Effect of Bronchodilators on Bronchial Gland Cell Proliferation After Inhalation and Burn Injury in Sheep

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The objective of this study was to measure the temporal changes in bronchial submucosal gland (SMG) cell proliferation in sheep after smoke inhalation and burn (S+B) injury, and to assess the effect of bronchodilators on the proliferative response. Archived main bronchial airways from sheep after S+B injury were immunostained for Ki67, and the percentage of ciliated duct and SMG cells expressing nuclear localization of Ki67 was determined for uninjured sheep and in sheep 24, 48, 72, and 96 hours after injury. A semiquantitative measure of lining epithelial exfoliation was made for each tissue. Bronchial tissues from sheep at 48 hours after S+B injury that had been nebulized with albuterol or tiotropium bromide (tiotropium) were examined to assess the effect of bronchodilators on the proliferative response. At 48 through 96 hours after injury, both ciliated duct and SMG cell proliferation were significantly increased compared with that of uninjured animals and animals 24 hours after injury, \( P < .05 \). A small increase in proliferation was seen in the SMG cells of albuterol-treated sheep compared with nebulized saline controls, \( P = .048 \). SMG cells of tiotropium-treated animals showed a significant increase in Ki67 nuclear staining compared with their study controls, \( P = .001 \). Extensive injury to the lining epithelium is associated with a proliferative response in both ciliated duct and SMG cells 24 hours after injury. The increase in proliferation in sheep treated with bronchodilators suggests that therapies for inhalation injury modify the glandular proliferative response. Further study to assess the ability of bronchodilators to enhance epithelial repair is warranted. (J Burn Care Res 2013;34:386–393)
lung environment. Previous studies have shown that restoration of sheep tracheal lining epithelium after a severe inhalation injury requires approximately 18 days and that repair is associated with proliferation of airway gland cells. This observation is in agreement with studies showing that the glandular epithelium provides a niche for stem/progenitor cells that are important in airway repair. Although the reparative process is essential for respiratory health, this response may be modified by bronchodilators used in the clinical management of inhalation injury as both adrenergic and muscarinic receptors are present on gland cells and each receptor-mediated pathway is associated with cell proliferation.

The current study uses archived bronchial tissue from sheep after a standardized S+B injury to measure the degree of bronchial epithelial exfoliation and temporal changes in proliferation of the ciliated duct epithelium and deeper submucosal gland (SMG) cells. The study has been extended to tissues from S+B injured animals that had been treated with nebulized albuterol (ALB), a β-2 adrenergic receptor agonist, and tiotropium bromide (tiotropium), a long-acting M1 and M3 muscarinic receptor antagonist, to test the hypothesis that bronchodilator therapies modify the proliferative response of the gland epithelium after injury.

**METHODS**

This is a retrospective study using bronchial tissues that were systematically sampled, formalin fixed, and processed to paraffin blocks from sheep after S+B injury and from injured sheep treated with ALB and tiotropium. The initial studies were conducted in compliance with the guidelines for the care and use of laboratory animals of the National Institutes of Health and the Association for the Assessment and Accreditation of Laboratory Animal Care.

S+B injury was induced after a standardized protocol in surgically prepared animals. All study animals were female, range-breed Merino sheep, between 30 and 40 kg in weight. The details of surgical preparation and S+B injury have been described. Briefly, under deep anesthesia, animals received a 40% TBSA burn and an inhalation injury induced by 48 breaths of cooled cotton smoke delivered with a modified bee smoker. After injury, animals were resuscitated with Ringer lactate solution (4 ml/percent TBSA per kilogram body weight per 24 hours), and both injured and sham animals received 100% inspiratory oxygen at a respiratory rate of 30 breaths/min for 3 hours to induce rapid clearance of carboxyhemoglobin. After 3 hours, ventilation and fraction of oxygen in inspired air were adjusted according to blood gas analysis to maintain a partial pressure of CO₂ in the arterial blood between 25 and 30 mm Hg and the arterial oxygen saturation above 90%. Slight hyperventilation to maintain partial pressure of CO₂ in the arterial blood below a normal level for sheep (30–35 mm Hg) during mechanical ventilation is required in awake sheep to prevent a panting response. Typically, in the ovine model of inhalation injury, arterial oxygen tension begins to decrease between 12 and 24 hours after injury, and virtually all animals are receiving 100% oxygen at 48 hours after injury.

