Optical imaging of subacute airway remodeling and adipose stem cell engraftment after airway injury

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Abstract: Acquired airway injury is frequently caused by endotracheal intubations, long-term tracheostomies, trauma, airway burns, and some systemic diseases. An effective and less invasive technique for both the early assessment and the early interventional treatment of acquired airway stenosis is therefore needed. Optical coherence tomography (OCT) has been proposed to have unique potential for early monitoring from the proliferative epithelium to the cartilage in acute airway injury. Additionally, stem cell therapy using adipose stem cells is being investigated as an option for early interventional treatment in airway and lung injury. Over the past decade, it has become possible to monitor the level of injury using OCT and to track the engraftment of stem cells using stem cell imaging in regenerative tissue. The purpose of this study was to assess the engraftment of exogenous adipose stem cells in injured tracheal epithelium with fluorescent microscopy and to detect and monitor the degree of airway injury in the same tracheal epithelium with OCT. OCT detected thickening of both the epithelium and basement membrane after tracheal scraping. The engraftment of adipose stem cells was successfully detected by fluorescent staining in the regenerative epithelium of injured tracheae. OCT has the potential to be a high-resolution imaging modality capable of detecting airway injury in combination with stem cell imaging in the same tracheal mucosa.

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1. Introduction

Acquired airway injury is frequently caused by endotracheal intubations, long-term tracheostomies, trauma, airway burns, and some systemic diseases [1]. The mechanism involves mucosal abrasion caused by cuff pressure or an over-sized tube for endotracheal intubation, resulting in mucosal inflammation, ulceration, and necrosis [2].
Several methods have been used for treating acquired airway injury. However, the management of severe tracheal injury in many patients continues to be challenging [3]. In cases of tracheal stenosis resulting from airway injury, procedures such as balloon dilation, laser vaporization, and stenting are less invasive compared with surgical methods. They, however, are ineffective and sometimes cause recurrence, thus cause poor clinical outcomes [4,5].

There is emerging interest in imaging methods with high resolution for research into early detection in airway injury models as well as early intervention to prevent serious stenosis using stem cell therapy. Optical coherence tomography (OCT) is a recently developed technology capable of providing real-time, noninvasive, high-resolution (micron-level) imaging of tissues such as trachea and bronchi to depths up to 2 mm below the tissue surface. To date, there have been multiple studies using OCT to detect acute airway inflammation and chronic subglottic stenosis in rabbit models [6,7]. Recently, with respect to early intervention, adipose stem cells have been studied as a potential alternative solution to ameliorate airway or lung injury. Adipose stem cells (ASCs) have been observed to display immunomodulatory properties [8]. ASCs can differentiate into tracheal epithelial cells in rabbit lungs injured by toxic inhalant, and the engraftment of ASCs may suppress inflammation and deposition of collagen in damaged lung tissue [9,10]. However, there have been no studies using ASCs in tracheal stenosis models.

To address both early monitoring using OCT and intervention using ASCs, in this study, we first performed the engraftment of ASCs in an airway stenosis model in rabbit. In addition, based on a stem cell model, we assessed airway changes after a scraping injury in rabbits.

2. Methods

All animal procedures were conducted in accordance with the guidelines published in the Guide for the Care and Use of Laboratory Animals (DHEW publication NIH 85–23, revised 2010, Office of Science and Health Reports, DRR/NIH, Bethesda, MD, USA). The study protocol was approved by the Committee on Animal Research of the College of Medicine at Kosin University. Figure 1 depicts the experimental procedure.

![Fig. 1. Experimental procedure. (A) General anesthesia. (B) Tracheal scraping. (C) Adipose stem cell injection through the peritoneum. (D) Tracheal preparation after resection. (E) OCT scanning. (F) Microscopic examination.](image-url)
2.1 Surgical procedure for the tracheal stenosis model

