Corticosteroid-Induced Apoptosis of Airway Epithelium*

A Potential Mechanism for Chronic Airway Epithelial Damage in Asthma

Steven R. White, MD; and Delbert R. Dorscheid, MD, PhD, FCCP

Damage to the airway epithelium is one prominent feature of chronic asthma. Mucosal damage includes gap openings, partial denudation, and loss of ciliated cells. Apoptosis of the airway epithelium is increas-

*From the Section of Pulmonary and Critical Care Medicine (Dr. White), Department of Medicine, University of Chicago, Chicago, IL; and the iCAPTURE Center (Dr. Dorscheid), University of British Columbia, Vancouver, BC, Canada. This research was supported by grants HL-60531 and HL-63300 from the National Heart, Lung, and Blood Institute, and by grant CIHR 43898 from the Canadian Institute of Health Research. Correspondence to: Steven R. White, MD, Section of Pulmonary and Critical Care Medicine, The University of Chicago, 5841 S Maryland Ave, MC6076, Chicago, IL 60637; e-mail: swhite@medicine.bsd.uchicago.edu

ingly recognized as a potential mechanism by which damage may occur. Corticosteroids (CSs) induce apoptosis in inflammatory cells, which in part explains their ability to suppress airway inflammation. However, CS therapy does not necessarily reverse epithelial damage. We examined whether CS therapy actually could induce airway epithelial apoptosis using culture models of primary airway epithelial cells and cell lines. The administration of CSs in low-micromolar concentrations induces apoptosis that involves the disruption of mitochondrial polarity, the activation of caspases, and the involvement of Bel-2. Clear differences exist between CS-induced apoptosis in the cultured epithelium vs cultured hematopoietic cells in regard to time course and resistance to apoptosis. Our data suggest that the use of CSs, in concentrations that could be attained in vivo with the inhalation of potent preparations or with systemic administration, may be one factor in the Airways remodeling and epithelial damage that is seen in many patients with chronic, persistent asthma.

(CHEST 2002; 122:278S–284S)

Key words: airway epithelium; asthma; apoptosis; corticosteroids; remodeling

Abbreviations: ASL = airway surface liquid; CS = corticosteroid; GR = glucocorticoid receptor; TUNEL = terminal deoxyuridineyl transferase mediated dUTP

The airway epithelium is a target of inflammatory and physical stimuli in the patient with asthma. Injury to the epithelium is a common finding in the patients with asthma, even when the clinical state is mild.1,2 Epithelial damage correlates with airway hyperreactivity in asthmatic patients,3 is seen in about half of subjects with mild asthma and in almost all subjects with persistent asthma,4 and may be seen in patients with newly diagnosed asthma.5 This suggests that occult inflammation has already initiated the process of mucosal damage and remodeling. Asthmatic subjects shed fourfold as many epithelial cells as do healthy subjects after bronchial washing.6 Many of these are ciliated epithelial cells, which are shed preferentially.7,8 The epithelial layer disruption that has been described in bronchial biopsy specimens obtained during fiberoptic bronchoscopy has been attributed in part to biopsy artifacts.9 However, the increased expression of CD44 cells in the areas of damage10 and epithelial growth factor receptor expression (c-erbB-1)11 as markers of repair,12 along with extensive denudation of the epithelium that is seen in patients with severe and fatal asthma,13 indicates that epithelial damage does occur in vivo. While asthma is an inflammatory disorder of the airways, inflammation alone is insufficient to explain both the chronic nature of the disease and its progression over time. Inflammation must occur in an airway that is susceptible to damage. Epithelial damage, along with other changes such as smooth muscle hypertrophy, basement membrane thickening, and submucosal fibrosis, are cardinal features of chronic airway remodeling, which is the culmination of this combination of inflammation plus structural changes in patients with long-standing, persistent asthma.
Corticosteroids (CSs) elicit apoptosis in inflammatory cells such as eosinophils and T lymphocytes, and suppress the production and release of both inflammatory eicosanoids and cytokines. This in part explains the potent antiinflammatory effects of CSs in asthma patients. However, CS therapy does not necessarily reverse the epithelial damage seen in patients with asthma. While CS therapy clearly suppresses inflammation and the release of epithelial cell-derived inflammatory mediators, subjects with severe asthma receiving regular inhaled CS therapy still have substantial epithelial destruction at all levels. Epithelial cells are seen in the BAL fluid of asthmatic patients with varying severities of the disease, regardless of CS therapy history. Adequate treatment with CSs may not completely prevent airway inflammation, and some patients may continue to have asthma symptoms and a long-term decline in lung function despite their use. Since epithelial restitution occurs quickly after a single insult, the mechanism by which epithelial denudation persists after repeated injury, particularly an injury that has been treated with CSs, suggests several possibilities, as follows: (1) the incomplete suppression of inflammation may allow continued damage to the mucosa; (2) suppressing inflammation does not suppress an underlying defect in the epithelial-mesenchymal unit that permits disordered repair to continue; and (3) CS therapy itself has effects that facilitate or continue the process of disordered repair.