Sham animals received instrumentation and anesthesia without burn or inhalation injury and were studied for 48 hours. Fluid resuscitation in the sham animals was 2 ml/kg/hr. At study termination, the lungs were removed and the right lower lobe was systematically sampled for histological analysis. Per protocol, one paraffin block contains a cross-section of the main bronchus that was sampled approximately 5 cm distal to the right main bronchial opening from the carina. This single histological block from each animal was used for analysis of gland cell proliferation.

To assess the temporal changes in lining epithelial injury and gland cell proliferation after S+B injury, archived main bronchial tissue from sheep 24 (n = 6), 48 (n = 6), 72 (n = 3), and 96 hours (n = 4) after injury was used. Main bronchial tissue from sham, uninjured but surgically prepared animals (n = 5) served to provide baseline data on the degree of lining epithelial injury and gland cell proliferation compared with injured animals. The sham group was limited to animals that were instrumented, anesthetized, and ventilated for 48 hours after mock injury. As with the injured animals, these animals received 3 hours of 100% oxygen, followed by 21% oxygen for the remainder of the 48-hour study period.

Assessment of the effects of ALB on ciliated duct and SMG cell proliferation was accomplished on S+B injured sheep that had received 40 mg/hr of ALB in saline that was continuously nebulized via a MiniHEART, low-flow nebulizer (Westmed, Tucson, AZ) for 48 hours (n = 5) and on S+B injured sheep that received continuously nebulized saline as experimental controls (n = 5). To assess the effects of tiotropium on ciliated duct and SMG proliferation, main bronchial tissues from sheep with S+B injury and those nebulized with tiotropium (Boehringer Ingelheim Pharmaceuticals Inc. Ridgefield, CT), 18 µg nebulized every 4 hours for 48 hours (n = 6), and in injured sheep that had received an equal volume of nebulized saline as experimental controls (n = 6) were used. In our assessment of the effects of bronchodilator therapies on gland cell proliferation,...
cell proliferation, no tissues from uninjured, sham-treated sheep were included.

Immunolocalization of Ki67, a marker of cell proliferation, was accomplished after an established protocol. From each study animal, 4-μm sections from paraffin blocks that contain the cross-section of the main bronchus were prepared. Sections were deparaffinized and placed in antigen retrieval buffer (Biogenex, San Ramon, CA) and maintained near boiling in a steamer for 20 minutes and then allowed to cool for 20 minutes before further processing. Sections were then pretreated with 0.06% hydrogen peroxide in 100% methanol to reduce endogenous peroxidase activity and incubated for 1 hour in normal horse serum to reduce nonspecific staining. Sections were then incubated with 1:1000 diluted monoclonal antibody against human Ki67 antigen (Biogenex) overnight at 4°C. After primary antibody incubation, sections were incubated with biotinylated horse antimouse antibody and avidin-biotin-peroxidase complex for 1 hour each following the manufacturer’s recommended protocol (Vector Laboratories, Burlingame, CA). Visualization of the bound antibody complex was achieved using diaminobenzidine (DAB) as a chromogen (Vector Laboratories) and the tissues counterstained with hematoxylin.

Quantification of bronchial lining injury and gland cell proliferation was accomplished by a microscopist unaware of the sample groups. For each slide, an estimate of main bronchial epithelial exfoliation between 0 and 100% was made. To assess ciliated duct cell proliferation, the ring of main bronchus was scanned, and ciliated duct epithelial cells within the lamina propria (between the airway lumen and smooth muscle) were scored as either positive or negative for nuclear Ki67 staining. Typically, between 50 and 200 cells per animal were scored. To assess SMG cell proliferation, only gland cells deeper than the airway smooth muscle, including epithelial cells of the acini, collecting duct, and secretory tubules were included in the analysis. The percentage of cells positive for nuclear localization of Ki67 was determined by examining the first 500 cells starting at the 12 o’clock position of the main bronchus and moving clockwise. After identification of the extent of Ki67 nuclear staining, slides were unmasked, and lining epithelial exfoliation and proliferation scores from each animal were tabulated into their respective groups. Group mean and SD for injury and proliferation of ciliated duct and SMG cell populations were determined.