As shown in Fig. 1(C), twelve male New Zealand white rabbits (Taesung Laboratory Animal Science, Busan, Korea) weighing 3.0 to 3.7 kg were used for the experiment. The twelve rabbits were divided into normal control (n = 4), sham-treated group (saline injection, n = 4), and experimental group (stem cell injection, n = 4). The rabbits were each intramuscularly anesthetized with 35 mg/kg ketamine and 5 mg/kg xylazine. Each rabbit was placed in the supine position on a heated operating table, and body temperature was maintained at 39°C by monitoring rectal temperature. Heart rate and respiratory rate were also monitored. The anterior neck of each rabbit was shaved and disinfected. To enhance analgesia, 2 ml of 1% lidocaine hydrochloride was injected into the subcutaneous area of the anterior neck. The surgical procedure was performed according to the methods described in a previous study [11]. After a midline skin incision in the anterior neck, the larynx and the trachea were exposed, with care taken not to injure the sternohyoid and sternothyroid muscles. The trachea was incised transversely along the tracheal cartilage with an incised length of two-thirds the circumference. The incision point was located 1.5 cm caudal to the bottom edge of the cricoid cartilage. A sheathed brush with a diameter of 1.5 mm was inserted into the trachea by way of the incised edge toward the mouth, and then the ten times of scraping injury to the tracheal mucosa with a brush were induced on anterior 120 degree. The scraping was carried out at a distance of 1.5 cm from the incision point. Oozing was stopped by applying pressure to the wound using gauze. After confirmation of hemostasis, the incised trachea was closed with four or five interrupted sutures. The degrees of airway injury were defined as normal, mild (hyperemic mucosa without nodular granulation), and moderate (hyperemic mucosa with nodular granulation) based on gross findings on the 10th day after procedure.

2.2 Optical coherence tomography system and probes

We constructed an 850 nm spectrometer-based OCT system as shown in Fig. 2. A broadband light source (Broadlighter D855, Superlum, Ireland) with a center wavelength of 850 nm and a full width at half maximum of 100 nm was used. The fringe pattern was collected by a line scan camera (Sprint spL4096-140km, Basler, PA, USA) with a line rate of 140 kHz and 4096 pixels. A two-axis scanner was customized using two galvanometers (6220H, Cambridge Technology, MA, USA). B-mode images were acquired at 10 fps for 1024 lateral pixels. The point spread function was measured and showed a depth resolution of 4 μm in air, a roll-off of 12 dB/mm, and a signal-to-noise ratio of 103 dB.
2.3 Adipose stem cell administration after airway injury

2.3.1 Isolation and culture of rASCs

Rabbit ASCs (rASCs) were isolated and cultured according to the methods described in a previous study [12]. Briefly, omental fat tissues from rabbit were processed to obtain a stromal vascular fraction (SVF). To isolate SVF, adipose tissues were washed extensively with equal volumes of phosphate-buffered saline (PBS; Gibco, Grand Island, NY, USA). Extracellular matrix (ECM) was digested at 37°C for 30 minutes with 0.075% type I collagenase (Sigma, Saint Louis, MO, USA). Enzyme activity was neutralized with Dulbecco's modified Eagle's medium: Nutrient mixture F-12 (DMEM/F12; GibcoBRL, Grand Island, NY, USA) containing 10% fetal bovine serum (FBS; Gibco, Grand Island, NY, USA) and centrifuged at 1200 g for 10 minutes to obtain a high density SVF pellet. The cellular pellet was resuspended in DMEM/F12 containing 10% FBS and filtered through a 100 μm mesh filter to remove cellular debris and incubated overnight at 37°C with 5% CO2 in the basic culture medium containing DMEM/F12, 10% FBS, 1% penicillin/streptomycin solution (Gibco, Grand Island, NY, USA), 10 ng/ml EGF (Sigma, Saint Louis, MO, USA), and 2 ng/ml bFGF (Sigma, Saint Louis, MO, USA). Following incubation, the plates were washed extensively with PBS to remove residual nonadherent red blood cells. To prevent spontaneous differentiation, ASCs were maintained at subconfluence levels. The cells were subcultured with 0.25% trypsin and 1 mm EDTA (Gibco, Grand Island, NY, USA) and passaged at a 1:4 ratio. Fifth to tenth passages of ASCs were used throughout the study.

2.3.2 Injection of rASCs

Cultured rASCs were harvested with trypsin, washed with PBS and DMEM, suspended at a density of 1x10⁷ cells/ml in PBS, and centrifuged at 1200g for 10 minutes to obtain a pellet. The rASCs were collected from the pellet and were labeled using PKH-67® (green fluorescence; MINI-67, Sigma, Saint Louis, MO, USA) according to the manufacturer’s instructions. Briefly, rASCs were centrifuged into a loose pellet, and the supernatant was removed. The prepared rASCs and the dye were immediately mixed by gentle pipetting. An equal volume of the complete medium was added to stop the staining reaction, and the cells were washed several times with the medium.

A 10 μl microsyringe (Hamilton, Reno, NV, USA) with a 31 G beveled needle was used to pierce the peritoneal cavity. The rASCs (approximately 2x10⁵/1.5 μl) were slowly injected into the peritoneal cavity of rabbit. Among the eight rabbits except normal control (n = 4), four were injected with rASCs, and the other four rabbits were injected with normal saline (Fig. 1(C)).

