

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.

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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 \pm SD, N=17.

| Postural stability index | Directional axis | Visual condition: eyes-open | Visual condition: eyes-closed |
|--------------------------|------------------|-----------------------------|-------------------------------|
| RMSdisp (cm) | ML | 0.51 \pm 0.17 | 0.73 \pm 0.25 |
| | AP | 0.53 \pm 0.19 | 0.71 \pm 0.21 |
| RMSvel (cm/sec) | ML | 1.54 \pm 0.62 | 3.02 \pm 1.40 |
| | AP | 1.40 \pm 0.61 | 2.56 \pm 1.13 |
| Traj Length (cm) | N/A | 85. \pm 38. | 156. \pm 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 \pm SE. Significance levels: *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$. The sham exposures did not reach statistical significance.

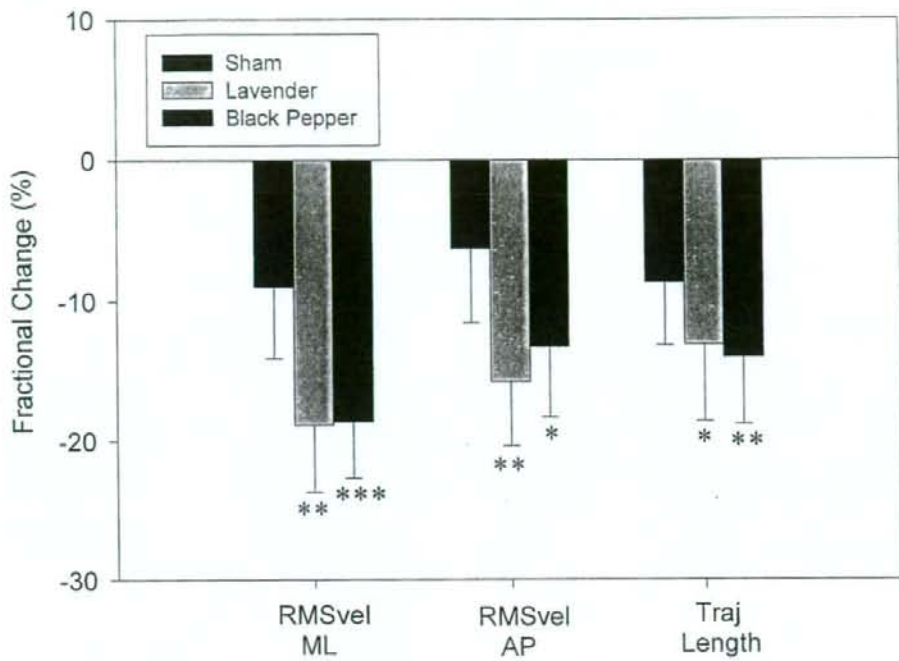


Figure 1.

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 $C_2/2$ 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 \pm 6.3 | 79.4 \pm 6.4 | n.s.* |
| MMSE (points) | 28.1 \pm 1.2 | 26.4 \pm 1.9 | n.s.* |
| LTSR (seconds) | 1.2 \pm 0.5 | 8.3 \pm 2.1 | < 0.001* |

Data are mean \pm 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.

