A novel model of aspiration in young and old guinea-pigs using LacZ gene transduction of adenovirus vector

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The effects of anaesthesia on upper airway reflexes in older animals have not been elucidated fully. We studied young (4-month-old) and old (28-month-old) guinea-pigs to examine the relationship between aspiration and altered upper airway reflexes during anaesthesia. We administered an adenovirus vector carrying *Escherichia coli* LacZ gene (Ad vector) intranasally to guinea-pigs with or without anaesthesia. LacZ gene expression was investigated in the nostrils and lungs of each animal under anaesthesia. No LacZ gene expression was found in the lungs of unanaesthetized animals given Ad vector. Thus intranasal administration of Ad vector was aspirated into the lower airways under anaesthesia. Next we examined the effect of age on anaesthesia-induced aspiration. At a lower concentration of halothane in 100% oxygen, greater LacZ gene expression in the lungs was measured in older than in younger animals, suggesting that older animals are liable to aspirate oropharyngeal contents into the lower airways during light anaesthesia. This novel animal model of aspiration using Ad vector may be useful to explore the mechanism of aspiration during and after anaesthesia in young and old animals.

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Upper airway reflexes, mucociliary transport (MCT) and phagocytosis by alveolar macrophages are important airway defence mechanisms to prevent pulmonary complications after thoracic and abdominal surgery.1–3 It has been reported that anaesthetics and sedatives alter the function of the airway defence mechanisms, including the swallowing reflex, cough reflex and MCT.4–10 Impairment of airway defence mechanisms caused by anaesthesia may contribute to postoperative pulmonary complications, including pneumonia and atelectasis. Because of the increasing age of the population, many patients presenting for surgery are elderly. Because silent aspiration, but not massive aspiration, plays a critical role in the pathogenesis of aspiration pneumonia and nosocomial pneumonia in older patients,11–15 and because age-dependent declines in upper airway reflexes have been reported,16–19 anaesthesia-induced impairment of reflexes during and after surgery may be particularly important in the development of aspiration pneumonia in older patients undergoing surgery. However, the relationship between postoperative aspiration and anaesthesia-induced impairment of upper airway reflexes in older patients has not been elucidated fully. Because the experimental approach for aspiration pneumonia in humans is ethically limited, an animal model of aspiration in association with anaesthesia is necessary to investigate the mechanism of postoperative aspiration and aspiration-induced pneumonia in older animals. However, as yet there is no appropriate animal model of silent aspiration.

We hypothesized that anaesthesia-induced depression of upper airway reflexes may cause aspiration and an aspirated bolus may reach the lower airways during anaesthesia. Furthermore, the aspirated bolus, including E1-deleted recombinant adenovirus carrying *Escherichia coli* (E. coli) LacZ gene (Ad-CMV-LacZ vector), may be seen in lungs by blue staining of LacZ expressing cells20–23 when intranasal administration of Ad-CMV-LacZ vector is aspirated into the lower airways and infects airway epithelial cells. This study was designed to develop a new animal model of aspiration which would be useful to investigate the relationship between aspiration and age-related impairment of upper airway reflexes.

Materials and methods
Specific pathogen-free (SPF) 3-month-old male guinea-pigs weighing 480–540 g were purchased from Charles Liver
Inc. (Shizuoka, Japan). The animals were maintained in a limited-access barrier under SPF conditions at the Animal Research Institute of Tokyo University. Male guinea-pigs were housed at 24±2°C under an alternating 12-h light and dark cycle, and given a commercial diet (Nihon CLEA, Tokyo, Japan) and water ad libitum. The experiments followed the guidelines for the care and use of laboratory animals of the NIH, and were approved by the Institutional Review Board for Lab Animal Care of the University of Tokyo. We studied young (4-month-old) male guinea-pigs weighing 600–720 g and old (28-month-old) male guinea-pigs weighing 950–1100 g.

Adenoviral vectors
Replication defective adenoviral vector based on human adenovirus 5 serotype (hAd5) were used. In the hAd5-CMV-LacZ, E1 and E3 sequences were deleted and replaced with a mini-gene containing the CMV promoter, and a cytoplasmic LacZ gene was inserted at the site of the E1 deletion of adenovirus. The Ad vector was propagated in 293 cells, purified by CsCl gradient ultracentrifugation and stored at −70°C until use for infection of endothelial cells. Ad vector titres (transducing unit (TU ml−1)) were determined by the number of LacZ gene expressing 293 cells per millilitre of vector by histochemical X-gal staining. The experiments used vector produced with titres of 1×1012 (TU ml−1). The ratio of TU to viral particle number (as measured by OD at 260 nm) was approximately 1:20. Heat-inactivated Ad vector were also used. Ad vector were inactivated by incubation in hot water at 65°C for 1 h.

