Abstract

Ameliorating postural instability is an important component of geriatric health care. The effect of olfactory stimuli (lavender and black pepper oils) on postural control in 17 older adults (78±6 years old) who had no apparent neurological deficits was studied. Measurements of center of pressure (CoP) trajectories were done with subjects standing quietly on a force plate. Control measurements were compared with olfactory interventions: brief exposure to sham (distilled water), lavender oil, and black pepper oil; experiments were repeated with eyes open and eyes closed. From the CoP data, the root mean square (RMS) displacement and velocity in mediolateral (ML) and antero-posterior (AP) directions, and the total trajectory length were computed. This study found that with eyes closed, olfactory stimulation with either lavender or black pepper oil significantly decreased both ML and AP RMS velocities and trajectory lengths compared with baseline. In contrast, little effect was observed under the eyes-open condition. Decreases in RMS displacements were small and mostly insignificant. The study suggests that olfactory stimulation may improve posture stability in older adults through decreasing the velocities of postural adjustments during normal sway.

1. Introduction

Increased postural instability in older adults is well established,[1,2] and increased postural sway has been associated with the incidence of falls in older adults.[3,4] Many factors contribute to loss in balance control, including diminished vision, muscle weakness, vestibular disorders, bone integrity, spinal injury, and somatosensory deficit.[5]

It has traditionally been thought that balance control occurs at an autonomic level, involving a complex interplay between vision and noncortical polysynaptic brainstem pathways associated with the vestibular apparatus and proprioception.[7,8] However, recent research has suggested that the cerebral cortex may be involved in controlling specific aspects of balance.[9] Odor is one of the strongest stimuli over a wide range of the cerebral cortex.[10] Moreover, there are brain diseases, such as Alzheimer's, schizophrenia, and certain types of stroke, which manifest both olfactory dysfunction and balance instability,[11-13] suggesting that both neuronal deficits may linked. However, data are lacking regarding the relationship between olfactory stimulation and postural stability. In order to investigate this, we gave odor stimuli using black pepper and lavender oils to subjects, and measured several indices of postural stability.

2. Methods

Subjects. We used posters to recruit community-dwelling older adult participants from communities surrounding Sendai, Japan. Seventeen subjects, age 78±6 years, male/female ratio of 13/4, completed the study. The experimental protocol (2007-192) was approved by the institutional ethics committee, and verbal informed consent was obtained from each subject. Criteria for participation included being medically stable, adequately comprehending instructions and the nature of the study, and being able to stand up and walk independently without a cane or assistance device.

Functional independence was assessed with the Barthel Index (subject range 80-95). Exclusion criteria included evidence of arthritis in the lower limbs, chronic back, knee or hip joint pain, evidence of Parkinson's disease, Meniere syndrome, cerebellar signs, cognitive deficits (Mini-Mental State Examination: MMSE<24), or peripheral neuropathy under standard neuropsychological assessment.

Protocol. Black pepper and lavender oils (#T03218 and #060706) were purchased from Yamamoto Perfumery Co., Osaka, Japan. Trials were conducted between 10:00am and 11:00am on separate days (minimum 2 day separation) to minimize a placebo or learning effect, as well as to insure stimulus clearance and a return to olfactory baseline. The order of the two oil and one sham trial exposures were randomly selected for each subject.

Subjects were instructed to stand with their feet slightly apart on a force plate. For each stimulus, the protocol was: 1 min. eyes-open control trial, 2 min. break, 1 min. eyes-closed control trial, 4 min. break, 1 min. eyes-open stimulus trial, 2 min. break, 1 min. eyes-closed stimulus trial. For the stimuli, an investigator held a paper stick, previously dipped in one of the oils or in distilled water, within a few centimeters of, but not touching, the right side of the subject's nose. The stick was re-dipped prior to each trial or corresponding break and held continuously from the time of exposure to the end of the eyes-closed stimulus trial. During eyes-open trials, subjects were instructed to look straight ahead. During the breaks, which were given to avoid fatigue, subjects were instructed to sit and relax. These were not blind studies, as the odor of the oils (and its absence in the shams) was more than sufficient for detection by all subjects.

Measurements. Movement of the body center of pressure (CoP) was measured with a force plate (Gravicoda GS-2000, Anima, Tokyo). Signals from its three force transducers were sampled at 100Hz, obtaining individual 4096 long data strings, over

41 sec periods during each trial. The data were filtered and compressed with a 9-point Gaussian filter using central binomial coefficient weighting. For each set of data, comprising 6 combinations of independent variables (3 interventions and 2 visual conditions), the mediolateral (ML) and antero-posterior (AP) components of the CoP were computed. We derived five indices from these data: the total trajectory length; the root mean square displacements (RMSdisp) of the CoP (ML and AP), and the RMS velocities (RMSvel) of the CoP (ML and AP).

Variations among the control measurements by interventions were assessed by ANOVA with the Tukey post-hoc test. Tests of the null hypothesis of no change relative to baseline were assessed with a paired two-tailed Student t-test. Significance was taken at $p=0.05$.

3. Results

Table 1 shows the population means±SD of the raw dimensional quantities measured. ANOVA revealed no significant differences in the control measurements with the different interventions, so these data were pooled.

Among the 30 combinations of measurements, the most consistent results were in the eyes-closed group, where we found, for both lavender and the black pepper stimuli, significant fractional decreases in RMSvel (ML and AP), and in total trajectory length. Figure 1 shows these results. With three exceptions, no other condition rose to statistical significance. These were: %change in RMSdisp (eyes-closed, AP, lavender); RMSdisp (eyes-open, AP, lavender); and RMSvel (eyes-open, AP, sham). However, as these follow no consistent pattern, we speculate that they may be secondary to the small sample size in this study, and are of little physiological significance. Surprisingly, and contrary to our initial expectations, we found no consistent pattern of significant decrease in the RMS displacement of the CoP.