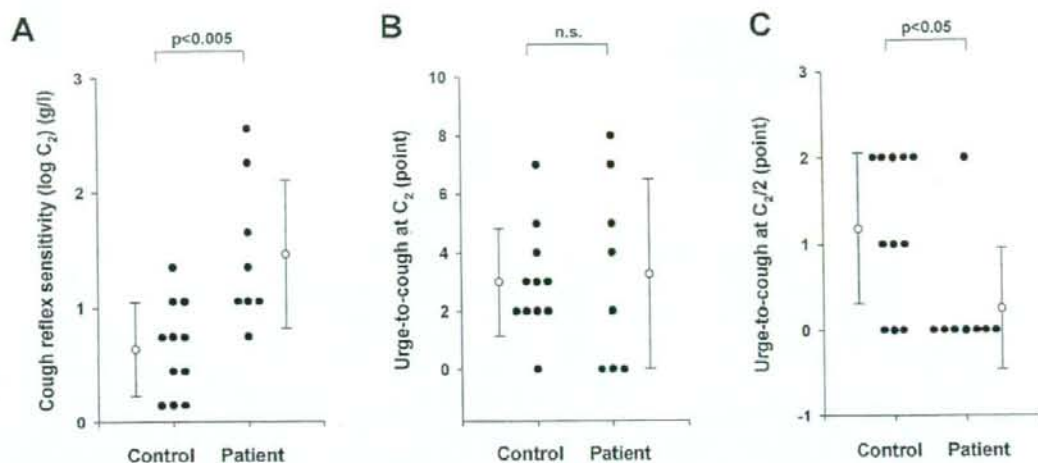


Figure 1
Comparisons of cough reflex sensitivity and urge-to-cough between control subjects (Control) and patients with aspiration pneumonia (Patient). (A) Cough reflex sensitivities expressed as the log transformation of the lowest concentration of citric acid that elicited five or more coughs (C_2). (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 ($0.5 \pm 0/9$ 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 co-workers 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

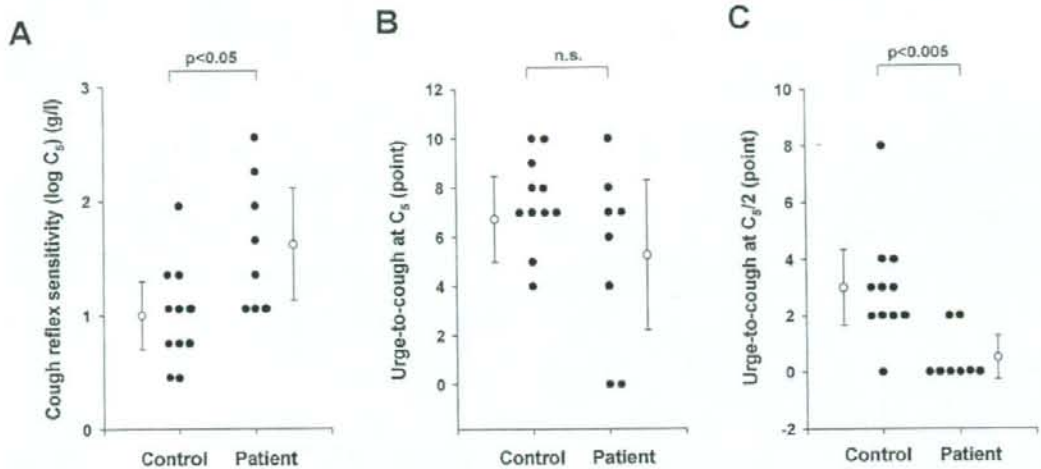


Figure 2

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 supramedullary 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 Angiogenesis¹

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 H₂O₂-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: Dil-acLDL, acetylated low-density lipoprotein-Dil 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 administering 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 width² × 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 H₂O₂ 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

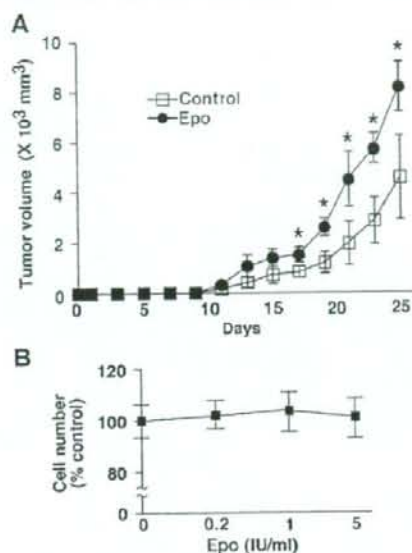


Figure 1. Epo accelerates tumor growth *in vivo* but not *in vitro*. (A) Mice were inoculated with LLCs on day 0 and treated with Epo or PBS from day 1 once a week. Erythropoietin significantly accelerated tumor growth ($n = 8$ per group, $*P < .03$). (B) Lewis lung carcinoma cells (5×10^3 cells) were 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].

