysis. In these analyses, we regarded the following data as covariates: sex, age (continuous variable), BMI (< 18.5, 18.5 to 22.9, ≥ 23.0), MOS scores (0–1, 2–4, 5–6), history of diagnosis of chronic disease (presence of cancer, stroke, myocardial infarction, kidney disease, or liver disease, or absence of these diseases), use of NSAIDs (Yes, No), total energy intake (continuous variable), smoking (never, former, currently smoking < 20 cigarettes per day, currently smoking ≥ 20 cigarettes per day), alcohol drinking (never, former, current drinker).

All statistical analyses were performed using SAS software, version 9.1 [28]. We used approximate variance formulae to calculate the 95% confidence intervals (CIs). All the statistical tests that we reported were two-sided. A p value of < 0.05 was accepted as statistically significant.

Results

Table I shows the characteristics of the study subjects by tertiles of plasma 8-iso-$PGF_{2\alpha}$ concentrations. Subjects in the lowest tertiles of plasma 8-iso-$PG_{2\alpha}$ were more likely to be women, be underweight, have no history of diagnosis of chronic disease, have highest total energy intake, and have never smoked. Age, physical functioning status, use of NSAIDs, and alcohol drinking were not apparently different among plasma 8-iso-$PGF_{2\alpha}$ concentration categories.

The ORs for high oxidative stress risk according to different plant foods are shown in Table II. Frequent intake of orange or other citrus fruits, or persimmon, strawberry, or kiwi fruit was associated with lower plasma 8-iso-$PGF_{2\alpha}$ concentrations, respectively. The multivariate ORs (95% CIs) for orange or other citrus fruits were 1.00 (reference), 0.66 (0.47, 0.92), and 0.58 (0.39, 0.87) (p for trend, 0.009). Corresponding ORs (95% CIs) were 1.00 (reference), 0.70 (0.53, 0.94), and 0.57 (0.35, 0.98) (p for

Table I: Characteristics of the study subjects by tertiles of the plasma 8-iso-prostaglandin $F_{2\alpha}$ concentrations.

Plasma 8-iso-prostaglandin $F_{2\alpha}$ concentrations.	< 18 pg/mL	18–22 pg/mL	> 22 pg/mL	*P* value (ANOVA or χ^2 test)
Plasma 8-iso-prostaglandin $F_{2\alpha}$ concentrations, mean (SD)	14.3 (2.3)	19.9 (1.5)	26.0 (2.7)	< .0001
No. of subjects	332	296	333	–
Women (%)	59.3	59.1	52.6	0.14
Age (years), mean (SD)	75.0 (4.5)	75.0 (4.8)	75.1 (5.1)	0.96
Body mass index (kg/m^2) (%)*				
< 18.5	6.9	5.7	3.6	
18.5–22.9	32.2	31.1	37.8	
≥ 23.0	60.8	63.2	58.6	0.16
Physical functioning status (%)†				
Able to perform vigorous activity	61.6	61.8	65.2	
Capable of moderate, but not vigorous activity	27.1	27.4	23.7	
Low physical ability	11.3	10.8	11.1	0.83
History of diagnosis of chronic disease (%)‡				
Absence	71.4	70.6	65.8	
Presence	28.6	29.4	34.2	0.24
Use of non-steroidal anti-inflammatory drugs (%)				
No	91.2	87.8	91.6	
Yes	8.4	12.2	8.4	0.19
Total energy intake per day (kcal), mean (SD)	1602.3 (404.1)	1580.0 (426.9)	1590.4 (395.6)	0.79
Smoking (%)				
Never smoked	57.2	56.0	51.5	
Former smoker	29.5	30.2	32.8	
Currently smoking < 20 cigarettes per day	9.9	8.9	11.7	
Currently smoking ≥ 20 cigarettes per day	3.4	4.8	4.0	0.73
Alcohol drinking (%)				
Never drank	39.4	37.6	44.3	
Former drinker	14.5	13.5	13.6	
Current drinker	46.1	48.9	42.1	0.47

Because of rounding, not all percentages add to 100.
ANOVA: analysis of variance, SD: standard deviation.
* Body mass index (BMI; weight in kilograms divided by height in meters squared) was calculated from participants' measured weight and height.
† The physical functioning status was assessed using the six-item physical functioning status measure of the Medical Outcomes Study (MOS) Short-form General Health Survey.
‡ Chronic disease includes cancer, stroke, myocardial infarction, kidney disease, or liver disease.

trend, 0.007) for persimmon, strawberry, or kiwi fruit. In other plant foods, no apparent association was found. No significant association was found in animal food intakes (data not shown).

In the nutrient analysis, a significantly decreased risk was found for vitamin C (Table III). In the highest intake category of vitamin C, the risk of being in the highest tertile of plasma 8-iso-$PGF_{2\alpha}$ concentrations was reduced by 39%. No other nutrients showed an apparent association.

