Effects of corticosteroid-induced apoptosis on airway epithelial wound closure in vitro

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Corticosteroids are potent anti-inflammatory agents in the treatment of asthma (10). This effect is a result, in part, of apoptosis induced in inflammatory cells such as eosinophils (34, 46) and T lymphocytes (8). However, long-term therapy using corticosteroids does not necessarily reverse epithelial damage seen in asthma. Epithelial destruction is seen in subjects with chronic asthma despite regular inhaled corticosteroid therapy (2). The mechanism by which epithelial denudation persists after repeated injury, even as corticosteroid treatment resolves inflammation, is not clear. There is no clear evidence to suggest that repair can begin if inflammation is suppressed.

We previously demonstrated (13) that corticosteroid treatment induces apoptosis of cultured airway epithelial cells. Corticosteroid treatment induces concentration- and time-dependent apoptosis that is associated with disruption of mitochondrial polarity, cytochrome c extrusion from mitochondria, and activation of caspase-9 and is associated with suppression of epithelial cell proliferation. These results suggest that in the in vitro setting the effects of these agents on airway epithelium differ greatly from the effects seen with other types of epithelial cells such as mammary gland cells, in which corticosteroids protect against apoptosis (19).

Repair of a damaged airway epithelium is an ordered process that begins soon after injury and involves progressive steps of plasma leakage into the site of injury from underlying venules (15), spreading of neighboring cells, migration of cells into the site of injury, proliferation of new cells to replace lost cells, and differentiation into required new cells (21, 27). Small wounds can be covered completely in <24 h by migration and spreading alone (21). In the asthmatic airway, epithelial damage is persistent; this may reflect ongoing damage due to environmental or inflammatory agents. Another potential mechanism for impaired repair may be induction of apoptosis and suppression of proliferation by glucocorticoid treatment. To examine this, we used an in vitro model that allowed us to examine spreading and migration of cells over collagen. Our data demonstrate that wound closure was significantly impairs epithelial cell migration. The addition of corticosteroids after injury does not slow migration, despite their ability to induce apoptosis in these cells.

epithelium; cell spreading

THE AIRWAY EPITHELIUM is a target of inflammatory and physical insults in asthma. Injury to the epithelium is a common finding in pathological studies of patients with asthma, even when the clinical state of disease is mild (3, 29). Epithelial damage correlates with airway hyperreactivity in asthmatic subjects (24), is seen in almost all subjects with persistent asthma (39), and may be seen in newly diagnosed asthma (30). Combined with evidence that the epithelium may also serve as an “effector” of airway inflammation (33, 36) and remodeling (23), the combination of epithelial cell damage, desquamation, and goblet cell hyperplasia (35) suggests a central role for the epithelium in this disease.

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MATERIALS AND METHODS

Materials. Penicillin, streptomycin, trypsin, epidermal growth factor (EGF), human placental collagen IV, sodium bicarbonate, formalin

solution, and dexamethasone were obtained from Sigma (St. Louis, MO). Fetal calf serum (FCS) was obtained from Hyclone (Logan, UT) and was heat denatured before use. Terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling (TUNEL) TACS II fluorescent assay kits were purchased from Trevigen (Gaithersburg, MD). Anti-Fas MAb (CH11) was purchased from MBL International (Watertown, MA). Dulbecco’s modified Eagle’s medium (DMEM) was purchased from Fisher. Geneticin was purchased from GIBCO BRL-Life Technologies.

**Cell culture.** The cell line 1HAEo−, a gift of Dieter Grunen (University of Vermont, Burlington, VT), are simian virus 40-transformed human airway epithelial cells (9) that have cell surface markers similar to primary airway basal epithelial cells (11). 1HAEo− cells stably transfected to overexpress either Bcl-2 (1HAEo−/Bcl-2−) or control empty insert (1HAEo−/neo−), previously created and validated (11), were used in this study. Cells were grown on collagen-coated plates or glass slides as previously described (11, 13).