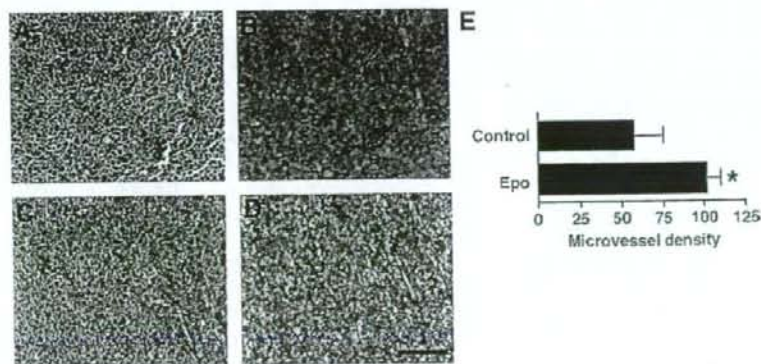


Figure 2. Epo increases tumor microvessel density *in vivo*. (A–D) Mice were inoculated with LLCs on day 0 and killed when the diameter of the tumors reached 1.2 cm. Control mice (A and B) were killed on day 21, and Epo-treated mice (C and D) were killed on day 19 (total three Epo injections). Sections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

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 europaeus* (Sigma-Aldrich,

St. Louis, MO). Two independent investigators evaluated the number of double-positive cells by counting three randomly selected high-power fields.

Methylcholanthrene-Induced Fibrosarcoma

Six-week-old male C57BL/6 mice were injected subcutaneously into the hind flank with 25 (low dose) or 100 μ g (high dose) of 3-MCA (Sigma-Aldrich) in 0.1 ml of maize oil. Development of the fibrosarcoma was assessed periodically for 100 to 150 days. Tumors more than 2 mm in diameter were recognized as positive. Tumor size was quantified as described in the Lewis Lung Carcinoma Cell Tumor Model section. For the MCA-induced fibrosarcoma cell isolation, when the diameter of 100- μ g MCA-induced fibrosarcoma reached 1 cm, the mice were killed and the tumors were removed aseptically. Tumors were cut into small pieces and treated with collagenase (Sigma type IV) at 37°C for 1 hour. Clumps were removed, and single cells were cultured in RPMI 1640 medium supplemented with 10% FCS and 2 mM L-glutamine.

Data Analysis

Data are shown as mean \pm SD. Statistical analysis was performed using analysis of variance with Fisher's least significant difference test. Statistical analysis of the overall survival of MCA-induced fibrosarcoma-bearing mice was performed using Mann-Whitney U test. *P* values < .05 were considered as significant.

Results

Erythropoietin Accelerates Tumor Growth In Vivo But Not In Vitro

To test the effect of Epo *in vivo*, we inoculated LLCs into mice subcutaneously on day 0 and injected Epo or PBS into the mice once a week starting at day 1. Erythropoietin significantly accelerated tumor growth (Figure 1A). To test whether this growth might reflect a direct effect of Epo on LLCs, we examined the response of LLCs to Epo *in vitro*. We found that Epo did not increase LLC proliferation *in vitro* (Figure 1B). Because the FCS might contain some growth factors that made high-growing background, we examined the response of LLCs to Epo under low-FCS culture medium condition that contained 5% FCS. We found that Epo did not increase LLC proliferation under this condition (data not shown). The effect of Epo on LLC tumor growth therefore seems to be indirect.

Erythropoietin Increases Tumor Microvessel Density In Vivo

To analyze the mechanism of tumor growth acceleration by Epo, we stained the tumors with H&E and found that the number of tumor blood vessels was higher in Epo-treated mice than in PBS-treated mice (Figure 2, A and C). To quantify tumor angiogenesis, we stained tumors with an antibody against the factor VIII-related antigen, a blood vessel marker (Figure 2, B and D) [1,26]. Erythropoietin significantly increased microvessel density in tumors (Figure 2E).