4. Discussion

This is the first study to investigate the relationship between olfactory stimulation and postural stability in older adults. In the current study, both black pepper and lavender improved the balance parameters such as RMSvel and trajectory length, suggesting that the posture stabilizing effect of olfactory stimulation is not odor specific. This is in contrast to the observation that black pepper odor improved the swallowing reflex in dysphagic older adults whereas lavender oil did not.[14]

It has been reported that the poor balance with closed eye was significantly associated with an increased risk of falls[1,15], suggesting that the improvement of the balance with olfactory stimulation that we observed in similar conditions may be extrapolated to improving risk of falling in older people. This is in addition to the observation that aromatherapy with lavender has successful application in the treatment of behavioural and psychological symptoms of dementia.[16]

The olfactory pathways project from the olfactory bulb widely throughout the cerebral cortex,[17,18] although these studies were done in macaque monkeys. While there are important species differences, the fact that these are primates suggests an analogous projection may exist in humans. Moreover, it has recently been noted that the cerebral cortex is involved in upright posture maintenance in humans, especially in balance compensation mechanisms.[5] For instance, the insular cortex is importantly involved in the processing and integration of sensory information for balance,[5] and is also activated by olfactory stimuli.[14] This suggests a mechanism by which odor stimulation may enhance cortical control of balance in older adults. This preliminary work thus suggests that odor stimulation may help ameliorate impairment of balance control in older adults, but further studies are necessary to apply these findings in practice.

Acknowledgments

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Conflict of interest statement.

We declare that there are no financial or other conflicts of interest in relation to this work.

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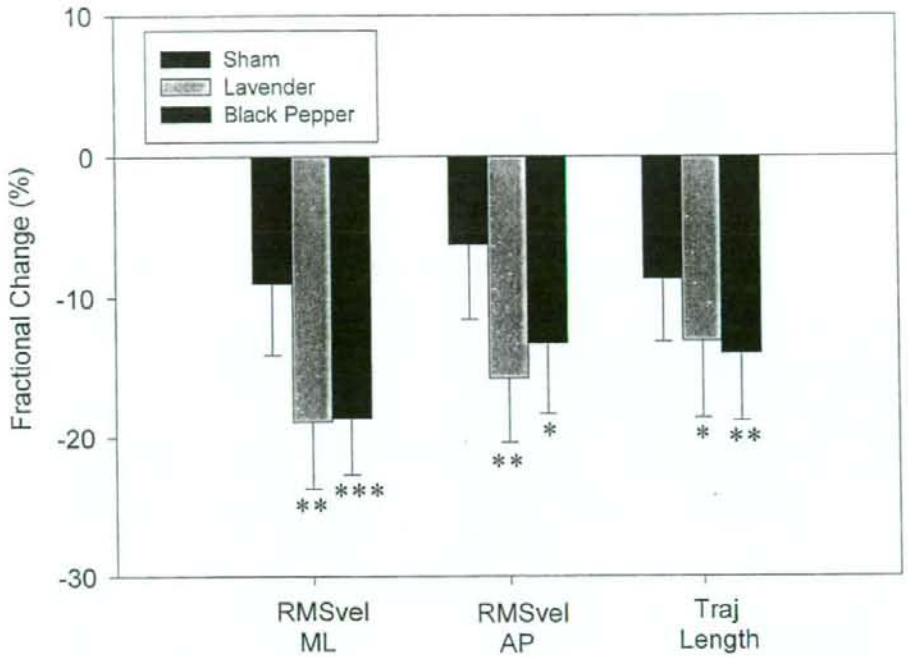
Table 1. Pooled control values for postural stability indices based on center of pressure (CoP) measurements. RMSdisp and RMSvel: respectively root mean square displacement and velocity of CoP along the indicated axis. Traj Length: total trajectory length of the CoP over the trial. ML: mediolateral, AP: antero-posterior. Data shown as means±SD, N=17.

Postural stability index	Directional axis	Visual condition: eyes-open	Visual condition: eyes-closed
RMSdisp (cm)	ML	0.51 ±0.17	0.73 ±0.25
	AP	0.53 ±0.19	0.71 ±0.21
RMSvel (cm/sec)	ML	1.54 ±0.62	3.02 ±1.40
	AP	1.40 ±0.61	2.56 ±1.13
Traj Length (cm)	N/A	85. ±38.	156. ±69.

Figure caption

Figure 1. Effects of odor stimulation on three posture stability indices under eyes-closed conditions. (With eyes open, neither olfactory stimulus showed any significant changes; data not shown). Fractional changes are shown for RMS velocity in the mediolateral (ML) and antero-posterior (AP) directions, and for total trajectory length over the trial (Traj Length). Data shown as means ±SE. Significance levels: *: $p<0.05$; **: $p<0.01$; ***: $p<0.001$. The sham exposures did not reach statistical significance.


10
0
-10
-20
-30
Fractional Change (%)
Sham
Lavender
Black Pepper
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RMSvel ML
RMSvel AP
Traj Length

Figure 1.

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Impaired urge-to-cough in elderly patients with aspiration pneumonia

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Abstract

Background: The down-regulation of the cough reflex in patients with aspiration pneumonia can involve both cortical facilitatory pathways for cough and medullary reflex pathways. In order to study the possible involvement of the supramedullary system in the down-regulation of cough reflex, we evaluated the urge-to-cough in patients with aspiration pneumonia.

Methods: Cough reflex sensitivity and the urge-to-cough to inhaled citric acid were evaluated in patients with at least a history of aspiration pneumonia and age-matched healthy elderly people. The cough reflex sensitivities were defined as the lowest concentration of citric acid that elicited two or more coughs (C_2) and five or more coughs (C_5). The urge-to-cough scores at the concentration of C_2 and C_5, and at the concentration of two times dilution of C_2 ($C_2/2$) and C_5 ($C_5/2$) were estimated for each subject.