The associations of orange or other citrus fruits, or persimmon, strawberry, or kiwi fruit showed in Table II were attenuated after further adjustment for vitamin C intake. The vitamin C-adjusted multivariate ORs (95% CIs) for orange or other citrus fruits were 1.00 (reference), 0.71 (0.50–1.00), and 0.70 (0.44–1.12) (p for trend, 0.11). Corresponding ORs (95% CIs) were 1.00 (reference), 0.75 (0.56–1.01), and 0.66 (0.38–1.15) (p for trend, 0.04) for persimmon, strawberry, or kiwi fruit.

Discussion

Our study demonstrated a reduced risk of being in the highest tertile of plasma 8-iso-$PGF_{2\alpha}$ concentrations with the increased consumption frequency of fruits, even after

Table II: Odds ratios (ORs) and their 95% confidence intervals (CIs) of being in the highest tertile of plasma 8-iso-prostaglandin $F_{2\alpha}$ concentrations according to frequencies of intake of different plant foods.

	Frequency of intake	No. of cases / No. of subjects*	Crude OR (95% CI)	*P* for trend†	Adjusted OR (95% CI)‡	*P* for trend†
Orange or other citrus fruits	< 1 time/week	94/224	1.00		1.00	
	1–6 times/week	162/490	0.68 (0.49, 0.95)		0.66 (0.47, 0.92)	
	≥ 1 time/day	71/238	0.59 (0.40, 0.86)	0.007	0.58 (0.39, 0.87)	0.009
Persimmon, strawberry or kiwi fruit	< 1 time/week	154/390	1.00		1.00	
	1–6 times/week	147/462	0.72 (0.54, 0.95)		0.70 (0.53, 0.94)	
	≥ 1 time/day	24/93	0.53 (0.32, 0.89)	0.003	0.57 (0.35, 0.98)	0.007
Fresh salad (including lettuce, julienne strips of cabbage)	< 1 time/week	46/137	1.00		1.00	
	1–6 times/week	204/574	1.09 (0.74, 1.62)		1.07 (0.72, 1.60)	
	≥ 1 time/day	77/238	0.95 (0.61, 1.48)	0.68	0.98 (0.62, 1.55)	0.86
Green leafy vegetables	< 1 time/week	22/71	1.00		1.00	
	1–6 times/week	224/616	1.27 (0.75, 2.16)		1.27 (0.73, 2.20)	
	≥ 1 time/day	78/261	0.95 (0.54, 1.68)	0.27	0.98 (0.54, 1.79)	0.37
Cabbage or Chinese cabbage	< 1 time/week	31/78	1.00		1.00	
	1–6 times/week	241/702	0.79 (0.49, 1.28)		0.77 (0.47, 1.26)	
	≥ 1 time/day	55/173	0.71 (0.41, 1.23)	0.24	0.73 (0.41, 1.30)	0.37
Carrot or pumpkin	< 1 time/week	18/55	1.00		1.00	
	1–6 times/week	243/683	1.14 (0.63, 2.04)		1.08 (0.59, 1.97)	
	≥ 1 time/day	66/215	0.91 (0.48, 1.72)	0.35	0.95 (0.49, 1.84)	0.61
Tomato, tomato ketchup or stewed dish of tomato	< 1 time/week	20/50	1.00		1.00	
	1–6 times/week	146/400	0.86 (0.47, 1.57)		0.80 (0.43, 1.47)	
	≥ 1 time/day	160/499	0.71 (0.39, 1.29)	0.10	0.69 (0.37, 1.28)	0.18
Potato (including all kinds of potatoes)	< 1 time/week	28/71	1.00		1.00	
	1–6 times/week	216/615	0.83 (0.50, 1.38)		0.80 (0.47, 1.34)	
	≥ 1 time/day	83/266	0.70 (0.41, 1.20)	0.14	0.70 (0.40, 1.24)	0.23
Bread	< 1 time/week	58/161	1.00		1.00	
	1–6 times/week	162/465	0.95 (0.65, 1.38)		0.96 (0.66, 1.41)	
	≥ 1 time/day	107/327	0.86 (0.58, 1.28)	0.43	0.85 (0.56, 1.28)	0.38
Rice	≤ 1 cup/day	58/187	1.00		1.00	
	2 cups/day	163/444	1.29 (0.90, 1.86)		1.40 (0.96, 2.04)	
	≥ 3 cups/day	112/330	1.14 (0.78, 1.68)	0.67	1.23 (0.81, 1.86)	0.48
Miso soup	≤ 1 cup/day	157/447	1.00		1.00	
	2 cups/day	132/393	0.93 (0.70, 1.24)		0.96 (0.71, 1.29)	
	≥ 3 cups/day	43/120	1.03 (0.68, 1.57)	0.93	1.02 (0.66, 1.59)	0.97

* Because of missing data, not all subjects add to 961.

† Trend tests were performed by including the ordinal variable in a linear regression analysis.

‡ Adjusted for sex, age (continuous variable), body mass index (< 18.5, 18.5–22.9, ≥ 23.0), physical functioning status (MOS scores 0–1, 2–4, 5–6), history of diagnosis of chronic disease (presence of cancer, stroke, myocardial infarction, kidney disease, or liver disease, or absence), use of non-steroidal anti-inflammatory drugs (yes, no), total energy intake (continuous variable), smoking (never, former, currently smoking < 20 cigarettes per day, currently smoking ≥ 20 cigarettes per day), and alcohol drinking (never, former, current).

adjustment for a variety of possible confounders including sex, age, BMI, physical functioning status, history of diagnosis of chronic disease, use of NSAIDs, total energy intake, smoking, and alcohol drinking among community-dwelling elderly Japanese. The associations were partly explained by vitamin C intake. To our knowledge, this is the first study to examine the association between dietary intakes and oxidative stress status among Asian population with a reasonably large-scale sample size.