**Wound repair assay.** We described this assay previously (43, 45). Briefly, pretreated monolayers were incubated for 0–48 h in dexamethasone and serum-containing DMEM (10% FCS). Dexamethasone treatments were replaced every 24 h. Before mechanical wounding, cell monolayers were washed and placed in serum-free medium. Wounds were made in the confluent monolayer with a rubber stylet to remove cells without disturbing the underlying protein matrix. Dexamethasone-pretreated and control monolayers were immediately treated as indicated, and wound closure was followed for 24 h by serial digital photography. The area of the remaining wound in each image was measured with either NIH Image or ImagePro Plus software and expressed as a percentage of the original wound area, so that each wound served as its own control.

**Apoptosis assay.** Linear wounds ∼1–1.5 mm in width, or ovoid wounds ∼1 mm² in diameter, were created in confluent 1HAEo−/neo− cell monolayers grown in two chamber glass slides coated with collagen IV. Wound width or size was chosen to ensure that closure would not be complete in 24 h. Cells were then treated with sham vehicle, 1 μg/ml of the CH11 MAb, or 10 μM budesonide for 24 h and fixed in 10% neutral buffered formalin. Apoptotic cells were demonstrated by labeling free 3′-hydroxyl groups of DNA with a TUNEL TACS II fluorescent assay kit (Trevigen), with a method we described previously (13, 44). Counting of apoptotic cells for linear wounds was done in two sets for each well: four regions within 50 μm of the wound edge and four regions at least 1 mm from the wound edge. Each set of regions was averaged to generate a single n for that set. For ovoid wounds, at least four regions per well at least 1 mm away from the wound edge were counted and averaged to generate a single n.

**Data analysis.** Data are expressed as means ± SE. Differences between groups were analyzed with either Student’s t-test or analysis of variance. When significant differences were found by F-test, post hoc analysis was done with Fisher’s protected least significant difference test. Differences were considered significant when P < 0.05.

**RESULTS**

**Wound closure after corticosteroid treatment.** Corticosteroid treatment induces apoptosis in airway epithelial cells (13), and it is possible that this cell death impairs repair after injury. To test this, we examined wound closure in 1HAEo−/neo− cell monolayers treated with 0–10 μM budesonide. The initial wound area in these experiments was 0.99 ± 0.03 mm² (n = 38 monolayers). Wound closure was >90% complete in <24 h in virtually all experiments. Treatment with any concentration of budesonide did not significantly slow closure (Fig. 1A). After 12 h, the remaining wound area in monolayers treated with 10 μM budesonide was 25 ± 3% vs. 18 ± 3% for untreated control monolayers (P = not significant NS; n = 8 in each group). We then examined closure in separate experiments in which initial area was greater: 2.15 ± 0.16 mm² (n = 16 monolayers). In these experiments, remaining wound area referenced back to initial area was greater per unit time, but velocity of closure (mm²/h) was similar in both sets of experiments (data not shown). Treatment with 10 μM budesonide did not significantly slow closure velocity. The remaining wound area after 12 h in monolayers was 39 ± 4% vs. 43 ± 3% for untreated control monolayers (P = NS; n = 8 in each group) (Fig. 1B).

We then asked whether other corticosteroids might inhibit closure. In these experiments, cells were treated with 0, 3, or 10 μM dexamethasone for 24 h after mechanical injury. The initial wound area in these experiments was 1.29 ± 0.10 mm² (n = 21 monolayers). As in the experiments with budesonide, neither concentration of dexamethasone inhibited repair (Fig. 1C). We then examined the effect of either budesonide or dexamethasone in the presence of a growth factor that accelerates wound closure (24). Addition of 40 ng/ml EGF accelerated wound closure modestly compared with control monolayers. In monolayers treated with EGF, concomitant treatment with either corticosteroid did not slow the rate of wound closure (data not shown).

**Prior exposure to dexamethasone inhibits wound closure.** Daily use of inhaled corticosteroids, as commonly prescribed for patients with persistent asthma, would result in chronic exposure of airway epithelial cells to these agents. We asked whether the addition of dexamethasone before injury might inhibit subsequent wound closure. In these experiments, 1HAEo−/neo− cells were pretreated with 0.3–10 μM dexamethasone for either 24 or 48 h, continuing for 24 h after injury. Controls included monolayers not pretreated with dexamethasone and monolayers treated with 10 μM dexamethasone for either 24 or 48 h followed by injury and wound closure in the absence of corticosteroid.