Results: Both C_2 and C_5 in the control subjects were significantly greater than those for patients with aspiration pneumonia. There were no significant differences in the urge-to-cough at C_2 and C_5 between control subjects and patients with aspiration pneumonia. However, the urge-to-cough scores at both $C_2/2$ and $C_5/2$ in patients with aspiration pneumonia were significantly lower than those in control subjects. The number of coughs at $C_5/2$ was significantly greater in the control subjects than those in the patients with aspiration pneumonia whereas the number of coughs at $C_2/2$ did not show a significant difference between the control subjects and the patients with aspiration pneumonia.

Conclusion: The study suggests the involvement of supramedullary dysfunction in the etiology of aspiration pneumonia in the elderly. Therefore, restoration of the cough motivation system could be a new strategy to prevent aspiration pneumonia in the elderly.

Background

Morbidity and mortality from aspiration pneumonia continues to be a major health problem in the elderly. A marked depression of cough reflex sensitivity is reported in elderly patients with aspiration pneumonia who show cerebral atrophy and lacunar infarction in the brain [1]. The risk of aspiration pneumonia in post-stroke patients is known to intimately correlate with the inhibition of the cough reflex [2,3].

Cough is usually referred to as a reflex defense mechanism mediated at the brainstem level, where sensory information arising from airway sensory receptors in response to an appropriate stimulus is processed by the medullary respiratory network to produce the motor pattern of cough. However, there is accumulating evidence indicating that human cough is under voluntary control and that higher centers such as the cerebral cortex or subcortical regions have an important role in both initiating and inhibiting reflexive cough [4,5]. Although the cough reflex is certainly subjected to influence originating from cortical or subcortical brain regions [6], understanding of the nature and function of such influences is still limited.

Cough is typically preceded by an awareness of an irritating stimulus and is perceived as a need to cough, termed the urge-to-cough [7]. In a capsaicin cough challenge test, the urge-to-cough occurred at a lower capsaicin concentration than that eliciting a motor cough, suggesting that the cough cognitive sensory process precedes the cough motor event [8]. A recent functional magnetic resonance imaging study revealed that the urge-to-cough was associated with activations in a variety of brain regions, including the insula cortex, anterior midcingulate cortex, primary sensory cortex, orbitofrontal cortex, supplementary motor area, and cerebellum [9]. The down-regulation of cough reflex in patients with aspiration pneumonia could be mediated by both cortical facilitatory pathways for cough and medullary reflex pathways [4]. However, there have been no studies investigating the cortical involvement of the down-regulation of cough reflex in patients with aspiration pneumonia. In order to study the possible involvement of the supramedullary system in the down-regulation of the cough reflex, we evaluated the urge-to-cough in patients with aspiration pneumonia.

Methods

Subjects

Cough reflex sensitivity and the urge-to-cough to inhaled citric acid were evaluated in patients with at least one history of aspiration pneumonia and age-matched healthy elderly people.

Patients were prospectively and consecutively recruited from those referred and admitted to the Geriatric Unit, Tohoku University Hospital for treatment of pneumonia from May 2007 to April 2008. Pneumonia was diagnosed by the presence of pulmonary infiltration on chest radiograph and computed tomography (CT) and according to systemic inflammation as determined according to white blood cell (WBC) count and C-reactive protein (CRP). The criteria for pneumonia were established according to the pneumonia guidelines of the Japan Respiratory Society [10]. In the current study, aspiration was defined according to the Japanese Study Group on Aspiration Pulmonary Disease as pneumonia in a patient with predisposition to aspiration because of dysphagia or swallowing disorders [11]. In our unit, all the elderly patients (> 75 years old) with pneumonia had fasted at the time of admission. When they recovered after treatment such as antibiotics drip infusion, we considered letting them start eating with their alert consciousness. We estimated their swallowing reflex before making the decision to start eating. 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 [12]. If the latency of swallowing reflex was > 5 seconds, we regarded the patients as suffering from impaired swallowing function, e.g. aspiration pneumonia.

During the entry period, 41 patients with pneumonia without an apparent past- and present-history of stroke were admitted to our 20 bed geriatric unit, and 34 patients (83%) were diagnosed as aspiration pneumonia. We performed simple chest X-ray in all of them. Among 34 patients, we performed chest CT scan in 30 patients. All 34 patients showed characteristic images of infiltrates compatible with aspiration pneumonia in the posterior segment of any of the lobes and/or lower lobe by simple chest X-ray and/or CT scan. Of 34 patients, 2 patients died and 3 patients eternally tracheostomized. Of 29 recovered patients, due to the difficulty of urge-to-cough estimation, we excluded patients with dementia using the mini-Mental State Examination (MMSE). Of 29 patients who recovered from aspiration pneumonia, 18 subjects with a MMSE score < 24 were excluded. Three patients with apparent paralysis were excluded. Finally, 8 patients (3 men) with aspiration pneumonia (70–88 years old) were enrolled for this study. From 6 patients among 8, we obtained brain images with non-contrast CT scan. The CT scan revealed that 2 patients had infarct in the deep region of middle cerebral artery territory, 2 patients in the superficial region (cortical or adjacent subcortical infarcts) of middle cerebral artery territory, and 1 patient in both the

deep and superficial region of middle cerebral artery territory. One patient had infarct in the superficial region of the posterior cerebral artery territory. The diameters of all infarcts were within 1 cm.

Eleven age and sex-matched healthy elderly people (72–84 years old) as control subjects were recruited from the community by advertisement. None of the subjects were demented (MMSE scores > 23). All control subjects were never-smokers, and had no previous history of pneumonia and other respiratory diseases. None of the patients or controls were taking medication which might affect cough sensitivity such as antitussives, narcotics, or ACE inhibitors. A CT scan was obtained from only one control subject.