Several methodological limitations should be considered in the interpretation of our results. First, our study had a cross-sectional design, and therefore no temporal relationship between dietary intakes and oxidative stress status can be inferred. Second, we used a FFQ to estimate the dietary intakes, which probably made our estimation less precise than methods such as the dietary record method. However, we used sufficiently validated FFQ (0.66 for fruits and 0.60 for vitamin C), thus the degree of

Table 3: Odds ratios (ORs) and their 95% confidence intervals (CIs) of being in the highest tertile of plasma 8-iso-prostaglandin $F_{2\alpha}$ concentrations according to tertiles of intake of macronutrients and vitamins.

	Intake	Cases/ No. of subjects*	Crude OR (95% CI)	*P* for trend†	Adjusted OR (95% CI)‡	*P* for trend†
Carbohydrate (g/day)	< 184.9	109/320	1.00		1.00	
	184.9–228.6	110/320	1.01 (0.73, 1.41)		1.10 (0.76, 1.59)	
	> 228.6	114/321	1.07 (0.77, 1.48)	0.70	1.20 (0.74, 1.95)	0.45
Protein (g/day)	< 57.8	111/320	1.00		1.00	
	57.8–70.7	113/320	1.03 (0.74, 1.42)		1.07 (0.74, 1.54)	
	> 70.7	109/321	0.97 (0.70, 1.34)	0.85	1.09 (0.69, 1.73)	0.70
Fat (g/day)	< 38.7	116/320	1.00		1.00	
	38.7–51.0	108/320	0.90 (0.65, 1.24)		0.91 (0.63, 1.31)	
	> 51.0	109/321	0.90 (0.65, 1.25)	0.54	0.99 (0.63, 1.55)	0.96
Vitamin A (IU/day)	< 1900.6	115/319	1.00		1.00	
	1900.6–3058.9	108/319	0.91 (0.66, 1.26)		0.92 (0.67, 1.30)	
	> 3058.9	107/319	0.90 (0.65, 1.24)	0.51	0.91 (0.64, 1.29)	0.60
Vitamin C (mg/day)	< 84.3	131/319	1.00		1.00	
	84.3–120.4	104/319	0.69 (0.50, 0.96)		0.68 (0.49, 0.95)	
	> 120.4	95/319	0.61 (0.44, 0.85)	0.003	0.61 (0.43, 0.88)	0.007
Vitamin E (mg/day)	< 4.92	122/320	1.00		1.00	
	4.92–6.45	112/320	0.87 (0.63, 1.21)		0.81 (0.57, 1.15)	
	> 6.45	99/321	0.72 (0.52, 1.00)	0.05	0.70 (0.46, 1.05)	0.09

* Because of missing data, not all subjects add to 961.

† Trend tests were performed by including the ordinal variable in a linear regression analysis.

‡ Adjusted for sex, age (continuous variable), body mass index (< 18.5, 18.5–22.9, ≥ 23.0), physical functioning status (MOS scores 0–1, 2–4, 5–6), history of diagnosis of chronic disease (presence of cancer, stroke, myocardial infarction, kidney disease, or liver disease, or absence), use of non-steroidal anti-inflammatory drugs (yes, no), total energy intake (continuous variable), smoking (never, former, currently smoking < 20 cigarettes per day, currently smoking ≥ 20 cigarettes per day), and alcohol drinking (never, former, current).

misclassification may be small. Third, we stored blood samples for a few hours before the plasma fraction was removed and stored at –80 °C for later 8-isoprostane assay, which may have caused some auto-oxidation. However, to minimize *in vitro* formation of isoprostanes, care was taken with plasma sample preparation. The blood samples were collected in tubes containing indomethacin and aprotinin, which are inhibitor of isoprostanes-inducing enzyme cyclooxygenase or kallikreins, respectively. Finally, this study used EIA [29] rather than gas chromatography/mass spectrometry (GC/MS), the gold standard for isoprostane analysis, because large numbers of samples had to be processed in a timely manner. It might be worth noting that a less precise EIA [30] would result in a random misclassification of the levels and that would bias us toward the null hypothesis, that is, tend to weaken associations between variables and plasma 8-iso-$PGF_{2\alpha}$ concentrations. Because of this limitation, we cannot fully exclude any association between dietary intakes other than fruits and oxidative stress status.