Our laboratory has focused on the last possibility, that CSs may damage the airway epithelium. CSs have long been associated with the suppression of inflammatory cells, and this is mediated in part by apoptosis. CSs elicit rapid, synchronous apoptosis in lymphocytes that is mediated by the glucocorticoid receptor (GR). An activation-deficient mutant, the GR is capable of inducing apoptosis in lymphocytes, suggesting that the primary effect of the GR in mediating apoptosis is via transcriptional repression rather than through activation. In contrast, the effect of CSs on epithelial cells derived from various tissues is variable. CSs impair keratinocyte repair after injury but also protect mammary gland epithelial cells from apoptotic stimuli and inhibit apoptosis induced by Fas ligation in alveolar epithelial cells. In cultured corneal keratinocytes, low concentrations (ie, approximately 10^{-8} mol/L) of dexamethasone, a potent CS, elicit increased proliferation, whereas higher concentrations (ie, approximately 10^{-6} mol/L) increased apoptosis. It is not clear from these studies what the predicted effect of CSs would be on the airway epithelium.

**The Airway Epithelium Undergoes Apoptosis When Stimulated To Do So**

The airway epithelium has death receptors and can undergo apoptosis when these receptors are stimulated. We have demonstrated the expression of the death receptors Fas and its ligand, FasL, in 1HAEo− cells, primary human bronchial epithelial cell in culture, and airway tissue sections from healthy human subjects. In addition, we have demonstrated that airway epithelial cells in culture undergo apoptosis on the cross-ligation of Fas. This occurs in ≈ 24 h, can approximate 30% of all cells, and can be blocked with an appropriate antibody against Fas. While both Fas and FasL are expressed in subjects with fatal asthma and severe, nonfatal asthma, there are no significant differences in either group compared to those with nonasthmatic subjects. This suggests that mechanisms other than Fas ligation may be different in subjects with asthma and may be activated during allergen challenge or environmental insult. In support of this idea, house dust mite cysteine proteinases can induce airway epithelial cell apoptosis. Oxidant-induced apoptosis may be substantially greater in epithelial cells collected from asthmatic subjects compared to cells collected from healthy subjects (DE Davies, PhD; University of Southampton, UK; personal communication). This suggests the presence of at least quantitative, and perhaps qualitative, differences between the normal and asthmatic epithelium.

The asthmatic airway epithelium also may be apoptotic, although the examination of this may depend, in part, on the disease state and its duration. We have recently reviewed a series of subjects who died of asthma and subjects with severe asthma who died of other causes. Terminal deoxynucleotide nick-end labeling (TUNEL) staining of the airway epithelium demonstrates the presence of a substantial proportion of apoptotic cells compared to the presence of very few such cells in the epithelium of...
nonasthmatic subjects (Fig 1). In contrast, one recent article\(^3\) suggested that in patients with mild asthma few apoptotic epithelial cells can be demonstrated to be present, regardless of the CS treatment. These divergent data suggest that, at least in some asthmatic patients, the ongoing damage to the epithelium may be due to an apoptotic loss of cells. The contribution of CSs may be to reduce inflammation, which may reduce damage. Conversely, CSs might induce damage and contribute to the genesis of long-term airway damage.

CSs Elicit Apoptosis of the Airway Epithelium

With this in mind, we recently initiated a study\(^3\) to examine CS-induced apoptosis in the airway epithelium. We originally hypothesized that CSs would protect the airway epithelium from apoptotic stimuli. This hypothesis fit the available data, which suggested that CS therapy would aid in the restitution of a damaged airway epithelium\(^3\) without eliciting damage itself.\(^3\) To our surprise, CS treatment induced apoptosis in central airway epithelial cells.