Cough reflex sensitivity and urge-to-cough

Cough reflex and urge-to-cough was examined more than 3 months after negative conversion of C reactive protein after pneumonia had responded to antibiotics treatment (median 24 days, range 13–30). At the time of evaluation, the subjects were in a stable state until at least 3 months before. Simple standard instructions were given to each subject.

We evaluated the cough reflex sensitivities using citric acid because we had previously used this method to observe depressed cough in the elderly [1,3]. Cough reflex sensitivity to citric acid was evaluated with a tidal breathing nebulized solution delivered by an ultrasonic nebulizer (MU-32, Sharp Co. Ltd., Osaka, Japan) [5]. The nebulizer generated particles with a mean mass median diameter of 5.4 μm at an output of 2.2 ml/min. Citric acid was dissolved in saline, providing a two-fold incremental concentration from 0.7 to 360 mg/ml. Based on "cough sound", the number of cough was counted both audibly and visually by laboratory technicians who were unaware of the clinical details of the patients and the study purpose. Each subject inhaled a control solution of physiological saline followed by a progressively increasing concentration of citric acid. Increasing concentrations were inhaled until five or more coughs were elicited, and each nebulizer application was separated by a 2-min interval. The cough reflex sensitivities were estimated by both the lowest concentration of citric acid that elicited two or more coughs (C_2) and the lowest concentration of citric acid that elicited five or more coughs (C_5).

Immediately after the completion of each nebulizer application, the subject made an estimate of the urge-to-cough. The modified Borg scale was used to allow subjects to estimate the urge-to-cough [7]. The scale ranged from "no need to cough" (rated 0) and "maximum urge-to-cough" (rated 10). The urge-to-cough scale was placed in front of the subjects and the subject pointed at the scale number, which was recorded by the experimenter. To assess the intensity of the urge-to-cough, subjects were recommended to ignore other sensations such as dyspnea, burning, irritation, choking and smoke in the throat. Subjects were told that their sensation of an urge-to-cough could increase, decrease, or stay the same during the citric acid challenges, and that their use of the modified Borg scale should reflect this.

Data analysis

The study protocol was approved by the local ethics committee and informed consent was obtained from all subjects. Data are expressed as mean (SD) except where specified otherwise. The Mann-Whitney *U* test or the chi-square test were used to compare patients with controls. A p value of < 0.05 was considered significant.

Results

All 19 subjects completed the experiments without any difficulty or side effects. Among the 8 patients with aspiration pneumonia, 3 patients had a history of recurrent pneumonia (2–3 episodes). All subjects were leading an independent life. The characteristics of subjects are summarized in Table 1. There was no significant difference in gender, age and MMSE scores between the control subjects and patients with aspiration pneumonia.

As shown in Figure 1A, the cough reflex threshold to citric acid, as expressed by log C_2, in patients with aspiration pneumonia (1.5 ± 0.6 g/l) was significantly higher than those of control (0.6 ± 0.4 g/l, $p < 0.05$). The urge-to-cough scores at the concentration of C_2 and at the concentration of two times dilution of C_2 ($C_2/2$) were estimated for each subject. There were no significant differences in the urge-to-cough at C_2 between control subjects (3.0 ± 1.8 points) and patients with aspiration pneumonia (3.3 ± 3.0 points) (Figure 1B). However, the urge-to-cough scores at $C_2/2$ in patients with aspiration pneumonia (0.3 ± 0.7 points) were significantly lower than those in control subjects (1.2 ± 0.8 points) (Figure 1C). There was no difference in the number of coughs at $C_2/2$ between the

Table 1: Comparison of characteristics between control and patients with aspiration pneumonia

	Control	Aspiration pneumonia	P-value
Number	11	8	
Male/Female	5/6	3/5	n.s.**
Age (years)	77.3 ± 6.3	79.4 ± 6.4	n.s.*
MMSE (points)	28.1 ± 1.2	26.4 ± 1.9	n.s.*
LTSR (seconds)	1.2 ± 0.5	8.3 ± 2.1	< 0.001*

Data are mean ± S.D. *P-values by the Mann-Whitney *U* test. **P-value by chi-square test. MMSE denotes mini-mental state examination. LTSR denotes the latent time of swallowing reflex. n.s. denotes not significant.

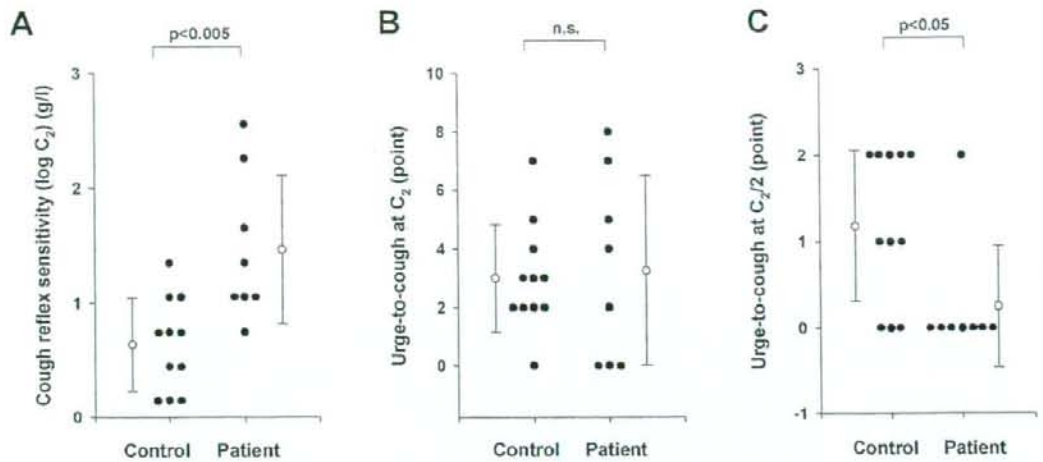

Figure 1

Comparisons of cough reflex sensitivity and urge-to-cough between control subjects (Control) and patients with aspiration pneumonia (Patient). (A) Cough reflex sensitivities expressed as the log transformation of the lowest concentration of citric acid that elicited five or more coughs (C_2). (B) The urge-to-cough estimated by the Borg scores at C_2 of each subject. (C) The urge-to-cough estimated by the Borg scores at the concentration of two times dilution of C_2 ($C_2/2$) of each subject. Closed circles indicate the value of each subject. Open circles and error bars indicate the mean value and the standard deviation in each group, respectively. n.s. denotes not significant.

control subjects (0.1 ± 0.3 times) than in patients with aspiration pneumonia (0.0 ± 0.0 times). At $C_2/2$, only one control subject coughed among all subjects.