### Statistical Analysis

To compare differences in the mean degree of exfoliation and mean levels of cell proliferation between study groups, Sigma Stat software (SPSS, Chicago, IL) was used. A one-tailed t-test was used to assess differences in mean degree of proliferation in ALB-treated sheep compared with the control group. Comparison of group means in the temporal assessments in Ki67 nuclear labeling and in the study of tiotropium compared with the control group was accomplished using a two-tailed t-test. P < .05 was used to define statistically significant differences.

### RESULTS

Changes in the degree of main bronchial exfoliation and the percentage of ciliated duct and SMG cells showing nuclear expression of Ki67 in sham animals and in bronchial tissue at different times after S+B injury are shown in Table 1. Mean and SD scores for the degree of lining epithelial exfoliation were significantly increased in all injured groups compared with the sham, uninjured group, P < .05. The increase in Ki67 nuclear labeling at 24 hours after injury was not significantly different from that of sham animals (P = .22). Temporal changes in SMG cells showed a pattern similar to ciliated duct cells. The mean score for the sham group was 0.4 ± 0.2. Scores from sheep 24, 48, and 72 hours after injury were all significantly increased compared with the sham group, P < .05. Scores from sheep 48 and 96 hours after injury were significantly greater than those of the 24 hours postinjured animals, P < .05. Micrographs showing cells of the ciliated duct with nuclear staining of Ki67 from a sham animal and from an animal 48 hours after S+B injury are shown in Figure 1A, 1B respectively.

Assessing the degree of ciliated duct and SMG cells expressing nuclear Ki67 in ALB-treated animals and their respective control tissues 48 hours after injury showed a trend of a greater percentage of cells with nuclear Ki67 staining in ALB-treated sheep compared with controls. The mean percent of Ki67 nuclear labeling in the ciliated duct region of S+B
controls was 30.4 ± 16.4 compared with 36.1 ± 7.5 in the ALB-treated animals, \( P = .5 \). Proliferation scores of the deeper SMG cells were 7.4 ± 4.9 and 14.4 ± 4.9 for control and ALB-treated sheep, respectively. Using a one-tailed \( t \)-test, the difference in mean score of the ALB-treated animals was significantly greater than that of controls, \( P = .048 \). Graphical presentation of the degree of Ki67 nuclear staining in both ciliated duct and SMG cells from animals in the ALB study are presented in Figure 2.

Assessment of proliferation of ciliated duct and SMG cells from animals 48 hours after injury and treatment with nebulized tiotropium, compared with that of the study’s control group, is shown in Figure 3. The mean scores for ciliated duct cells were 53.4 ± 24.3 and 60.1 ± 15.6 for control and tiotropium-treated animals, respectively. The difference in Ki67 percent staining in the ciliated duct region was not significantly different, \( P = .60 \). In contrast to the ciliated duct cells, SMG cells of the tiotropium-treated animals showed a statistically significant increase in the percentage of cells showing Ki67 nuclear staining compared with the injured and untreated controls. The mean score from control animals was 9.9 ± 4.4 compared with 37.6 ± 6.8 in tiotropium-treated animals, \( P = .001 \). Representative micrographs showing the change in the degree of Ki67 nuclear staining in control and tiotropium-treated animals are shown in Figure 4A, 4B.

### DISCUSSION

Airway gland cells are a large epithelial cell population within the walls of trachea and bronchi that are essential in the innate airway defense system and for maintaining a sterile lung environment. However, in airway diseases such as chronic obstructive pulmonary disease, asthma, and chronic bronchitis, hyperplasia of gland cells and excessive gland secretion contribute significantly to morbidity and mortality. Sheep have an airway submucosal gland system most similar to humans, which has allowed us to study this cell population in the pathogenesis of the acute reaction to inhalation injury. In previous

<table>
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<th>Time Postinjury</th>
<th>Main Bronchial Exfoliation</th>
<th>Ciliated Duct Cell Proliferation</th>
<th>Submucosal Gland Cell Proliferation</th>
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<tr>
<td>0 (n = 5)</td>
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<td>5.4 ± 5.9</td>
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<td>24 (n = 6)</td>
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<td>48 (n = 7)</td>
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<td>68.3 ± 9.5†</td>
<td>12.7 ± 5.7†</td>
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<td>72 (n = 3)</td>
<td>78.3 ± 25.6*</td>
<td>60.1 ± 12.3*</td>
<td>12.3 ± 10.1*</td>
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<tr>
<td>96 (n = 4)</td>
<td>100 ± 0.0*</td>
<td>61.8 ± 20.3*</td>
<td>12.3 ± 1.5†</td>
</tr>
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Proliferation was assessed by quantification of nuclear expression of Ki67 as described in “Methods.” Data represent means ± SDs. 0 time represents sham, instrumented but uninjured animals.