Intranasal administration of Ad vector with or without anaesthesia
We administered a total volume of 100 μl of Ad-CMV-LacZ (Ad vector) or 100 μl of phosphate-buffered saline (PBS) intranasally to 4-month-old guinea-pigs with or without anaesthesia (pentobarbital sodium (Abbott Laboratories, North Chicago, IL, USA), 5 mg per 100 g body weight i.p.). For the sham control, Ad vector was administered intranasally after administration of PBS i.p. instead of pentobarbital.

In addition, to determine which of the blue cells seen with X-gal staining were a result of LacZ gene expression by Ad vector or up-regulation of endogenous β-galactosidase, we administered heat-inactivated Ad vectors carrying LacZ gene intranasally in three young and three old animals.

To investigate the effect of age on the frequency of aspiration in guinea-pigs, halothane (Takeda Chemical Industries Ltd, Osaka, Japan) was used to determine the depth of anaesthesia. Young (4-month-old) and old (28-month-old) guinea-pigs were housed in airtight clear glass chambers, measuring 30 cm wide×30 cm deep×17.5 cm high. Halothane in 100% oxygen (fresh gas flow 5 litre min−1) entered the chamber at one end and was vented at the other end. Halothane was delivered from a dedicated, calibrated vaporizer, and the anaesthetic concentration in the chamber was monitored continuously with a crystal sensor (ICOR Anaesthetic Agent Monitor; ICOR AB Co., Sweden). After 15 min of administration of 0.5–4% of the inhaled anaesthetic in 100% oxygen, a steady-state level of anaesthesia was obtained, and animals were given Ad vector through the nostrils. A total of 100 μl of Ad vectors were administered intranasally to the young and old guinea-pigs. Furthermore, to test the effect of age alone on the susceptibility of the airway epithelia to adenoviral transduction, regardless of anaesthesia, we administered the Ad vector intranasally to young and old animals after administration of PBS i.p. instead of pentobarbital. Swallowing was identified by visual observation of the characteristic laryngeal/neck movement and by signs of bronchospasm and wheezing.

Assessment of distribution of aspirated solution in airways using LacZ gene expression of Ad vectors
To assess the distribution of LacZ gene expression of Ad vectors in airways, X-gal staining was performed on tissues from the nostrils and lungs. LacZ gene expressing cells were detected in situ as blue-stained cells by X-gal staining. One day after transnasal administration of Ad vector, animals were killed by exsanguination under anaesthesia, and the nostrils and lungs were removed. Lungs were inflated with 2% paraformaldehyde (PFA) in PBS for 1 h and then sectioned in the frontal plane at the depth of the hilum. The nostrils were also fixed with 2% PFA in PBS for 1 h. After fixation, tissues were washed twice with cold PBS with MgCl2 1 mmol litre−1, and stained with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) for 4 h at 37°C. Tissue sections were counterstained with haematoxylin–eosin after X-gal staining. A positive section expressing the LacZ gene from the nostrils and lungs was judged by more than 1% of cells expressing the LacZ gene in 20 randomly selected fields in the tissue section. For more quantitative analysis of LacZ gene expression in the lung, we measured the frequency of LacZ gene expression in the lung of each guinea-pig under halothane anaesthesia. The frequency of LacZ gene expression in the lung was determined as the percentage of LacZ gene expressing fields in the tissue section under the microscope (Nikon Microphot, EPI-FL3). More than 50 fields were counted in each section.

Statistical analysis
Data are presented as mean (SEM). The chi-square test was used to compare the frequency of airway LacZ gene expression. Analysis of variance (ANOVA) with Fisher’s protected least significant difference method was used for comparing data of the frequency of LacZ gene expressed in young and old animals under anaesthesia. Analyses were performed using the software package Stat View 4.0 (Abacus Concepts, Inc., CA, USA). P<0.05 was considered statistically significant.
Fig 1 Nostril and lung histologies of guinea-pigs after intranasal administration of E1-deleted adenoviral vector (Ad-CMV-LacZ) with or without pentobarbital anaesthesia. Young=4-month-old guinea-pigs; aged=28-month-old guinea-pigs. Those animals that were not anaesthetized (anaesthesia –) were given the adenoviral vector intranasally after i.p. injection of phosphate-buffered saline (PBS) instead of pentobarbital; those anaesthetized (anaesthesia +) were given adenoviral vector intranasally after i.p. injection of pentobarbital 5 mg/100 g body weight. Nose=nostril histology; lung=lung histology. Histological sections were stained with X-gal and haematoxylin–eosin. Blue-stained cells were considered to be expressed LacZ gene. (Original magnification ×200.)