As shown in Figure 2A, the cough reflex threshold to citric acid, as expressed by log C_5, in patients with aspiration pneumonia (1.6 ± 0.5 g/l) was significantly higher than those of control (1.0 ± 0.4 g/l, $p < 0.05$). The urge-to-cough scores at the concentration of C_5 and at the concentration of two times dilution of C_5 ($C_5/2$) were estimated for each subject. There were no significant differences in the urge-to-cough at C_5 between control subjects (7.5 ± 1.8 points) and patients with aspiration pneumonia (5.3 ± 3.4 points) (Figure 2B). However, the urge-to-cough scores at $C_5/2$ in patients with aspiration pneumonia (0.5 ± 0/9 points) were significantly lower than those in control subjects (3.0 ± 1.9 points) (Figure 2C). The number of coughs at $C_5/2$ was significantly greater in the control subjects (2.3 ± 1.4 times) than in patients with aspiration pneumonia (0.75 ± 1.4 times, $p < 0.05$). Actually, 6 patients (75.0%) with aspiration pneumonia did not cough at all at $C_5/2$ whereas 2 control subjects (18.2%) did not.

In the present study, C_2 and C_5 are same value in 1 subject in control group and 5 subjects in the patients with aspiration pneumonia.

Discussion

This study shows, for the first time to our knowledge, that the urge-to-cough is significantly attenuated in elderly patients with aspiration pneumonia. It has been suggested that the aspiration pneumonia is, at least in part, a consequence of cough reflex impairment. Sekizawa and coworkers demonstrated a marked depression of the cough reflex in elderly patients with aspiration pneumonia [1]. Nakajoh and colleagues demonstrated that the greater the derangement of the cough reflex, the greater the risk of pneumonia [3]. In this study, we also showed a heightened cough reflex threshold in patients with aspiration pneumonia who did not have cognitive dysfunction and apparent paralysis. Although cough is usually referred to as a reflex controlled from the brainstem, cough can be also controlled via the higher cortical center and be related to cortical modulations. Therefore, the impairment of cough reflex could be due to the disruption of both the cortical facilitatory pathway for cough and the medullary reflex pathway. Since that the urge-to-cough is a brain component of the cough motivation-to-action system, depressed urge-to-cough suggests the impairment of supramedullary pathways of cough reflex [13].

Although we did not observe significant difference in the urge-to-cough at C_2 and C_5, this might be due to too small sample number in this preliminary study. However, as the

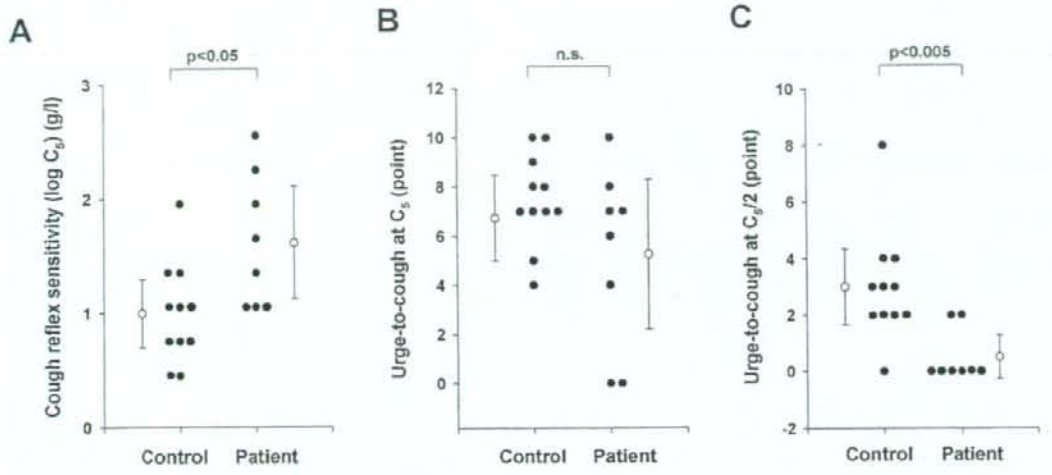

Figure 2
Comparisons of cough reflex sensitivity and urge-to-cough between control subjects (Control) and patients with aspiration pneumonia (Patient). (A) Cough reflex sensitivities expressed as the log transformation of the lowest concentration of citric acid that elicited five or more coughs (C_5). (B) The urge-to-cough estimated by the Borg scores at C_5 of each subject. (C) The urge-to-cough estimated by the Borg scores at the concentration of two times dilution of C_5 ($C_5/2$) of each subject. Closed circles indicate the value of each subject. Open circles and error bars indicate the mean value and the standard deviation in each group, respectively. n.s. denotes not significant.

urge-to-cough precedes the actual cough [7], the difference may become smaller in the point of actually coughing. This could be the reason why the difference in urge-to-cough at C_2 was not significant between groups. Moreover, the actual cough has possibility to affect the urge-to-cough. In the study, all patients with aspiration pneumonia did not cough at $C_2/2$, and 6 of 8 did not at $C_5/2$. If the actual cough has ameliorating effect on the depressed urge-to-cough in the patients with aspiration pneumonia, the urge-to-cough scores at C_2 and C_5 became not different between groups. Well-designed and larger sample studies are warranted to clarify this.