2.4 Pathology and fluorescence microscopy

2.4.1 Pathology

Each rabbit was euthanized using CO2 gas. Trachea including injured site was removed, transversely cut into pieces, fixed with 10% neutral buffered formalin (NBF), and embedded in paraffin. Four-micrometer-thick serial sections were stained with hematoxylin and eosin (H&E) and examined by microscopy.

2.4.2 Cryosectioning

The tracheas were fixed in 4% paraformaldehyde (PFA) for 2 hours, washed with PBS, and transferred to 30% sucrose in PBS overnight before cryosectioning. The fixed tracheas were embedded in optimal cutting temperature compound (Tissue-Tek®, Tokyo, Japan), and 7-10 μm frozen sections were made using a cryostat (Leica, Germany).
2.4.3 Fluorescence microscopy

The stained specimens were observed using a fluorescence microscope (Nidek, Japan). The injected cells that have been labeled with PKH-67® show green fluorescence. Therefore, the incorporated rASCs expressed green.

![Fluorescence microscopy images](image)

Fig. 3. Gross findings of normal (A), mild (B), and moderate (C) injuries on the tenth day after airway scraping. A. Normal finding. B. Mildly injured airway epithelium showed hyperemic and hemorrhagic spot (white circle). C. Moderately injured epithelium showed hyperemic nodular granulation (white circle) on the tenth day after airway scraping.

3. Results

3.1 Gross observation on the 10th day after airway injury

The twelve rabbits were sacrificed on the tenth day after the scraping. The tracheas of the twelve rabbits were removed on the day of death, and histologic examination was performed. Based on gross finding, four tracheas from normal rabbits showed non-specific finding (Fig. 3(A)) and four tracheal mucosa (two in saline, two in stem cell group) showed mild hyperemic change, while four tracheal mucosa (two in saline, two in stem cell group) showed moderate granular change on the tenth day after scraping (Figs. 3(B), 3(C)).

![Gross observation images](image)

Fig. 4. OCT findings according to the degree of the airway injury. OCT findings on the tenth day after scraping (A-C) and corresponding pathologic findings (D-F). (A,D) OCT finding of normal airway wall and normal pathology. (B,E) OCT and pathology finding of mild airway injury. The thickness of epithelium and basement membrane become slightly increased. (C,F) OCT and pathology finding of moderate airway injury. The thickness of epithelium and basement membrane become significantly increased. EP: Epithelium, BM: Basement membrane, SM: Submucosa, C: Capillary.
3.2 OCT findings

Notable structural changes were observed in injured tracheas by OCT on the tenth day after scraping. Marked epithelial thickening became apparent in those animals that received scraping. Particularly in the basement membranes of injured tracheas, high scattering density was observed with band-like widening in comparison with normal basement membranes of uninjured tracheal epithelia (Fig. 4). Three dimensional OCT images for normal, mild, and moderate cases were shown in Fig. 5. Four sites were chosen from normal, mild, and moderate cases, respectively. Thicknesses were measured and averaged at nine points chosen uniformly over each site. The mean thicknesses of the basement membranes according to the degree of injury (normal, mild, moderate) were $33 \pm 2.0 \mu m$, $71 \pm 2.5 \mu m$, $105 \pm 2.7 \mu m$ ($p<0.01$). The mean thicknesses of the epithelia according to the degree of injury (normal, mild, moderate) were $48 \pm 2.1 \mu m$, $74 \pm 8.6 \mu m$, $152 \pm 3.2 \mu m$ ($p<0.01$) (Fig. 6).

Fig. 5. Three-dimensional OCT images for (a) normal, (b) mild, and (c) moderate cases. Each image was reconstructed using 512 two-dimensional images.

Fig. 6. OCT Measurement of the thickness of epithelium and basement membrane in normal and injured airways. Statistically significant increases of the thicknesses of epithelial layer and basement membrane were seen according to the degree of injury ($p<0.01$). EP: Epithelium, BM: Basement membrane.
3.3 Histologic observation

Histologic examination showed various degrees of inflammatory tissue changes. Examination of moderately injured trachea showed significant granulation reaction with epithelial hyperproliferation, submucosal hypertrophy caused by proliferation of fibroblasts, thickened collagen fibers, and increased number of capillaries (Fig. 4). In addition, mildly injured trachea showed regenerative epithelial thickening without granulation tissue and infiltration of inflammatory cells was seen in the submucosal lesion; most of the inflammatory cells were neutrophils until 10 days after the scraping, after which lymphocytes and histiocytes accounted for the majority of inflammatory cells.