The inverse association between fruits intake and plasma 8-iso-$PGF_{2\alpha}$ concentrations are in general agreement with a previous large-scale data study. Block and colleagues from the US demonstrated that the mean plasma F_2-isoprostanes concentrations were 0.055, 0.052, 0.049, and 0.045 ng/mL from lowest to highest quartile of fruits intake category (p for trend, 0.015) [13]. They found a significant association only in fruits intake among the foods. Another large study by Lasheras and colleagues from Spain indicated different results. The ORs for being in the highest tertile of plasma malondialdehyde (MDA) concentrations were 1.00 (reference), 1.54 (0.54–4.45), 1.62 (0.99–2.63), 1.06 (0.75–1.51), from lowest to highest quartile of vitamin C-rich fruits intake category [14]. They found an inverse association for cooked vegetables or wine, while finding a positive association for potatoes or egg intake. We cannot fully explain this discrepancy, but we believe that differences of lifestyle factors and/or genetic backgrounds among populations should be carefully considered in future studies [31].

In conclusion, our findings suggest that frequent intake of fruits may be associated with lower oxidative stress in elderly Japanese. This is partly explained by vitamin C intake.

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Granulocyte colony-stimulating factor promotes tumor angiogenesis via increasing circulating endothelial progenitor cells and Gr1+CD11b+ cells in cancer animal models

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Abstract

Recombinant granulocyte colony-stimulating factor (G-CSF) is used for cancer patients with myelosuppression induced by chemotherapy. G-CSF has been reported to progress tumor growth and angiogenesis, but the precise mechanism of tumor angiogenesis activated by G-CSF has not been fully clarified. N-terminal-mutated recombinant human G-CSF administration increased WBCs and neutrophils in peripheral blood and reduced bone marrow stromal cell-derived factor-1 in mice, indicating its biological relevance. Mice were inoculated with Lewis lung carcinoma cells (LLCs) or KLN205 cells and treated with G-CSF. G-CSF accelerated tumor growth and intratumoral vessel density, while it did not accelerate proliferation of LLCs, KLN205 cells or human umbilical vein endothelial cells *in vitro*. In the absence of tumors, G-CSF did not increase circulating cells that displayed phenotypic characteristics of endothelial progenitor cells (EPCs). In the presence of tumors, G-CSF increased circulating EPCs. In addition, G-CSF treatment increased immune suppressor and endothelial cell-differentiating Gr1+CD11b+ cells in tumor-bearing mice. We conclude that G-CSF promotes tumor growth by activating tumor angiogenesis via increasing circulating EPCs and Gr1+CD11b+ cells in cancer animal models.

Introduction

Neutropenia and the resultant infection are life-threatening side effects of cancer chemotherapy. The use of dose-intensive chemotherapeutic regimens has made the control of myelosuppression increasingly important. Granulocyte colony-stimulating factor (G-CSF) is commonly used to treat these patients to ameliorate neutropenia and prevent severe infections.

G-CSF can shorten the duration of chemotherapy-induced neutropenia, but recent reports show that it has no benefit in reducing the rate of hospitalization of febrile neutropenia (1, 2) or in prolonging survival (3), and question the preventive effects of G-CSF on neutropenia.

Although a few earlier reports show the inhibitory effect of G-CSF on solid tumor growth (4), many recent reports show their accelerating effects on tumor growth (5–7). Since blood supply is essential for solid tumors, growth is highly dependent on angiogenesis and the formation of new capillaries from pre-existing blood vessels. In the conventional view, angiogenesis is mediated by the local proliferation and migration of vessel wall-associated endothelial cells (ECs) that emerge from their resting state in response to angiogenic growth factor (8). However, recent works suggest that circulating cells with the potential to differentiate into mature ECs, the so-called circulating endothelial progenitor cells (EPCs), may also contribute to tumor angiogenesis (9, 10).

Granulocyte macrophage colony-stimulating factor and vascular endothelial growth factor (VEGF) have been reported to mobilize EPCs into peripheral circulation from the bone marrow and promote angiogenesis (11–14). Although it was reported that G-CSF administration promotes tumor growth

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and angiogenesis (7), the effect of G-CSF on EPCs in cancer animal models has not been investigated. On the other hand, a recent study demonstrated that Gr1+CD11b+ cells in spleens contribute to tumor angiogenesis by directly differentiating into ECs (15). But the relationship between Gr1+CD11b+ cells and G-CSF has not been investigated. In this study, we investigated the effect of G-CSF on tumor growth and angiogenesis. To clarify how G-CSF activates tumor angiogenesis, we investigated the effect of G-CSF on circulating EPCs and Gr1+CD11b+ cells. Our findings suggest that G-CSF promotes tumor angiogenesis by increasing circulating EPCs and Gr1+CD11b+ cells in tumor-bearing mice.

Methods

Cell culture

Lewis lung carcinoma cells (LLCs) and squamous carcinoma KLN205 cells were purchased from American Type Culture Collection (Manasass, VA, USA). LLCs were cultured in high-glucose DMEM containing 10% FCS and 100 μg ml^{-1} kanamycin. KLN205 cells were cultured in MEM containing 10% FCS, 1% non-essential amino acids and 100 μg ml^{-1} kanamycin. Human umbilical vein endothelial cells (HUVECs) were purchased from Kurabo (Osaka, Japan), and were cultured in HuMedia-MvG (Kurabo).