The initial wound area in the 24-h pretreatment experiments was 1.11 ± 0.03 mm² (n = 48 monolayers). There was no difference in initial wound area between any of the treatment groups. In monolayers pretreated with 0.3–10 μM dexamethasone for 24 h, wound closure after injury was significantly slowed compared with untreated monolayer wounds (Fig. 2A). At 12 h after injury, closure in monolayers pretreated with 10 μM dexamethasone was 61 ± 2% of time 0 area vs. 35 ± 5% for untreated monolayer wounds (P = 0.001; n = 8). However, by 18 and 24 h, wound closure was equivalent in all groups. In monolayers pretreated with 10 μM dexamethasone in which the agent was removed immediately after wounding, there was significant inhibition of closure compared with control at 12 h (56 ± 2% vs. 35 ± 2% for control; P < 0.0001) but not at 24 h.

In monolayers pretreated for 48 h before injury, slowing of wound repair in the first 12 h was more pronounced. The initial wound area in these experiments was 1.01 ± 0.03 mm² (n = 48 monolayers). There was no difference in initial wound area between any of the treatment groups. In monolayers pretreated with 0.3–10 μM dexamethasone for 48 h, wound closure after injury was significantly slowed compared with untreated monolayer wounds (Fig. 2B). At 12 h after injury, closure in monolayers pretreated with 10 μM dexamethasone was 64 ± 2% of time 0 area vs. 49 ± 2% for untreated monolayer wounds (P = 0.0005; n = 8). By 18 and 24 h, wound closure was equivalent in all groups. In monolayers pretreated with 10
In which the agent was removed immediately after wounding, there was significant inhibition of closure compared with control at 12 h (66 ± 2% vs. 49 ± 2% for control; *P < 0.0001) but not subsequently.

Resistance to apoptosis is not found in cells at wound edge. Given these data, one potential explanation for the lack of slowed wound repair in corticosteroid-treated monolayers is that cells at the leading edge of repair are resistant to apoptosis. To test this, wounds were created in 1HAEo−.neo cell monolayers, followed by treatment with 0 or 10 μM dexamethasone. Apoptotic cells could be visualized at and near the wound edge and in cells away from the wound edge. After treatment for 24 h, the proportion of apoptotic cells at the wound edge and at points away from the wound was similar and <1% in control wounds (Fig. 3A). In wells treated with 10 μM budesonide,
apoptosis at and away from the wound edge was increased and similar at both locations (Fig. 3A). Apoptotic cells were distributed in random order at the wound edge and were not clustered. In additional experiments, experiments were repeated using the CH11 MAb to activate the Fas receptor. This receptor is found on airway epithelium (22) and induces apoptosis in several cell types, including airway and alveolar epithelium (13, 20). Wounds were created in 1HAEo neo cell monolayers, followed by treatment with 1 μg/ml of the CH11 MAb. As in experiments using budesonide, apoptotic cells

![Fig. 3. A: proportion of apoptotic cells, as demonstrated by terminal deoxynucleotidyltransferase-mediated dUTP biotin nick end labeling (TUNEL), in airway epithelial monolayers 24 h after wound creation. Monolayers were treated with 0 or 10 μM budesonide for 24 h after wound creation, after which cells were fixed and labeled. \*P = 0.001 vs. control at wound edge (edge); \$P = 0.0002 vs. control away from wound edge (away). Differences for edge vs. away within each treatment group were not significant; n = 5 for control monolayers and 7 for monolayers treated with budesonide. B: proportion of apoptotic cells, as demonstrated by TUNEL, in airway epithelial monolayers 24 h after wound creation. Monolayers were treated with 1 μg/ml of the CH11 MAh to activate Fas or sham vehicle for 24 h after wound creation, after which cells were fixed and labeled. \*P = 0.0001 vs. appropriate control edge. Differences for edge vs. away within each treatment group were not significant; n = 6 for each group. C: proportion of apoptotic cells, as demonstrated by TUNEL, in airway epithelial monolayers 24 (left) and 48 (right) h after pretreatment with 0.3–10 μM dexamethasone, followed by wounding and treatment for an additional 24 h, after which cells were fixed and labeled. Control monolayers were not pretreated, and additional monolayers were pretreated with 10 μM dexamethasone but not treated with this agent after wound creation (pre-treat). Cells away from wound edges only are presented; cells at the wound edge were similar. \*P = 0.01, \$P = 0.0005, \#P < 0.0001 vs. control; \*\*P = 0.02, \$\$P = 0.0005 vs. 10 μM dexamethasone throughout the experiment; n = 4 for each group.]