Table 1 Frequency of LacZ gene expression in tissues of guinea-pigs. Nostril and lung histologies of guinea-pigs after intranasal administration of E1-deleted adenoviral vector (Ad vector) with or without pentobarbital anaesthesia. Those animals that were not anaesthetized (anaesthesia –) were given the adenoviral vectors, phosphate-buffered saline (PBS) or heat-inactivated vector intranasally after i.p. injection of PBS instead of pentobarbital; those anaesthetized (anaesthesia +) were given adenoviral vectors, PBS or heat-inactivated vector intranasally after i.p. injection of pentobarbital 5 mg/100 g body weight. Ad-LacZ=E1-deleted adenoviral vector (Ad vector) carrying E. coli; heat-inactivated Ad vector=Ad vector inactivated by incubation in hot water at 65°C for 1 h. Nose=nostril histology; lung=lung histology. Values in parentheses indicate the percentage of LacZ gene expressing animals to the total animals in the group. **P<0.01 compared with animals administered PBS intranasally during anaesthesia.

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Results

Effect of anaesthesia on aspiration detected by LacZ gene expression in young and old guinea-pigs

LacZ gene expression in the nostrils was measured in each guinea-pig given Ad vector transnasally with or without anaesthesia. While LacZ gene expression in the lungs was recognized as blue staining in animals who were anaesthetized, no LacZ gene expression was found in the lungs of unanaesthetized animals (Fig. 1). In guinea-pigs given PBS without Ad vector transnasally, there was no LacZ gene expression in the nostrils or lungs. The results of LacZ gene expression after intranasal administration of Ad vector, PBS or heat-inactivated Ad vector are summarized in Table 1. Every anaesthetized animal given Ad vectors exhibited LacZ gene expression in the lungs. In contrast, there was no LacZ gene expression in the nostrils or lungs of anaesthetized animals administered heat-inactivated Ad-CMV-LacZ vector or PBS intranasally (Table 1).

Effect of age on anaesthesia-induced aspiration in the animal model

LacZ gene expression in the nostrils was measured in each animal who was or was not anaesthetized, irrespective of age (Fig. 1). However, there was no LacZ gene expression in the lungs of any unanaesthetized guinea-pig. Therefore, there was no effect of age alone on the susceptibility of the airway epithelia to adenoviral transduction, regardless of whether or not the animals were anaesthetized. However, LacZ gene expression in the lungs was detected in young...
and old animals during halothane anaesthesia. There was a relationship between depth of anaesthesia and the ratio of the number of LacZ gene expressing animals to the number of total animals (Fig. 2). LacZ gene expression in the lungs was investigated in all guinea-pigs anaesthetized with 4% halothane in 100% oxygen, irrespective of age. However, a higher percentage of LacZ gene expression in the lungs was measured in older guinea-pigs at a lower concentration of halothane compared with the concentration in young guinea-pigs (Fig. 2). For more quantitative analysis of LacZ gene expression in the lungs, we measured the frequency of LacZ gene expression in the lungs of each guinea-pig and compared the percentage of LacZ gene expressing fields in the tissue section under the microscope. The percentage of LacZ gene expressing fields in the lung tissue section was significantly greater in older than in young animals after anaesthesia with 1–3% halothane (Fig. 3). This suggests that lighter anaesthesia can depress upper airway reflexes in older animals compared with younger ones.

Discussion

Many animal models of aspiration pneumonia have been reported. Although models are useful to investigate aspiration of gastric contents or hydrochloric acid, such as in Mendelson’s syndrome, there is no animal model of silent aspiration. Because silent aspiration and aspiration-associated pneumonia were often investigated in postoperative elderly patients, the effect of anaesthetics–sedatives on age-related impairment of upper airway reflexes may have been important. However, the effect of anaesthesia on the swallowing reflex and cough reflex in aged animals has not been elucidated fully.