In vivo tumor models

LLCs were injected (3×10^5 cells per animal) subcutaneously into the flank of male 6- to 9-week old C57BL/6 mice on day 0. KLN205 cells were injected (5×10^5 cells per animal) subcutaneously into the flank of male 6- to 9-week old BDF1 mice on day 0. Tumor size was quantified daily as width^2 × length × 0.52 (16). For tumor growth rate models, N-terminal-mutated human recombinant G-CSF (nartograstim, Kyowa Hakko Kogyo, Tokyo, Japan; 8 μg kg^{-1} body weight for G-CSF low and 50 μg kg^{-1} body weight for G-CSF high) was injected into mice subcutaneously for 3 days daily from day 10, and every following week, G-CSF was injected daily for 3 days. Mice inoculated with LLCs were sacrificed on day 28 (total of nine G-CSF injections per mouse). Mice inoculated with KLN205 cells were sacrificed on day 36 (total of 12 G-CSF injections per mouse). For culture assay of EPCs, G-CSF was injected subcutaneously for 3 days daily from day 25, and the mice were sacrificed on day 28. For tumor growth inhibition models by SU1498 (Calbiochem, San Diego, CA, USA), mice were inoculated with LLCs on day 0. From day 9 and every following week, mice were injected intramuscularly with SU1498 three times a week. G-CSF (8 μg kg^{-1}) was injected into mice subcutaneously daily for 3 days from day 10, and every following week, G-CSF was injected daily for 3 days (total of nine G-CSF injections per mouse). Mice were treated with 400 μg of SU1498 dissolved in 100 μl dimethyl sulfoxide (DMSO) for each injection. Control mice were treated with DMSO. Mice were sacrificed on day 28. For an analysis of Gr1+CD11b+ cells in spleens, mice were inoculated with LLCs on day 0. From day 19, G-CSF (8 μg kg^{-1}) was injected into mice subcutaneously daily for 3 days and spleens were isolated on day 22. G-CSF dissolved in 100 μl PBS was used for each injection, and control mice were injected subcutaneously with PBS.

Cell growth assays

Cell proliferation assay was performed as previously described (17). Briefly, LLCs (5×10^3 cells), KLN205 cells (5×10^3 cells) or HUVECs (3×10^3 cells) were plated onto 96-well plates and incubated with 0, 0.1, 1 or 10 ng ml^{-1} of G-CSF. LLCs were cultured for 48 h. KLN205 cells and HUVECs were cultured for 72 h. Then the cell number was determined by water-soluble terazolium (WST) assay using a Cell Counting kit (Dojindo, Tokyo, Japan).

Culture assay of circulating EPCs

Mononuclear cells were isolated, cultured and characterized as previously described with some modifications (18). First, mononuclear cells were isolated from 700 μl of peripheral blood from each mouse using density gradient centrifugation with lymphosepar II (IBL, Fujioka, Japan). Following isolation, 4×10^6 cells were plated on dishes coated with human fibronectin (Becton Dickinson, Bedford, MA, USA) and maintained in HuMedia-MvG (Kurabo), supplemented with 20% FCS and 100 ng ml^{-1} recombinant mouse VEGF (R&D, Minneapolis, MN, USA). The medium was changed on day 4. On day 7, medium was changed and cells were washed with PBS. Adherent cells were incubated with 2.4 μg ml^{-1} acetylated low-density lipoprotein–DiI complex (DiI-acLDL, Molecular Probes, Eugene, OR, USA) for 1 h. Cells were fixed in 2% PFA for 10 min and stained with 10 μg ml^{-1} FITC-labeled lectin from *Ulex europaus* (Sigma) for 1 h. Fluorescent microscopy identified double-positive cells as EPCs. Two independent investigators evaluated the number of double-positive cells in each well by counting three randomly selected high-power fields.

Flow cytometry

FITC-labeled anti-CD34, purified rat anti-CD144 (VE-cadherin) and APC-labeled anti-Gr1 antibodies were purchased from BD PharMingen (San Diego, CA, USA), and control rat IgG2a and FITC-labeled anti-CD11b were purchased from eBioscience (San Diego, CA, USA). For staining of EPCs, mononuclear cells in peripheral blood were isolated and cultured for 7 days as shown in Culture Assay of Circulating EPCs. Then the cells were washed with PBS and adherent cells were scraped off with a cell scraper (Iwaki, Tokyo, Japan) and suspended in PBS. For staining of splenocytes, single-cell suspensions were made from spleens. The cells were first incubated with unlabeled anti-CD16/32 mAb (eBioscience) to block non-specific binding to the FcγR. After washing, the cells were incubated on ice with FITC-, APC- or non-labeled mAbs. The cells incubated with anti-CD144 were then incubated with FITC-labeled anti-rat IgG (H + A) (eBioscience). After washing again, the cells were subjected to flow cytometry on a FACScan (BD Bioscience, San Jose, CA, USA) and the data were analyzed with CellQuest software (BD Bioscience). For all samples, dead cells were excluded from the analysis by propidium iodide staining.