* Significantly different than the mean of the sham group, \( P < .05 \).

† Significantly different than the 24-hour group (\( P < .05 \)).

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Figure 1. A, Micrograph showing Ki67 nuclear staining of a cell (arrow) in the ciliated duct of an uninjured sheep. B, Micrograph showing abundant Ki67 nuclear staining of ciliated duct cells from a sheep 48 hours after S+B injury. The mean difference in Ki67 staining was significantly increased in the 48-hour sheep compared with sham-treated animals, \( P < .05 \). Bar = 50 µm.
studies, we have shown that inhalation injury causes almost total depletion of gland cell cytoplasmic stored mucins and that inhalation injury causes these cells to produce numerous inflammatory cytokines and growth factors.

Although SMG cells are important in the acute pathology of inhalation injury, the frequency of gland ducts that open to the airway lumen, approximately 1 duct/mm², allows this cell population to contribute rapidly to airway lining epithelial repair. Studies have shown that gland cells contain both α and β adrenergic receptors and M1 and M3 muscarinic receptors such that bronchodilator therapies used in the acute management of inhalation injury may modify the proliferative and inflammatory response of these cells. To our knowledge, no studies have examined the effects of bronchodilators on this unique cell population in either normal or pathologic conditions.

The first aim of this study was to characterize the degree of main bronchial airway exfoliation after S+B injury and to measure the dynamics of gland cell proliferation. The second aim of the study was to assess the effects of ALB and tiotropium bronchodilator therapies on the proliferative response of gland cells compared with respective control animals that were injured and received an equal volume of nebulized saline as the treated animals.

Assessment of the degree of main bronchial epithelial exfoliation after injury showed significant levels of exfoliation compared with sham animals. At 24 hours after injury, the mean score for exfoliation

Figure 2. Graph depicting the mean percent (±SEM) of ciliated duct and submucosal gland (SMG) cells exhibiting Ki67 nuclear staining in smoke inhalation and burn-injured sheep that were nebulized with saline (S+B) and injured sheep that were treated with 40 mg/hr nebulized albuterol (S+B+ALB). Statistical increase in the mean degree of cell proliferation of the submucosal gland cells of injured sheep treated with ALB compared with the mean of the control animals using a one-tailed t-test, P = .048.

Figure 3. Graph depicting the mean percent (±SEM) of ciliated duct and submucosal gland (SMG) cells exhibiting Ki67 nuclear staining in smoke inhalation and burn-injured sheep that were nebulized with saline (S+B) and injured sheep that were treated with nebulized tiotropium bromide (S+B+TB). No statistically significant difference in the degree of proliferation was detected in the ciliated duct cell population, P = .60. The mean number of Ki67 nuclear labeling in the SMG population of the S+B+TB group was statistically increased compared with the mean score from the injured and untreated group (S+B), P = .001.

Figure 4. A, Micrograph showing Ki67 nuclear staining of SMG cells (arrows) of an untreated, S+B injured sheep. B, Micrograph showing abundant Ki67 nuclear staining of SMG cells (arrows) from a tiotropium bromide–treated sheep 48 hours after S+B injury.
was 64%. At 96 hours after injury, all four animals showed complete exfoliation of the columnar lining epithelium with focal areas of reparative, nondifferentiated epithelial cells of one to three cell layers. Assessment of Ki67 nuclear labeling found that both ciliated duct and deep SMG cells show an increase in the number of nuclear stained cells beginning 24 and 48 hours after injury, respectively. The increase in the number of cells staining was sustained in both cell populations through 96 hours after injury.