In the present study, we estimated the cough reflex sensitivity using C_2 and C_5. C_5 is considered as a clinically superior value based on better reproducibility compared to C_2 [14]. However, Mazonne et al. assessed urge-to-cough at the concentration of $C_2/2$ in order to avoid the effect of actual cough on the result [9]. In the present study, the number of coughs is significantly greater in control groups than patients with aspiration pneumonia at $C_5/2$ whereas there is no significant difference in the number of cough between controls and patients with aspiration pneumonia at $C_2/2$. Therefore, the urge-to-cough at $C_2/2$ may more purely reflect the supramedually involvement of urge-to-cough.

Due to a lack of flow monitoring, we could not accurately distinguish between cough reflex and expiration reflex, both of which are defensive reflexes to remove foreign substances from the airway by producing the expiratory airflow. However, the latency from stimuli to induce expiration reflex was much shorter than that of cough reflex, suggesting that cortical involvement is unlikely in the expiration reflex [15]. Therefore, the urge sensation investigated here was to be the sensation for cough reflex, not for expiration reflex.

In stroke patients, an impaired cough capacity is now regarded as one of the main factors accounting for the increased prevalence of aspiration pneumonia [16-18]. The underlying mechanism of this phenomenon is still not fully understood. It is conceivable that ischemic brain damage may spread to influence the brainstem cough pathway, a phenomenon commonly referred to as 'brainstem shock'. Alternatively, it may be that ischemic brain damage of the supramedullary area causes a loss of cortical neuro-transmission to the brainstem cough mechanism that is facilitatory to cough [19]. In this study, although our subjects did not have an obvious history of stroke, they were old enough to have silent cerebral infarction. The prevalence of silent infarction in the age group in this study was more than 15% [20,21]. Indeed, all 6

patients who had brain CT scan imaging in the present study revealed a silent cerebral infarction at various levels. A further systematic and larger sample study is required to elucidate the relationship between brain lesions and depressed urge-to-cough in the elderly.

Since it has been proposed that initiation of a reflex cough response requires the urge-to-cough to facilitate it [13], the depressed urge-to-cough could be the cause for impairment of cough reflex response in patients with aspiration pneumonia. The present study may suggest that there might be a population whose cough is impaired due to cortical or subcortical lesions rather than medullary lesions.

Conclusion

This study suggests the involvement of supramedullary dysfunction, at least in a part, in the etiology of aspiration pneumonia in the elderly. Therefore, the restoration of the cough motivation system could be a new strategy to prevent aspiration pneumonia in the elderly.

Abbreviations

MMSE: mini-Mental State Examination; C_2: the lowest concentration of citric acid that elicited five or more coughs; $C_2/2$: The urge-to-cough scores at the concentration of C_2 and at the concentration of two times dilution of C_2; C_5: the lowest concentration of citric acid that elicited five or more coughs; $C_5/2$: Urge-to-cough scores at the concentration of C_5 and at the concentration of two times dilution of C_5.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

SY, SE and TE participated in the design of the study, collected and analyzed data, and drafted the manuscript. MY, TA, MA and KU participated in the design of the study and collected the data. HA participated in design of the study and helped to draft the manuscript. All the authors read and approved the final manuscript.

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Erythropoietin Promotes the Growth of Tumors Lacking Its Receptor and Decreases Survival of Tumor-Bearing Mice by Enhancing Angiogenesis[1]

Abstract

Erythropoietin (Epo), a known hematopoietic growth factor, has been reported to promote tumor growth and angiogenesis in Epo receptor (EpoR)–positive tumors, but its effects on EpoR-negative tumors have not been clearly shown. Here, we show that Epo accelerates the growth of EpoR-negative tumors by promoting tumor angiogenesis. Mice were inoculated with Lewis lung carcinoma cells and treated with Epo. Erythropoietin accelerated tumor growth and increased intratumoral microvessel density, although it did not accelerate Lewis lung carcinoma cell tumor proliferation *in vitro*. To observe the direct effect of Epo on endothelial cells, we examined human dermal microvascular endothelial cells (HMVECs) that expressed EpoR. Erythropoietin induced the proliferation of HMVECs and protected them from H_2O_2-induced cell death. Erythropoietin activated the extracellular signal–regulated kinase signaling pathway and up-regulated the expression of the downstream antiapoptotic protein Bcl-xL in HMVECs. Moreover, in both the absence and presence of tumors, *in vivo* treatment of mice with Epo increased circulating endothelial progenitor cells. To investigate the role of Epo in a primary tumor model, we inoculated the chemical carcinogen methylcholanthrene (MCA) subcutaneously into mice at two doses, a high or a low dose, which induced fibrosarcoma, and treated them with Epo. Erythropoietin promoted tumor growth after MCA inoculation at both doses and decreased the overall survival of the mice inoculated with the high-dose MCA. However, Epo did not increase the incidence of fibrosarcoma at either dose. Lewis lung carcinoma cells and MCA-induced fibrosarcomas did not express EpoR. These results suggest that Epo accelerates the growth of tumors that lack EpoR expression by promoting tumor angiogenesis.