Immunohistochemistry

When the diameter of the tumor became ~1 cm, tumor tissues were fixed in 10% formalin, embedded in paraffin and sectioned. Control mice were sacrificed on day 21, and the

G-CSF (8 μg kg^{-1})-treated mice were sacrificed on day 19 (total of six G-CSF injections). They were blocked with 10% normal goat serum and incubated with polyclonal anti-human factor VIII-related antigen antibody (DAKO, Carpinteria, CA, USA). Subsequently, the sections were incubated with biotinylated goat anti-rabbit IgG (Vector, Burlingame, CA, USA), and then with ABC kit (Vector), and were detected by 3-amino-9-ethylcarbazole (Vector) and counterstained with hematoxylin.

Determination of microvessel density

The intratumoral microvessel density (MVD) was determined as previously described (19, 20). In brief, the intratumoral vessels were stained immunohistochemically with anti-human factor VIII-related antigen antibody. The image that contained the highest number of microvessels was chosen for each section by an initial scan at 100× magnification. Then the vessels were counted in the selected image at 200× magnification.

Peripheral blood analysis

G-CSF (8 μg kg^{-1}) was injected into mice subcutaneously daily for 3 days. Eight hours after the last injection, 700 μl of peripheral blood was collected from each mouse. Total WBC numbers and differential leukocyte counts were obtained using hematology analyzer LH750 (Beckman Coulter, Fullerton, CA, USA).

Cytokine ELISA

The concentrations of stromal cell-derived factor-1 (SDF-1) in serum and bone marrow were determined using a murine SDF-1 ELISA kit (R&D) according to the manufacturer's recommendation. G-CSF (8 μg kg^{-1}) was injected into mice subcutaneously for 1 or 3 days daily. Then the inferior vena cava of the mouse was punctured, peripheral blood was collected and the serum was isolated. Bone marrow was obtained by a single flush of a right femur with 500 μl of PBS. After centrifugation, the supernatant was subjected for ELISA. The concentration of VEGF in serum was determined using a murine VEGF ELISA kit (R&D) according to the manufacturer's recommendation. G-CSF (8 μg kg^{-1}) was injected into mice subcutaneously for 3 days daily. PBS was injected into control mice. Then the serum was isolated and subjected for ELISA. For tumor models, LLCs were injected into mice on day 0. From day 25, G-CSF (8 μg kg^{-1}) was injected into mice for 3 days daily. PBS was injected into control mice. On day 28, serum was isolated and subjected for ELISA.

Data analysis

Statistical analysis of the results was performed using analysis of variance with Fisher's least significant difference test for multiple comparisons. Values of $P < 0.05$ were considered to be significant.

Results

G-CSF treatment increased WBCs and neutrophils in peripheral blood and reduced the bone marrow SDF-1 level

We treated mice with N-terminal-mutated recombinant human G-CSF, nartograstim (8 μg kg^{-1}), and examined the number of total WBCs and neutrophils in peripheral blood to confirm its biological effects. Three days of G-CSF treatment significantly increased both WBCs and neutrophils in peripheral blood (Fig. 1A). G-CSF has been reported to reduce SDF-1 level in bone marrow without changing the serum SDF-1 level, and mobilizes leucocytes from bone marrow to peripheral circulation (21). We examined the SDF-1 level in serum and bone marrow after 1 or 3 days of G-CSF treatment. G-CSF treatment did not change the serum SDF-1 level (Fig. 1B), but

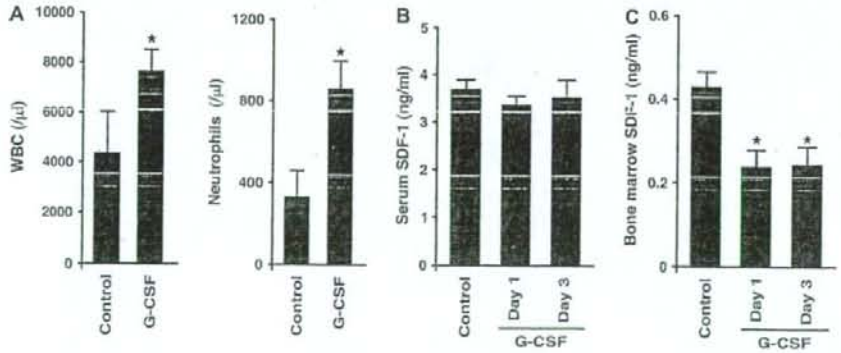

Fig. 1. G-CSF treatment increased WBCs and neutrophils in peripheral blood and decreased the bone marrow SDF-1 level. (A) Effect of G-CSF treatment on peripheral blood cell counts. G-CSF (8 μg kg^{-1}) was injected into mice daily for 3 days. Eight hours after the last injection, peripheral blood was collected. The numbers of WBCs and neutrophils were determined using a hematology analyzer. G-CSF treatment significantly increased WBCs and neutrophils (*$P < 0.05$). Results are indicated as mean ± SD of eight mice in each group. (B) Mice were treated with G-CSF for 1 or 3 days, and the serum SDF-1 level was determined by ELISA. Results are indicated as mean ± SD of eight mice in each group. (C) Mice were treated with G-CSF for 1 or 3 days. Bone marrow was obtained by flushing a right femur bone with 500 μl of PBS. After centrifugation, the supernatant was subjected for ELISA. G-CSF treatment significantly reduced the bone marrow SDF-1 level (*$P < 0.01$). Results are indicated as mean ± SD of eight mice in each group.

significantly reduced the bone marrow SDF-1 level (Fig. 1C) as previously shown (21). These results indicated that G-CSF at this dose would be sufficient to exert its biological effects.