Results of our assessment of ALB treatment on cell proliferation after S+B injury did not indicate any significant difference in the degree of proliferation of the ciliated duct cells. The respective mean scores of 30 and 36% for the control and ALB-treated sheep are less than the 68% score seen in tissues 48 hours postinjury of the temporal assessment, and less than the 53% mean score in the control sheep from the tiotropium study. Given that both the control and ALB-treated sheep received continuous saline nebulization, one can conclude that saline nebulization may reduce ciliated duct cell proliferation. This possibility would be important in the design of future studies to compare the regenerative potential of different pharmacological agents. With respect to the deeper SMG cells, a trend of increased Ki67 nuclear labeling was seen in the ALB-treated sheep relative to the control animals. Using a one-tailed t-test provides statistical significance difference between the means with a P value of .048. As previously presented, tissues for this assessment of proliferation derived from the study by Palmieri et al6 included only five animals per group. Possibly, a larger sample size would more easily allow identification of β-2 agonist receptor promotion of SMG cell proliferation after inhalation injury, which would be consistent with in vitro studies showing β-2 adrenergic receptor–induced proliferation of specific epithelial cell lines through a mitogen-activated kinase pathway.22

In contrast to the results seen in the ALB assessment above, tiotropium-treated animals showed a significant increase in SMG proliferation, approximately 4-fold, compared with their respective control animals. The increase in SMG proliferation with tiotropium suggests that acetylcholine (ACH) may have an inhibitory function in gland cell proliferation after inhalation injury. However, this conclusion contradicts the paradigm that ACH and muscarinic receptor activation is promitogenic. Nonneuronal ACH and muscarinic receptor activation have been shown to be involved in cancer cell proliferation.37–40 In part, the induced proliferation is mediated by activation of muscarinic receptors coupled to mitogen-activated protein kinase and transactivation of the epidermal growth factor receptor.42–44 Studies in airway tissues show that ACH and M3 receptor activation acts synergistically with platelet-derived growth factor to induce airway smooth muscle mitogenesis.20 However, a study of ACH signaling in a murine embryonic cell line found that muscarinic receptor activation decreased cell proliferation but increased cell viability.45 The authors concluded that ACH and its receptors may have different and/or opposing functions depending on the cell types and processes involved. Although ACH may have a direct effect on gland cell proliferation, several studies show involvement of muscarinic processes in inflammation46 and that muscarinic antagonists suppress ACH-induced release of cytokines and leukotrienes.47–49 Data from a recent study using tiotropium therapy showed that the degree of airway submucosal gland neutrophilia, a measure of airway inflammation, was significantly decreased in tiotropium-treated sheep compared with the injured controls (unpublished data). Conceptually, a reduction in inflammatory cell–induced oxidative stress in the tiotropium-treated sheep may reduce protein and DNA damage within gland cells to allow these cells to more effectively participate in airway repair. Support for this concept is found in the study by Jope et al,50 which showed that oxidative stress oppositely modulated a process stimulated by muscarinic receptors.

Although not a direct part of this study, the increased degree of Ki67 nuclear staining seen with tiotropium treatment prompted the study of two uninjured animals given nebulized tiotropium (18 µg every 4 hours for 48 hours). The degree of Ki67 nuclear labeling in the SMG cells of these animals was 1.2 and 2.4%, similar to the degree of labeling seen in our sham animal (1.7 ± 1.8). We conclude from this assessment that the increase in proliferation seen with tiotropium treatment appears to be restricted to animals with injury to the airway lining epithelium. Also included in our Ki67 immunostaining procedure was archived bronchial tissue from a 40% TBSA, burn-only injured sheep that had been studied for 48 hours. The percent of SMG cells showing Ki67 nuclear localization was 0.02, suggesting that burn injury alone does not contribute to an increase in SMG proliferation.

In conclusion, this retrospective study using archived tissues demonstrates that both ciliated duct and SMG cells of sheep show an increase in Ki67 nuclear labeling after inhalation injury. Increased Ki67 nuclear localization was present from 48 hours in both the ciliated duct region and in the deeper...
SMG cells, and was maintained through 96 hours after injury. Significant increases in proliferation were seen in the SMG cells of ALB- and tiotropium-treated sheep suggesting that bronchodilator therapies used to combat the acute bronchoconstrictive response after an inhalation injury may modify the proliferative response of the glandular epithelium. Future studies using the ovine model of inhalation injury to compare the efficacy of different classes of bronchodilators on the airway epithelial regenerative processes are warranted. Improved mechanistic understanding of airway epithelial repair after inhalation injury is essential for future clinical research that can significantly reduce the respiratory associated morbidity and mortality of burn victims with inhalation injury.

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