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Abbreviations: DiI-acLDL, acetylated low-density lipoprotein–DiI complex; EC, endothelial cell; EPC, endothelial progenitor cell; Epo, erythropoietin; EpoR, Epo receptor; ERK, extracellular signal–regulated kinase; ESA, erythropoiesis-stimulating agent; FCS, fetal calf serum; G-CSF, granulocyte colony-stimulating factor; H&E, hematoxylin and eosin; HMVEC, human dermal microvascular endothelial cell; HUVEC, human umbilical vein endothelial cell; Jak, Janus-associated kinase; LLC, Lewis lung carcinoma cell; M-CSF, macrophage colony-stimulating factor; MCA, methylcholanthrene; PBS, phosphate-buffered saline; Stat, signal transducer and activator of transcription; VEGF, vascular endothelial growth factor; WST, water-soluble tetrazolium

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Introduction

Hematopoietic growth factors are often used in intensive cancer chemotherapy to help overcome myelosuppression, a reduction in blood cell numbers caused by anticancer treatment. However, recent studies in mice suggest that hematopoietic growth factors such as macrophage colony-stimulating factor (M-CSF) and granulocyte colony-stimulating factor (G-CSF) can cause tumor growth by promoting angiogenesis [1–3], which supplies blood to solid tumors [4,5]. Erythropoietin (Epo) is one of the hematopoietic growth factors often used in cancer treatment. It normally regulates the proliferation, survival, and differentiation of the erythroid lineage, but recent studies have shown that Epo can act on nonhematopoietic organs including solid tumors [6]. The effect of Epo on the survival rate of cancer patients seems variable: Epo decreases the survival of cancer patients with head and neck, metastatic breast, or non–small cell lung cancer [7–9], but by contrast, erythropoiesis-stimulating agents (ESAs), which include Epo, does not reduce the survival rate of patients with small cell lung cancer [10]. In 2007, the Food and Drug Administration convened a meeting to discuss the risks of administrating ESAs to cancer patients. However, no clear conclusion was reached, and the Food and Drug Administration simply advised caution in the use of ESAs [10]. A clearer understanding of Epo's effect on tumor growth is therefore urgently needed to help clinicians decide whether to prescribe Epo to their cancer patients.

One way in which Epo could trigger tumor growth is by acting directly on the tumor cells because many tumor cells express the Epo receptor (EpoR). However, the response of EpoR-expressing tumor cells to Epo varies. Some studies found that Epo treatment can increase tumor cell numbers *in vitro* [11–13], although others found no effect [6]. *In vivo* studies found that blocking Epo function can inhibit the progression of certain tumors [13,14], although other studies found that Epo treatment had no effect on tumor growth [15]. Whereas the effect of Epo on EpoR-positive tumors is still controversial, even less is known about the effects of Epo on EpoR-negative tumors.

Several studies suggest that Epo could act indirectly on tumor growth. For instance, Epo has been reported to act on tumor angiogenesis [6,16] and some studies have shown a direct effect of Epo on some endothelial cells (ECs) [17,18]. Moreover, Epo can increase the number of circulating endothelial progenitor cells (EPCs) in tumor-free humans and mice [19,20]. However, although EPCs have recently been found to contribute to tumor vessel formation [21,22], the effect of Epo on circulating EPCs has not been reported in cancer models. In addition, because most previous studies used EpoR-positive tumors, they could not distinguish between a direct effect of Epo on tumors and an indirect effect through angiogenesis.

In the present study, we examined the effect of Epo on two types of EpoR-negative tumors: implanted Lewis lung carcinoma cells (LLCs) and primary fibrosarcoma induced by a chemical carcinogen methylcholanthrene (MCA) [23]. We found that Epo could trigger growth of these tumors by stimulating angiogenesis and examined which pathway responded to Epo in the ECs.

Materials and Methods

Cell Culture

Lewis lung carcinoma cells, H9c2 cells, and KLN 205 cells were purchased from American Type Culture Collection (Manassas, VA). Lewis lung carcinoma cells were cultured in a high-glucose Dulbecco's modified Eagle's medium containing 10% fetal calf serum (FCS) and 100 μg/ml kanamycin. H9c2 cells were cultured in a high-glucose Dulbecco's modified Eagle's medium containing 10% FCS, 100 U/ml penicillin, and 0.1 mg/ml streptomycin. H9c2 cells were differentiated to H9c2 myotubes as previously shown [24]. KLN 205 cells were cultured in minimum essential medium containing 10% FCS, 1% nonessential amino acids, and 100 μg/ml kanamycin. Normal adult human dermal microvascular endothelial cells (HMVECs), originally derived from foreskins, and human umbilical vein endothelial cells (HUVECs) were purchased from Kurabo (Osaka, Japan) and were cultured in HuMedia-MvG medium (Kurabo).

Lewis Lung Carcinoma Cell Tumor Model

Lewis lung carcinoma cells (3×10^5 cells per mouse) were injected subcutaneously into the hind flank of male C57BL/6 mice (6–9 weeks old) on day 0. Tumor size was quantified daily as width^2 × length × 0.52 [1]. For tumor growth rate models, human recombinant Epo (epoetin beta, 200 IU/kg; Chugai Seiyaku, Tokyo, Japan) was injected into mice subcutaneously once a week from day 1. The mice were killed on day 25 (total four Epo injections per mouse). For the culture assay of EPCs, Epo was injected subcutaneously for 3 days daily from day 18, and the mice were killed on day 21. Controls were subcutaneously injected with phosphate-buffered saline (PBS).

Histology

When the diameter of the tumors reached approximately 1.2 cm, tumors were fixed in 10% formalin, embedded in paraffin and sectioned [1]. Control mice were killed on day 21, and the Epo-treated mice were killed on day 19 (total three Epo injections). The sections were stained with hematoxylin and eosin (H&E). The intratumoral microvessel density was determined as previously described by immunohistochemical staining with polyclonal anti–human factor VIII–related antigen antibody (DakoCytomation, Carpinteria, CA) [1,25,26].

Cell Proliferation and Cell Death Assays

The assays were performed as previously shown [27]. In short, LLCs (5×10^3 cells) were incubated with 0, 0.2, 1, or 5 IU/ml Epo for 48 hours. Human dermal microvascular endothelial cells (3×10^3 cells) were incubated with 0, 0.008, 0.04, 0.2, or 1 IU/ml Epo for 36 or 48 hours. Then, the cell numbers were determined by water-soluble tetrazolium (WST) assay using a Cell Counting Kit (Dojindo, Tokyo, Japan). For cell death assays, HMVECs were incubated with 0, 0.04, 0.2, or 1 IU/ml Epo for 16 hours. Then, the cells were stimulated with H_2O_2 for 8 hours. The WST assay determined the cell viability.