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could be visualized at and near the wound edge and in cells away from the wound edge. After treatment for 24 h, the proportion of apoptotic cells at the wound edge and at points away from the wound was similar and <1% in control wounds (Fig. 3B). In wells treated to activate Fas, apoptosis at and away from the wound edge was increased and similar at both locations (Fig. 3B). As in experiments using budesonide, apoptotic cells were distributed in random order at the wound edge and were not clustered. These data suggested that cells at and near the wound edge are not relatively protected from cell death regardless of the apoptotic stimulus.

We also evaluated whether changes in wound closure in cells pretreated with dexamethasone were due to apoptosis. In these experiments, 1HAEo− cells used in the preceding wound repair experiments, pretreated for 24 or 48 h with 0.3–10 μM dexamethasone, were labeled for the presence of apoptotic cells at the conclusion of the 24-h observation point after wounding. Areas at least 1 mm away from any wound edge were imaged after TUNEL labeling. As shown in Fig. 3C, there was substantial apoptosis in cells pretreated with up to 10 μM dexamethasone for either 24 or 48 h. The proportion of apoptotic cells was substantially greater with pretreatment compared with cells treated only after wounding (compare to Fig. 3A). Removal of dexamethasone after wounding, after the pretreatment period, attenuated significantly the proportion of apoptotic cells in both pretreatment groups. However, there were no differences in remaining wound area at 24 h in either pretreatment group (Fig. 2) despite the marked degree of apoptosis seen (Fig. 3C). This suggested that apoptosis, even when substantial, did not have a significant effect on epithelial cell migration.

We then examined wound closure in monolayers of 1HAEo−.Bcl-2+, a cell line relatively resistant to the apoptosis-inducing effect of corticosteroids (13). The initial wound area in these experiments was 1.60 ± 0.12 mm² (n = 20 monolayers). Treatment with 10 μM dexamethasone did not affect the rate of wound closure (Fig. 4A). Addition of 40 ng/ml EGF modestly accelerated wound repair: at 18 h, the remaining wound area was 21 ± 3% in control cells and 13 ± 4% in cells treated with EGF. However, the addition of 10 μM dexamethasone to cells treated with EGF did not slow repair (Fig. 4B).

To ensure that the inhibition was not a result of the increasing apoptosis as noted with corticosteroid pretreatment (Fig. 3C), we carried out the same pretreatment experiments in the Bcl-2-overexpressing cell line 1HAEo−.Bcl-2+ (13). The initial wound area in the 1HAEo−.Bcl-2+ monolayers was 1.55 ± 0.11 mm² (24-h pretreatment) and 1.51 ± 0.08 mm² (48-h pretreatment) (n = 24 monolayers for each pretreatment group). After dexamethasone pretreatment and injury, the Bcl-2-overexpressing monolayers (Fig. 5) had similar wound closure inhibition relative to the nontreated dexamethasone-treated 1HAEo−.neo cells (Fig. 2). Pretreatment for 48 h before injury dramatically inhibited closure relative to the untreated control (Fig. 5B). At 12 h after injury, closure in the 1HAEo−.Bcl-2− monolayers pretreated with 10 μM dexamethasone was 60 ± 3% of time 0 area vs. 33 ± 3% for untreated monolayer wounds (P = 0.004; n = 4). This dexamethasone effect on wound closure was observed up to 24 h after wounding. These data suggested that blocking apoptosis by the overexpression of Bcl-2 did not alter the inhibitory response of dexamethasone on epithelial cell migration.