Erythropoietin Promotes Proliferation and Survival of HMVEC In Vitro, Activates ERK Signaling, and Increases Bcl-xL Expression

Because Epo promoted tumor angiogenesis, we examined effect of Epo on ECs more precisely *in vitro*. We examined EpoR expression

on two types of ECs using FACS analysis. We found EpoR expression on HMVECs (Figure 3A) but not on HUVECs (data not shown). Because HMVECs expressed EpoR, we next examined HMVEC proliferation after various periods of exposure to Epo. Erythropoietin significantly promoted proliferation of HMVECs at 36 and 48 hours of exposure (Figure 3B) but not at 24 hours (data not shown). Another hematopoietic growth factor, M-CSF, supports the survival of cardiomyocytes and skeletal muscle cells in the presence of the toxic dose of H₂O₂ [24]. Therefore, we examined the effect of Epo on the survival of HMVECs exposed to H₂O₂. Erythropoietin significantly protected HMVECs from H₂O₂-induced cell death (Figure 3C). In erythroid cells, Epo activates cell signaling pathways such as the Akt pathway, the Janus-associated kinase 2 (Jak2)-Stat5 pathway, and the ERK pathway [31,32]. To elucidate the molecular mechanisms of Epo-induced proliferation and survival, the activation status of the ERK, Akt, and Stat5 signaling pathways was investigated in HMVECs after treatment with Epo. Erythropoietin activated ERK, as indicated by ERK phosphorylation, although it did not affect the total ERK protein levels in cell lysates (Figure 3D). In contrast, Epo did not activate Akt or Stat5 (Figure 3D). Cell lysates derived from H9c2 myotubes stimulated with M-CSF and HeLa cells stimulated with IFN- α were used as positive controls for the activated form of Akt and Stat5 respectively (Figure 3D). Extracellular signal-regulated kinase activation by Epo up-regulates the antiapoptotic protein Bcl-xL in a leukemia cell line and in erythroid progenitor cells [33]. Therefore, we examined Bcl-xL expression in Epo-stimulated HMVECs. Low-level Bcl-xL expression was detected in HMVECs without Epo stimulation (Figure 3D). Erythropoietin up-regulated Bcl-xL expression after 12 and 24 hours of exposure (Figure 3D) before Epo has any effect on cell proliferation. At later time points, Bcl-xL levels kept increasing, but we cannot interpret this increase because of Epo's effect on cell proliferation. These results suggest that Epo induces the proliferation and survival of HMVECs by activating ERK and their survival by up-regulating Bcl-xL.

Erythropoietin Increases the Number of Circulating EPCs in Tumor-Bearing Mice

We hypothesized that Epo enhanced tumor angiogenesis by increasing the numbers of circulating EPCs in addition to directly stimulating ECs. To investigate the effects of Epo on the number of circulating EPCs, mice were injected with Epo for 3 days and circulating EPCs, characterized by Dil-acLDL uptake, lectin binding, and CD34 and VE-cadherin expression (Figure 4, A and B) [34-36], were counted. Erythropoietin significantly increased the number of circulating EPCs (Figure 4C). We next evaluated the effect of Epo on circulating EPCs in mice bearing LLCs. Injection of Epo once a week starting 1 day after LLC inoculation significantly increased the tumor volume after day 17 (Figure 1A). Previous study showed that a growing LLC tumor itself increased serum vascular endothelial growth factor (VEGF) level and the numbers of EPCs [1], which suggested that when the volume of LLC tumors increased, it could increase the number of circulating EPCs. To isolate circulating EPCs from mice bearing a similar volume of LLC tumors, we injected Epo from day 18 for three consecutive days and isolated EPCs on day 21. This treatment significantly increased the number of circulating EPCs (Figure 4D) without significantly affecting tumor size (data not shown).