Flow Cytometry

Fluorescein isothiocyanate–labeled anti-CD34 and purified rat anti-CD144 (VE-cadherin) antibodies were purchased from BD Pharmingen (San Diego, CA), PE-labeled anti-EpoR was purchased from DakoCytomation, and control rat IgG2a was purchased from eBioscience (San Diego, CA). Flow cytometry for EPCs was performed as previously shown [1]. For EpoR detection, the cells were

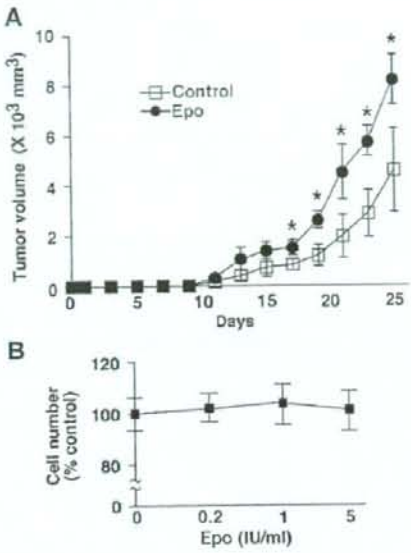

Figure 1. Epo accelerates tumor growth *in vivo* but not *in vitro*. (A) Mice were inoculated with LLCs on day 0 and treated with Epo or PBS from day 1 once a week. Erythropoietin significantly accelerated tumor growth ($n = 8$ per group, $^*P < .03$). (B) Lewis lung carcinoma cells (5×10^3 cells) were cultured with the indicated amounts of Epo for 48 hours. Water-soluble tetrazolium assay determined the cell number. Shown is representative of three independent experiments.

first incubated with unlabeled anti-CD16/32 mAb (eBioscience) and then with the anti-EpoR antibody. Flow cytometry was performed with a FACScan (BD Bioscience, San Jose, CA), and data were analyzed with CellQuest software (BD Bioscience) [1].

Western Blot Analysis

Western blot analysis was performed as previously shown [28]. Human dermal microvascular endothelial cells (1×10^6 cells) were stimulated with Epo (1 IU/ml) for indicated periods. Methylcholanthrene-induced fibrosarcoma cells (3×10^6 cells) or LLCs (2×10^6 cells) were plated onto a culture dish, cultured overnight, and lysed. H9c2 cells were differentiated to H9c2 myotubes, stimulated with M-CSF (100 ng/ml) for 10 minutes, and lysed as previously shown [24]. HeLa cell lysates were purchased from Cell Signaling Technology (Beverly, MA). The cell lysates were subjected to gel electrophoresis and transferred onto polyvinylidene difluoride membranes (Millipore, Billerica, MA). The membranes were blotted with antibodies to phospho–extracellular signal–regulated kinase (ERK), phospho-Akt, phospho–signal transducer and activator of transcription 5 (Stat5), Bcl-xL (Cell Signaling Technology), or EpoR (Santa Cruz Biotechnology, Santa Cruz, CA). The membranes blotted with antibodies to detect phosphorylation were then reblotted with antibodies to total ERK, Akt, and Stat5 (Cell Signaling Technology).

RNA Isolation and Reverse Transcription–Polymerase Chain Reaction

Methylcholanthrene-induced fibrosarcoma cells (3×10^6 cells), LLCs (2×10^6 cells), or KLN 205 cells (3×10^6 cells) were plated onto a culture dish and cultured overnight, and total RNA was isolated using RNAzol B reagent (Tel-Test, Friendswood, TX). Conventional reverse transcription–polymerase chain reaction (RT-PCR) was performed as previously shown [1] using primers as previously described [29].

Culture Assay of Circulating EPCs

Mononuclear cells were isolated, cultured, characterized, and counted as previously described [30] with some modifications [1]. Fluorescent microscopy identified cultured circulating EPCs as double-positive cells for acetylated low-density lipoprotein–Dil complex (Dil-acLDL; Molecular Probes, Eugene, OR) and fluorescein isothiocyanate-labeled lectin from *Ulex europaus* (Sigma-Aldrich,

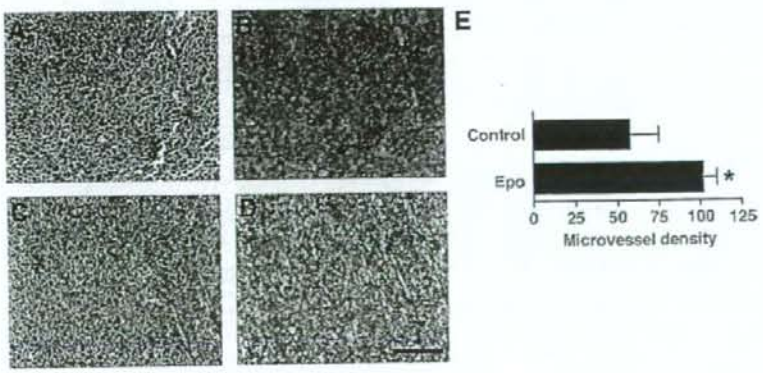

Figure 2. Epo increases tumor microvessel density *in vivo*. (A–D) Mice were inoculated with LLCs on day 0 and killed when the diameter of the tumors reached 1.2 cm. Control mice (A and B) were killed on day 21, and Epo-treated mice (C and D) were killed on day 19 (total three Epo injections). Sections were stained with H&E (A and C) and anti–factor VIII–related antigen antibody (B and D); scale bar, 100 μm. Images show one representative mouse of eight in each group. (E) Epo significantly increased microvessel density in tumors ($n = 8$ per group, $^*P < .01$).