G-CSF treatment accelerated the tumor growth rate in vivo, but not in vitro

LLCs were inoculated into the flank of C57BL/6J mice subcutaneously on day 0. From day 10, we injected G-CSF or PBS into the mice daily for 3 days, and every following week, we injected G-CSF or PBS daily for 3 days. As shown in Fig. 1, 8 μg kg^{-1} G-CSF (indicated as G-CSF low) had a biological effect, but this dose was relatively low. Therefore, we treated mice with 50 μg kg^{-1} G-CSF as a positive control (indicated as G-CSF high). Compared with PBS treatment, G-CSF treatment accelerated the tumor growth statistically significantly (Fig. 2A). There was no significant difference in tumor growth between high- and low-dose G-CSF treatment (Fig. 2A). Similar results were obtained from mice inoculated with KLN205 cells (Fig. 2B). Thus, we performed the following experiments using the low-dose G-CSF (8 μg kg^{-1}). We next examined the direct effect of G-CSF on the proliferation of LLCs and KLN205 cells *in vitro*. We cultured LLCs and KLN205 cells in various concentrations of G-CSF. G-CSF had no effect on cell proliferation *in vitro* (Fig. 2C). We also cultured LLCs and KLN205 cells with 0, 0.1, 1 or 10 ng ml^{-1} of G-CSF and counted the cells on days 3, 4, 5 and 6. We could not find an effect of G-CSF at any concentration on the cell proliferation rate either (data not shown).

G-CSF treatment induced the increase of vessel density in tumor tissues, but did not affect the proliferation of HUVECs

Hematoxylin and eosin staining of the tumor tissues revealed hyper-neovascularization in tumors from G-CSF-treated mice (Fig. 3A and C; total of six G-CSF injections). To confirm the vessels, we performed immunohistochemistry staining using an antibody against factor VIII-related antigen (Fig. 3B and D). Factor VIII-related antigen is a well-established cell-surface marker of vascular ECs (20). Compared with control mice, we found an increase of tumor vessel density in G-CSF-treated mice. The difference in MVD between control and G-CSF-treated mice was statistically significant (Fig. 3E). To investigate whether G-CSF induced the proliferation, differentiation and development of sprouts from pre-existing ECs, we examined the direct growth effect of G-CSF on HUVECs. We cultured HUVECs with various concentrations of G-CSF. G-CSF had no effect on the proliferation of HUVECs *in vitro* (Fig. 3F). We also cultured HUVECs with 1 or 10 ng ml^{-1} of G-CSF and counted the cells on days 2, 3, 4 and 5. We could not find an effect of G-CSF on the proliferation rate or morphological changes (data not shown).

G-CSF treatment to cancer animal models increased the circulating EPCs

As G-CSF did not have a direct growth effect on ECs, we hypothesized the enhanced neovascularization as the result of an increase of circulating EPCs. We cultured mononuclear cells and characterized EPCs as adherent cells double positive for DiI-acLDL uptake and lectin binding as previously described (Fig. 4A) (13, 14, 22). We further confirmed the expression of well-established murine endothelial-specific markers CD34 and VE-cadherin on adherent cells by FACS analysis (Fig. 4B) (22, 23). To investigate the effects of G-CSF on the increase of DiI-acLDL uptake and lectin-binding cells in peripheral blood, mice were injected with G-CSF for 3 days, and mononuclear cells in peripheral blood which displayed phenotypic characteristics of EPCs were counted. G-CSF treatment itself did not significantly increase the circulating EPCs (Fig. 4C). To further investigate the effect of G-CSF on the increase of circulating EPCs in cancer animal models, we inoculated LLCs into mice at day 0. At 25 days after inoculation, we treated them with G-CSF for 3 days. This treatment