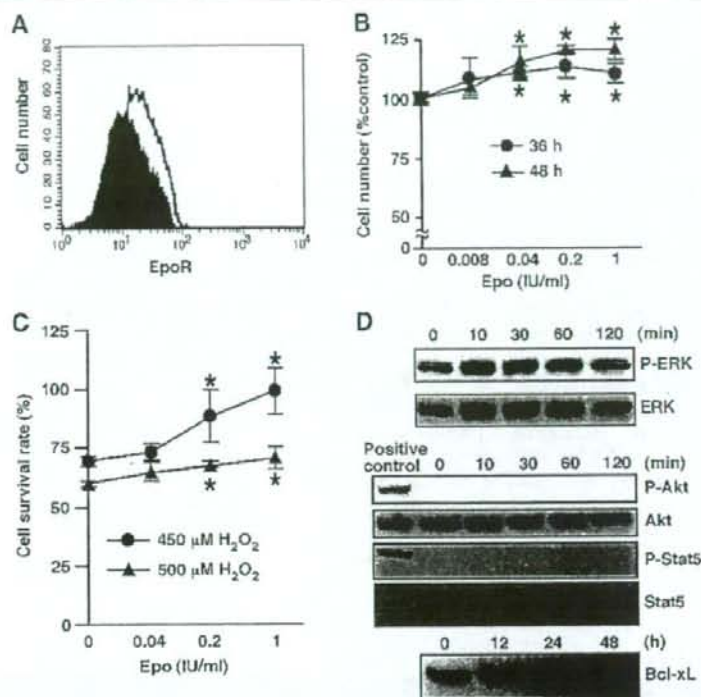


Figure 3. Epo promotes the proliferation and survival of HMVECs *in vitro*, activates ERK signaling, and increases Bcl-xL expression. (A) Human dermal microvascular endothelial cells expressed EpoR. The shaded histogram indicates staining with EpoR, and the blank histogram indicates staining with control IgG. Shown is representative of two independent experiments. (B) Human dermal microvascular endothelial cells were cultured with indicated amount of Epo for indicated periods, and WST assay determined the cell number. Erythropoietin (0.04, 0.2, and 1 IU/ml) significantly increased the number of HMVECs ($*P < .05$). (C) Human dermal microvascular endothelial cells were cultured with indicated amount of Epo for 16 hours and then stimulated with H_2O_2 for 8 hours. Erythropoietin (0.2 and 1 IU/ml) significantly protected HMVECs from H_2O_2 -induced cell death ($*P < .02$). (D) Human dermal microvascular endothelial cells were stimulated with Epo (1 IU/ml) for the indicated periods, and then cell lysates were blotted with antibodies specific for the activated form of ERK (phospho-ERK), Akt (phospho-Akt), or Stat5 (phospho-Stat5). The membranes were reblotted with antibodies to total ERK, Akt, or Stat5, respectively. Cell lysates derived from M-CSF-stimulated H9c2 myotubes and IFN- α -stimulated HeLa cells were used as positive controls for the activated form of Akt and Stat5, respectively (D). Bcl-xL expression was confirmed by the specific antibody. Shown is representative of two independent experiments.

Erythropoietin Promotes Tumor Growth But Does Not Increase the Incidence of MCA-Induced Fibrosarcoma and Impairs Overall Survival of Mice Inoculated with High-Dose MCA

We next investigated whether Epo impaired overall survival and increased the incidence of primary tumor development after treatment with the chemical carcinogen MCA. We injected 25 (low dose) or 100 μ g (high dose) of MCA into mice and injected Epo once a week for 100 to 150 days starting 1 day after MCA injection. Erythropoietin did not significantly increase tumor incidence at either dose of MCA (Figure 5, A and D). However, it significantly promoted tumor growth after the onset of the tumors at both doses (Figure 5, B and E). Erythropoietin significantly impaired the overall survival of mice inoculated with the high dose of MCA but not the lower dose (Figure 5, C and F). Western blot analysis showed EpoR in HMVECs but not in MCA-induced fibrosarcoma cells or in LLCs (Figure 5G), which was consistent with the result in Figures 1B and 2F. Conventional RT-PCR showed mRNA of EpoR in squamous

carcinoma cell line KLN 205 cells, which Epo significantly increased proliferation rate *in vitro* (data not shown), but not in MCA-induced fibrosarcoma cells or in LLCs (Figure 5H).