In our studies, we administered E1-deleted recombinant adenovirus carrying E. coli LacZ gene (Ad vector) intranasally to young and old guinea-pigs with or without anaesthesia. Because the Ad vector, which is a representative DNA virus, is known to infect airway epithelial cells and express the foreign gene (i.e. E. coli LacZ gene) in the epithelial or other cells, the fate of the aspirated bolus, including the Ad vector, may be identified as blue staining of LacZ gene expression in the airways. We demonstrated that deep anaesthesia caused aspiration of nasopharyngeal contents in both young and old animals and LacZ gene transduction of Ad vector was identified in the lower airways as blue staining (Fig. 1). However, unanaesthetized animals did not exhibit aspiration and LacZ gene expression was not found in the lungs of animals, irrespective of age. These observations suggest that intranasally administered Ad vector is aspirated into the lower airways because of impaired upper airway reflexes during anaesthesia and aspirated vector is internalized into the airway epithelial cells. Therefore, the animal model of aspiration using LacZ gene transfer provides a unique approach for both detection of the distribution of aspirated contents in the lung and distribution of virus infection. Although we described X-gal staining as a marker of LacZ gene expression by adenovirus vector, there is a possibility that the blue staining was caused by upregulation of endogenous β-galactosidase. We also administered heat-inactivated Ad-CMV-LacZ vectors to anaesthetized animals intranasally. Because no blue cells were found in the nostrils or lungs of anaesthetized animals administered PBS alone or heat-inactivated vector, the blue-stained cells were not...
caused by up-regulation of endogenous β-galactosidase after infection by the adenovirus or by an immune reaction vector proteins.

To further elucidate the relationship between aspiration and depression of upper airway reflexes caused by anaesthesia or age, we administered Ad vectors intranasally to young and old animals under anaesthesia in the presence of a volatile anaesthetic. At first, we tested the effect of age alone on the susceptibility of the airway epithelia to adenoviral transduction without anaesthesia. At 0% halothane anaesthesia in 100% oxygen (i.e. unanaesthetized condition), LacZ gene expression was measured in the nostrils but not in the lungs of every animal, irrespective of age. In contrast, LacZ gene expression was found in the nostrils and lungs of young and old animals during 4% halothane anaesthesia. Therefore, the susceptibility of the airway epithelia to adenoviral transduction was not affected markedly by age alone.

The effect of age on aspiration was investigated using different concentrations of halothane. Although LacZ gene expression in the lungs was investigated in all young and old animals at concentrations of halothane $\geq 4\%$, at lower concentrations of halothane, the percentage of LacZ gene expression in the lungs was greater in older than in younger animals (Figs 2, 3). These results suggest that aspiration occurs frequently in young and old animals during deeper anaesthesia, but lighter anaesthesia, which does not cause aspiration in young animals, may often cause aspiration in older animals. These observations may confirm that older animals are likely to aspirate oropharyngeal secretions during sedation and/or anaesthesia. This is in agreement with our clinical experience where elderly patients are liable to aspiration pneumonia after operation. Although the mechanisms of susceptibility of older animals to alterations in upper airway reflexes during light anaesthesia were not determined in our studies, several investigators have reported an age-related decline in upper airway reflexes in humans.16–19 This novel animal model of aspiration using transnasal administration of Ad-CMV-LacZ may provide an animal model for examining various interventions in relation to age-related impairment of upper airway reflexes.

Both wild-type adenovirus (wt-Ad) and E1-deleted adenoviral vectors are known to infect airway epithelial cells.23 26 31 However, differences in Ad infection with airway epithelial cells between young and old animals have not been described fully. Our study indicates that the transduction efficiency of Ad vectors in the nostrils and lungs was not very different between young and old guinea-pigs. Because pneumonia infiltration occurs mainly in the lower rather than the upper airways, Ad vector carrying LacZ gene can prove lower airway infection of small organisms from aspirated nasopharyngeal contents. Thus our new model of aspiration may also be useful for investigating the distribution of aspirated micro-organism infection in the lung. However, the transduction efficiency of Ad vector is known to be different between cells and species.23–26 32 33 Therefore, data from animals do not always predict results in humans. Further studies are needed to elucidate differences in aspiration in relation to anaesthesia between rodents and primates.

In summary, we have described an animal model of aspiration using an Ad vector. We believe that our findings that older animals were liable to aspiration during light anaesthesia may raise further concerns regarding the clinical use of anaesthetics–sedatives, particularly in elderly patients.

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