![Fig. 4. Effect of dexamethasone on wound closure in 1HAEo−.Bcl-2+ cell monolayers. Cells were treated with 0 or 10 μM dexamethasone for 24 h after wound creation (A; n = 6). Additional cells were also treated with 40 ng/ml epidermal growth factor (EGF) (B; n = 4). No points were significantly different from control at 12 h. Some error bars are within symbols.](http://ajplung.physiology.org/)

We finally asked whether inducing apoptosis with an agent other than a corticosteroid would affect wound repair. In these experiments, cells were treated with the CH11 MAb to activate Fas immediately after wound generation. The initial wound area in these experiments was 0.89 ± 0.04 mm² (n = 24 monolayers). Fas activation did not appreciably change wound closure or the rate of closure compared with nontreated cells (Fig. 6).

**DISCUSSION**

Corticosteroid therapy is a main controller therapy used to control asthma symptoms because of its anti-inflammatory effects. Corticosteroids elicit apoptosis of lymphocytes (8) and eosinophils (34, 47) and also suppress the production and release of inflammatory cell indicators (41). Corticosteroid therapy does not necessarily reverse epithelial damage seen in asthma. Although corticosteroid therapy clearly suppresses inflammation (1, 2) and release of epithelial cell-derived inflammatory mediators (42), subjects with severe asthma receiving regular inhaled corticosteroid therapy still have substantial...
Epithelial destruction at all levels (31). Epithelial cells are seen in bronchoalveolar lavage fluid of patients with asthma of varying severity, despite corticosteroid therapy (19). Because epithelial restitution occurs quickly after a single insult (16), the mechanism by which epithelial denudation persists after repeated injury in asthma is not clear.

Recent studies in our laboratory suggest that corticosteroids may elicit apoptosis of airway epithelium both in culture models (13) and in a mouse model (12). The question arises as to whether corticosteroid-induced cell death is deleterious in airway repair after injury. One previous study suggested that apoptosis of airway epithelium in endobronchial biopsies was an uncommon event in patients with mild asthma (40). In contrast, two more recent studies demonstrated increased numbers of apoptotic epithelial cells in endobronchial biopsies collected from asthmatic airways compared with normal airways (4, 37). In the first study, apoptosis persisted despite treatment with inhaled corticosteroids and clinical control of disease. This suggests that epithelial cell damage and apoptosis may not be ameliorated by corticosteroid treatment, even though inflammation is controlled.

The present study examined the potential effects of corticosteroids on wound closure in cultured epithelial cells. We also examined the possibility of an existing resistance to apoptosis by the cells at the wound edge, generally considered to be the cells that initially migrate and spread into a site of injury. Our data demonstrate that Fas activation or corticosteroids at the time of injury had no significant effect either on the time required for wound closure or on the rate of closure, despite the increased apoptosis seen in cells treated with either agonist both at and away from the wound edge. As the rate of apoptosis was relatively low, one explanation for these findings is that there are sufficient remaining cells near the wound edge unaffected by corticosteroid treatment to affect normal repair: until the rate of cell death reached a critical threshold, closure of small wounds would be unaffected. In sharp contrast, pretreatment of cells with corticosteroid slowed migration significantly after injury. This effect could not be reversed significantly by removing the corticosteroid after injury (Fig. 2), even though such removal did attenuate substantially the proportion of apoptotic cells (Fig. 3C). These data suggest that the presence of corticosteroids at the time of injury due to treatment before and during repair slows processes required to initiate closure of the wound.

Closure of epithelial wounds involves three steps, taken together and in sequence: spreading of adjacent cells into the wound region, migration of nearby cells further into the region, and proliferation of new cells (27). To our knowledge, our report is the first to demonstrate a significant effect of corticosteroids on airway epithelial cell migration. This effect is both...
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time dependent and reversible. The longer the exposure of corticosteroids to the airway epithelial cells, the greater the reduction in wound closure. If the corticosteroid was removed at the time of injury, even with 48 h of prior exposure, wound closure was less impaired. This timing suggests that it is not apoptosis of the airway epithelial cells that is responsible for the impaired closure of small wounds. This was later confirmed with a cell line that overexpresses the antiapoptotic protein Bcl-2, a cell line that was previously shown to be resistant to dexamethasone-induced apoptosis (13). Together, these data suggest that the effect of corticosteroids on slowing airway epithelial cell migration and wound repair is not related to their ability to induce apoptosis.