Discussion

In this study, we found that Epo accelerated the growth of EpoR-negative tumors *in vivo* by promoting angiogenesis, both in a primary tumor model (MCA-induced tumor) and a conventional tumor model (LLC inoculation). Erythropoietin directly increased EC proliferation and decreased EC death by activating ERK and up-regulating the downstream antiapoptotic protein Bcl-xL. In addition, Epo increased the number of circulating EPCs in tumor-bearing mice. In the MCA-induced primary tumor model, Epo impaired the overall survival of the 100- μ g MCA-inoculated mice but not of the 25- μ g MCA-inoculated mice. However, Epo did not increase MCA-induced tumor incidence.

We have previously reported that other hematopoietic growth factors other than Epo could accelerate tumor growth by promoting

angiogenesis. Macrophage colony-stimulating factor up-regulated serum concentration of VEGF, a potent angiogenic factor [1], and G-CSF increased the number of a type of endothelial cell precursors known as Gr1⁺CD11b⁺ cells [2] and promoted tumor angiogenesis and growth in mice. Unlike these hematopoietic growth factors, Epo did not increase VEGF or Gr1⁺CD11b⁺ cell numbers in mice (data not shown). However, we showed that Epo could act on ECs directly.

Erythropoietin significantly impaired the overall survival of mice inoculated with the higher dose but not the lower dose of MCA. This difference might be due to the insufficient number of tumor-positive mice in 25- μ g MCA low-dose groups. The high dose of MCA induced 5 to 6 tumor-positive mice among 10 mice, whereas the 25- μ g dose induced only 3 tumor-positive mice among 10 mice. Therefore, to analyze the overall survival, the number of tumor-positive mice in low-dose MCA-inoculated groups might be insufficient.

To our knowledge, our study is the first to examine the effect of Epo on HMVECs. Our observations suggest that different ECs respond to Epo in different manners. For instance, a previous study using EA.hy926 endothelial cells, which are derived from the fusion of HUVEC with A549 lung carcinoma cells, showed that Epo activated Jak2 [17]. Instead of Jak2, we examined its downstream effector Stat5 and found that it was not activated by Epo in HMVECs, suggesting that Epo does not activate Jak2 in these cells. Very recently, another study showed that Epo activated Akt and ERK in HUVECs

[18]. By contrast, we could only find evidence for ERK activation after Epo treatment of HMVECs. Interestingly, we could not detect EpoR on HUVECs we looked at by FACS analysis (data not shown), whereas we could detect it on HMVECs. Consistent with this finding, Epo treatment accelerated the proliferation of HMVECs but not of HUVECs (data not shown). Difference in culture conditions or in the source of HUVECs might explain why we failed to detect any response from HUVECs to Epo. Further experiments are required for pursuing the effect of Epo on ECs using other types of ECs.

Furthermore, our study shows that Epo increases the number of circulating EPCs in tumor-bearing mice. The role of circulating EPCs in tumor angiogenesis has been controversial. Using bone marrow transplantation, some studies reported large contributions of circulating EPCs to tumor angiogenesis, but other studies reported that these contributions were negligible [22]. Moreover, another study showed that bone marrow-derived cells mainly contributed to the formation of periendothelial vascular mural cells but not to the formation of endothelial cells [37]. On the basis of our observations, we suggest that both a direct effect of Epo on ECs and the mobilization of circulating EPCs by Epo contribute to Epo-induced tumor angiogenesis. However, we could not determine the extent of the contribution of circulating EPCs to Epo-induced tumor angiogenesis. Further study is required for identifying this issue.