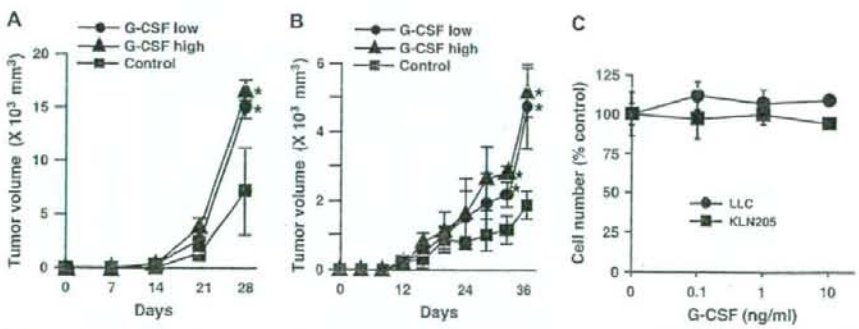

Fig. 2. G-CSF treatment accelerated the tumor growth *in vivo*, but not *in vitro*. G-CSF low indicates 8 μg kg^{-1} G-CSF treatment and G-CSF high indicates 50 μg kg^{-1} G-CSF treatment. (A) Mice were injected subcutaneously with LLCs on day 0 and treated with PBS or G-CSF. Results are indicated as mean ± SD of eight mice in each group, and the difference in tumor volume on day 28 between control and G-CSF-treated mice was statistically significant (*$P < 0.01$). (B) Mice were injected subcutaneously with KLN205 cells on day 0 and treated with PBS or G-CSF. Results are indicated as mean ± SD of eight mice in each group, and the difference in tumor volume on days 32 and 36 between control and G-CSF-treated mice was statistically significant (*$P < 0.01$). (C) LLCs and KLN205 cells (5×10^3 cells) were cultured with the indicated amounts of G-CSF. LLCs were cultured for 48 h, and KLN205 cells were cultured for 72 h. Cell number was determined by WST assay. Data are shown as mean ± SD of triplicate samples. Similar results were obtained from three independent experiments.

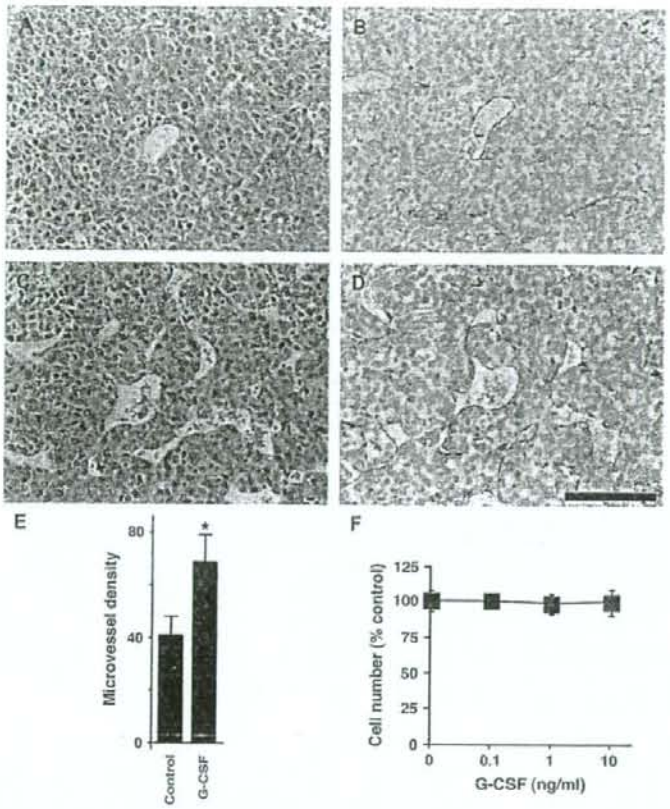

Fig. 3. G-CSF treatment induced an increase of vessel density in tumor tissues, but did not progress the proliferation of HUVECs. Mice were injected subcutaneously with LLCs on day 0. When the diameter of the tumors reached ~1 cm, mice were sacrificed. Control mice were sacrificed on day 21, and the G-CSF (8 μg kg^{-1})-treated mice were sacrificed on day 19 (total of six G-CSF injections). Tumors were resected and embedded in paraffin. Tumor tissues from control (A and B) and G-CSF-treated (C and D) mice are shown. (A and C) Paraffin sections were stained with hematoxylin and eosin. (B and D) Paraffin sections were stained immunohistochemically using an antibody against factor VIII-related antigen; scale bar, 100 μm. Pictures represent one of eight mice in each group. (E) MVD was determined. Results are indicated as mean ± SD of eight mice in each group. The difference in MVD between control and G-CSF-treated mice was statistically significant (*$P < 0.01$). (F) HUVECs (3×10^3 cells) were cultured with the indicated amounts of G-CSF for 72 h. Cell number was determined by WST assay. Data are shown as mean ± SD of triplicate samples. Similar results were obtained from three independent experiments.

significantly increased the number of circulating EPCs in G-CSF-treated mice (Fig. 4D). Similar results were obtained from mice inoculated with KLN205 cells (data not shown).

LLCs inoculation elevated the serum VEGF level, and VEGFR-2 kinase inhibitor SU1498 inhibited the tumor growth

VEGF has been shown to mobilize bone marrow-derived EPCs (12, 14). We examined the serum level of VEGF in mice after G-CSF treatment for 3 days. VEGF was not detectable in the serum from the control or G-CSF-treated mice (Fig. 5A). We next inoculated LLCs into mice and examined the serum VEGF level. Inoculation of the LLCs induced the elevation of the serum VEGF level in control and G-CSF-treated mice (Fig. 5A). VEGF has been reported to contribute to angiogenesis through activation of VEGFR-2 (VEGFR-2/KDR/Flk-1) and SU1498 is a potent and selective inhibitor of the VEGFR-2 tyrosine kinase (24). To investigate the possible role of VEGF on LLC tumor growth, we treated mice with SU1498 and G-CSF. SU1498 inhibited the tumor growth in mice both with and without G-CSF injection. However, tumor growth in mice treated with G-CSF had the SU1498-insensitive part (Fig. 5B). These results suggested that VEGF might not be involved in the differential tumor growth between G-CSF-treated and non-treated mice.