Although spreading of adjacent cells is useful for repairing small gaps created by the loss of a single or very few cells (as one example, repairing loss of cells due to apoptosis in retinal pigment epithelium; Ref. 26), spreading is less useful to close larger wound regions. A monolayer injury of ~1.0-mm² area and ~4-mm perimeter, given an average single cell width at the wound edge of ~20 μm and an average single cell area of ~400 μm², will have ~2,500 cells removed and 200 cells at the circumference. Under these circumstances, it is clear that spreading will have relatively little impact in wound closure, and most early (<24 h) closure occurs by migration of nearby cells.

Corticosteroids suppress proliferation of airway epithelial cells (13), and this may have a role in delaying repair of larger areas of damage. We are aware of one preliminary study that demonstrates such an effect in airway epithelium (32). In that study, initial wound area was larger (~3 mm²), and a longer time was required for closure. Treatment with up to 1 μM dexamethasone delayed closure significantly in their model. In contrast, initial wound area in our model was smaller (~0.7–1.6 mm²) such that near-complete closure was seen in ~24 h despite treatment with higher concentrations of corticosteroids. We previously demonstrated (43) that in such wounds cell proliferation as assessed by cyclin B₁ expression in cells at the wound edge is <3% in the first 24 h of closure. This is in accord with the work of Zahm et al. (47), which demonstrates that closure of small wounds in respiratory epithelium is done almost completely by cell spreading and migration. Our data on wound closure, combined with that of Luppi et al. on proliferation (32), suggest that the effects of corticosteroids on wound closure may result from effects on both proliferation and cell migration. Combined with our previous data demonstrating effects of cell survival (13), these data suggest that corticosteroids may have significant effects on airway epithelial repair after injury. Corticosteroid exposure may be affecting the expression of molecule(s) by the airway epithelial cells needed for repair. If persistent exposure of the airway epithelium to corticosteroids can impair the repair process, this observation may correspond with recent observations that intermittent corticosteroid use in asthmatic patients provided symptom relief similar to that with continuous use (6, 7). Continuous use of inhaled corticosteroids may suppress inflammation within the airway, but this effect may be offset, to an uncertain degree, by negative effects on the epithelium. In this regard, it is interesting that similar mechanisms are proposed to explain how corticosteroid treatment in inflammatory bowel diseases may impair intestinal epithelial wound restitution and proliferation (25). Despite the extensive use of these agents in the clinical therapy of asthma, there is no clear agreement on whether inhaled corticosteroids prevent asthma progression and airway remodeling (41).

The highest concentration of corticosteroid used in this study, 10 μM, is approximately the maximum predicted local concentration at the apical face of exposed central airway epithelial cells achieved by the highest doses used in clinical settings (13). Only two studies to date have attempted to identify the local concentrations of inhaled glucocorticoids. Distribution studies of fluticasone and budesonide in human subjects suggests airway mucosal and tissue concentrations in at least the high nanomolar range (17, 38). Our data suggest that such concentrations of corticosteroid, when present before injury, slow airway epithelial cell migration.

One potential concern in the present study is the use of cultured cells. Although some morphological features of the cells used in our study are not the same as the pseudostratified columnar epithelium seen in vivo, the monolayers are uniform and thus permit assessment of wound closure without the potential confounding events of difference between cell types (e.g., ciliated columnar cells or secretory cells) or of phenotype shift from one morphology to another. Basal epithelial cells are considered to be a stem cell for epithelial reconstitution in human airways (5, 18), and the cell line we use has characteristics of basal cells (11). We previously demonstrated in wound repair models (43, 45) and in epithelial cell apoptosis models (13) that cell lines provide a reasonable approximation of primary cells in standard submersion culture conditions.

In conclusion, we demonstrate that prior exposure to corticosteroids impairs airway epithelial cell migration and spreading in an in vitro assay of wound repair. Although corticosteroids induce apoptosis and reduce proliferation, such processes were not relevant in blocking small wound closure in our model. Our data suggest that corticosteroids may have an effect on the early changes in cell migration in epithelial repair after injury. How the effect of corticosteroids on wound repair may play a role in airway remodeling or the clinical treatment of asthma remains to be explored.

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