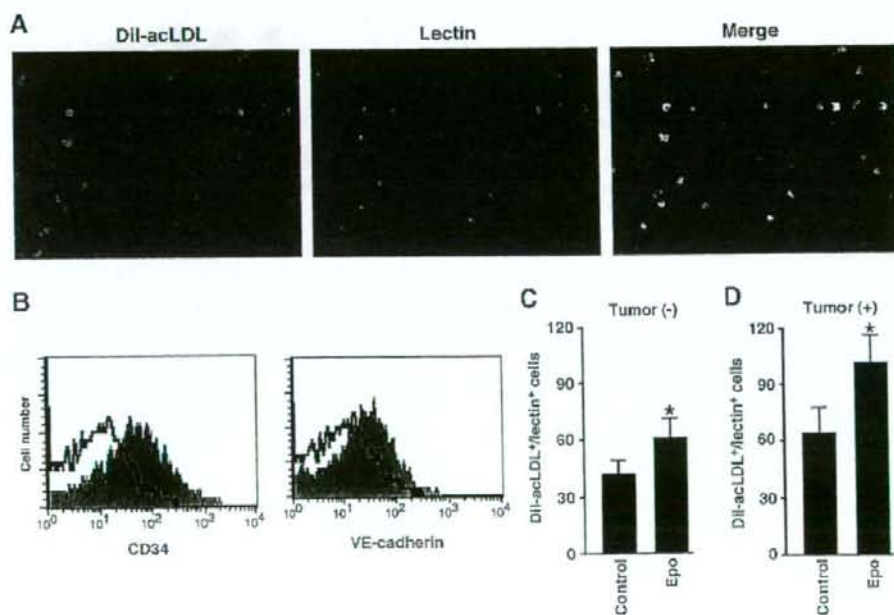


Figure 4. Epo increases the number of circulating EPCs in tumor-bearing mice. (A) Mononuclear cells were isolated from peripheral blood and cultured. Fluorescence microscopy determined Dil-acLDL uptake (left panel) and lectin binding (middle panel) of adherent cells. Double-positive cells (merge) were considered as cultured circulating EPCs (right panel). (B) Expression of CD34 and VE-cadherin on cultured circulating EPCs. The shaded histograms indicate staining with Abs to CD34 or VE-cadherin, and the blank histograms indicate background staining with control IgG. (C and D) Mice were injected with Epo for 3 days daily and were then killed (C). Mice were inoculated with LLCs on day 0, injected with Epo for 3 days daily from day 18, and killed on day 21 (D). Mononuclear cells (4×10^6 cells per mouse) were isolated from peripheral blood and cultured. Adherent Dil-acLDL and lectin double-positive cells were counted. Erythropoietin significantly increased the number of double-positive cells ($n = 8$ per group, $*P < .01$).