![Fluorescence microscopic findings](image)

Fig. 7. Fluorescence microscopic findings (A,B,C) and the corresponding pathologies (D,E,F). A,D. Fluorescence microscopic finding of the injured airway epithelium with stem cell injection and pathology (x400). A. Significant amount of green fluorescence was observed in the rASCs-transplanted tracheas (arrows). B,E. Fluorescence microscopic finding of the uninjured airway epithelium with stem cell injection and pathology (x400). C,F. Fluorescence microscopic finding of the injured airway epithelium with saline injection and pathology (x400). No green fluorescence was observed in the uninjured epithelium in stem cell group and the injured epithelium in saline group (B,C). The basement membrane showed auto-fluorescence in both experimental and control groups. EP: Epithelium, BM: Basement Membrane, SM: Submucosa.

3.4 Fluorescence microscopic finding of engraftment of rASCs

Fluorescent microscopic examination was performed under green fluorescence (FITC). Injected rASCs had been incorporated into tracheal epithelium in stem cell injected four
rabbits. A significant amount of green fluorescence was observed in the rASCs-transplanted tracheas, and the signal was scattered, particularly in the area of regenerative epithelium (Fig. 7). In contrast to the rASCs-injected tracheas, no green fluorescence was observed in the saline-injected tracheas and even in the uninjured epithelium of the rASCs-transplanted tracheas (Fig. 7). The basement membrane showed auto-fluorescence in both experimental and control groups. Thickening of the basement membrane was similar with OCT findings according to the degree of injury.

4. Discussion

Assessment of the presence and extent of airway stenosis is important for the selection of the proper treatment. Therefore, it is essential to develop quantitative, minimally invasive methods for detecting and assessing the extent of airway injury using new technologies such as OCT.

The OCT changes in the mucosa and submucosal region are apparent within 10 days following scraping injury. Increased thickening of basement membrane according to the epithelial thickening was apparent with following OCT monitoring. In several studies, the extent of injury has been quantitatively measured with this spectral-domain OCT system [13–15]. In this study, we first measured thickness of the basement membrane in injured tracheas. It is generally recognized that thickening of the basement membrane in a chronic airway disease like asthma is an important marker of morbidity and mortality. However, the clinical significance of basement membrane thickening in acute injured airway has not been reported. In clinical settings, airway stenosis usually develops 2-4 weeks after injury of the tracheal wall [16]. From this point of view, the capability of OCT to detect the earliest changes in airway epithelium and basement membrane after injury in a minimally invasive manner provides a new potential tool for early assessment of coming stenosis and early intervention with steroid treatment or stenting. In this study, the OCT measurements were all obtained ex vivo. Thicknesses of epithelium and basement membrane need to be confirmed by in vivo experiment using an endoscopic OCT probe in the future.

In many studies, ASC engraftments have been shown to attenuate lung injury and fibrosis [17,18]. In our study, rASCs were detected in the rASCs treatment group but not in the saline group. Furthermore, rASCs were exclusively detected in the area of the regenerating epithelial layer of granulation on 10th day after injury, which indicated rASCs immigrated to the layer and might play an important role in epithelial wound repair. The epithelial granulation is a well known area of tracheal stenosis after airway injury [19–21]. This result demonstrates the potential challenge of stem cell research in vivo in rescuing injured tracheal tissue in a clinical context. For clinical application, efficiency of transplantation of stem cells into the trachea is very important. We believe that using this engraftment model, the degree of amelioration in airway stenosis can be compared to the association with the decrease in inflammatory markers in future studies in animal models.

Recently, there have been several studies regarding stem cell imaging in tissue regeneration using biomedical engineering and stem cell biology [22–24]. In this study, we established a new fusion method for biomedical monitoring and stem cell engraftment in airway injury in rabbits. Histopathologic and OCT examination revealed that, while the exogenous adipose stem cells engrafted in the regenerative epithelium, thickening of the epithelium and basement membrane were simultaneously measured by OCT. To date, it has not been reported that ASCs contribute to regenerative and immunomodulatory processes in an airway stenosis model. In this study, we did not directly demonstrate the response to stem cells therapy. However, our model can contribute to further study of the immunomodulatory effects of ASCs in airway stenosis. In addition, future developments with ultrahigh-resolution imaging technologies may overcome these limitations. Such capabilities should improve the accuracy of diagnosis, regional variability assessment, and ability to differentiate the pathologic events occurring during the injury process.
5. Summary

This study demonstrates the feasibility of utilizing OCT for high-resolution detection and monitoring of the sub-acute changes in airway surface including the thicknesses of epithelial layer and basement membrane in a rabbit model with adipose stem cell engraftment after airway injury. Future studies will be needed to determine the correlation between the acute changes in thickness of the airway basement membrane and subsequent airway stenosis.

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