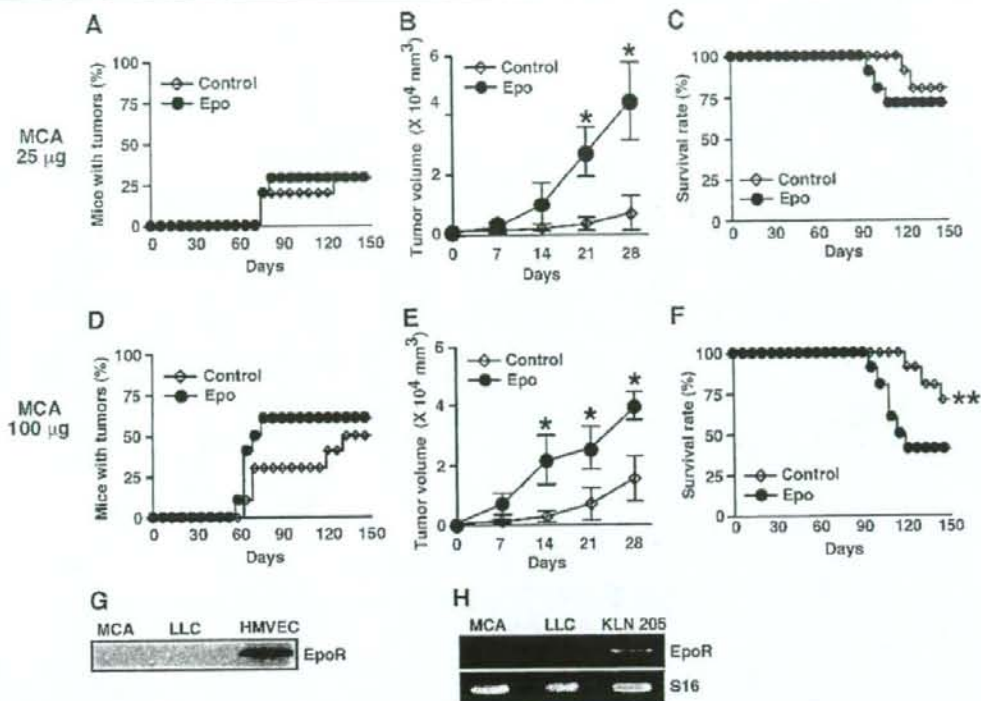


Figure 5. Epo promotes tumor growth but does not increase the incidence of MCA-induced fibrosarcoma and impairs overall survival of mice inoculated with high-dose MCA. Mice were subcutaneously inoculated with 25 (low dose) or 100 μ g (high dose) of MCA into the hind flank on day 0, treated with Epo or PBS from day 1 once a week, and observed for fibrosarcoma development during the course of 100 to 150 days ($n = 10$ mice per group). (A and D) Percentages of mice with tumors are shown. (B and E) Tumor volumes after the onset of tumor development are shown ($n = 3-6$ per group). Erythropoietin significantly accelerated tumor growth ($*P < .03$). (C and F) Overall survival of mice is shown. Erythropoietin significantly decreased overall survival of 100- μ g MCA inoculated mice but not of 25- μ g MCA-inoculated mice ($**P < .04$). (G) Cell lysates were isolated from MCA-induced fibrosarcoma, LLCs, or HMVECs and blotted with specific antibody for EpoR. Methylcholanthrene-induced fibrosarcoma or LLC did not express EpoR. (H) Total RNA was isolated from MCA-induced fibrosarcoma, LLCs, or KLN 205 cells, and conventional RT-PCR was performed for EpoR and S16. KLN 205 cells expressed mRNA of EpoR but MCA-induced fibrosarcoma or LLCs did not.

The effect of Epo on EpoR-negative tumors seems very dose-dependent. Whereas administering 200 IU/kg once a week increased the rate of tumor growth, administering 2000 IU/kg once a week had no detectable effect on tumor growth in our mice models (data not shown). A large dose of Epo can cause thromboembolic disease [10]. It is therefore possible that the 2000-IU/kg injection, which is 20 times the recommended dose, causes thromboembolism in tumor blood vessels, impairing blood supply and preventing acceleration of the tumor growth. The 200-IU/kg once a week injection is two times the recommended dose for humans. Therefore, we suggest proceeding with caution while administering Epo to cancer patients.

We performed immunohistochemical analysis of LLC tumors and MCA-induced fibrosarcomas but could not obtain clear staining. As a previous study reported limited use of anti-EpoR antibodies for immunohistochemical analysis [38], this analysis will be performed when an antibody suitable for mice EpoR immunohistochemical analysis is available.

In summary, Epo directly protected ECs, stimulated EC proliferation, and increased the number of circulating EPCs. Erythropoietin

promoted tumor growth and angiogenesis in EpoR-negative tumors and impaired overall survival of the primary tumor-bearing mice. The effect of Epo on tumor progression might include other mechanisms. However, our results suggest that clinicians should be careful while using Epo, even with patients carrying EpoR-negative tumors.

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