Figure 2. Left: A: apoptosis in primary human airway epithelial cells after CS treatment or after ligation of the Fas receptor, as demonstrated by TUNEL staining. Cells were treated with dexamethasone or 1 μg/mL CH11 Fas-ligating monoclonal antibody. Apoptosis increased through 24 h and at dexamethasone concentrations of ≥ 3 μmol/L (μM), and was equivalent to that seen with Fas ligation. *= p < 0.05 (vs control subjects); § = p < 0.02 (vs control subjects); † = p < 0.01 (vs control subjects); and ‡ = p < 0.005 (vs control subjects). Right: B: apoptosis in the 1HAEo airway epithelial cell line after treatment with beclomethasone, triamcinolone, or budesonide for 24 h. Apoptosis was significant after treatment with ≥ 3 μmol/L each CS. Ctl = control; CH11 = CH11 Fas-ligating monoclonal antibody. Reprinted with permission from Dorscheid et al.\(^\text{36}\)
cells. In both primary human airway epithelial cells and in the central airway epithelial cell line 1HAEo−, treatment with dexamethasone, budesonide, triamcinolone, or beclomethasone elicited concentration-dependent and time-dependent apoptosis (Fig 2). Little necrosis was present after CS treatment, as demonstrated by a lack of lactate dehydrogenase activity in culture medium after 48 h. Apoptosis occurred with CS concentrations (ie, ≥1 μmol/L) that are at the high end of the range generally used to treat patients with asthma.

Some consideration of the concentrations of glucocorticoids that are attainable in the airway will illustrate this point. CSs must dissolve in the periciliary layer of fluid atop the airway epithelium before entering cells and binding the GR. The average depth of the airway surface liquid (ASL) layer in the first 10 generations of human central airways generally is accepted as being approximately 5 to 25 μm,38,39 and the aggregate area of airways from the trachea to 1 mm diameter is approximately 1,000 cm².40 The total volume in these airways calculates then as approximately 0.5 to 2.0 mL, which matches the estimated values.41 The inhalation of 400 μg CS, assuming 10 to 60% deposition in the airways,42,43 leads to a local concentration of approximately 4 to 120 μg/mL (10 to 400 μmol/L) at the central airways epithelium, which is at and above the level that was used in our experiments. Even assuming a relatively low deposition (eg, 10%) of a single dose of inhaled CS (eg, 200 μg) with a thicker ASL of approximately 60 μm (as measured in vivo44) into an average ASL volume (eg, 6 mL) leads to a potential point concentration atop the airway epithelium of 3 μg/mL, or approximately 10 μmol/L. The concentration range of CSs used in

![Figure 3](https://www.chestjournal.org/cfch21986/)

**Figure 3.** Top, A: demonstration of mitochondrial membrane potential after the CS treatment of 1HAEo− airway epithelial cells. Cells were treated with control medium or 3 μmol/L dexamethasone (Dex) for 4 h. Cells then were loaded with 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolcarbocyanine iodide, and mitochondrial membrane potential was assessed by fluorescent microscopy. The preservation of polarity, demonstrated by aggregated (red) dye, was lost with permeability transition as demonstrated by the dye in monomeric form (green) in cells treated with dexamethasone. Bottom, B: cleavage of pro-caspase-9 in 1HAEo− airway epithelial cells after CS treatment, as demonstrated by Western blot. Cells were treated with 3 μmol/L dexamethasone for 0 to 12 h. Protein lysates were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, were transferred onto nitrocellulose, and were probed for caspase-9 using an antibody that recognized both the pro-form and the active fragments. Cleavage products became apparent within 6 h of treatment (arrows). Reprinted with permission from Dorscheid et al.36
culture-based experiments thus approximates the peak concentrations that may be attained in vivo. An additional consideration is the potency of the inhaled CS. The trifluorinated glucocorticoids that are used in current asthma therapy have increased potency and lipid solubility compared to dexamethasone.45 For these reasons, CS treatment in these cell culture-based experiments may actually underestimate the potential in vivo effect of inhaled (or parental) CS therapy on the airway epithelium.

Multiple apoptotic signals link to a signaling cascade of caspase proteases. These proteases are constitutively expressed as inactive zymogens that are cleaved when activated. Two distinct pathways exist for caspase activation, as follows: type I, in which caspases are activated early after death receptor stimulation46,47; and type II, in which increased mitochondrial permeability and loss of mitochondrial membrane polarity are required prior to caspase activation.48 We examined whether CS-induced apoptosis in airway epithelial cells would be associated with a disruption in mitochondrial polarity. To do this, we loaded epithelial cells with the vital fluorescent lipophilic dye Hoechst 33342, and then treated cells with 3 μmol/L dexamethasone for up to 24 h. Cells demonstrated loss of mitochondrial polarity as early as 1 h and significant disruption within 4 h (Fig 3). In mitochondrial-facilitated apoptosis, caspase cascade activation is triggered by the formation of a multimeric Apaf-1/cytochrome c complex to recruit and activate pro-caspase-9 with subsequent effector caspase activation.49 Airway epithelial cells treated with dexamethasone released a significant amount of cytochrome c from the mitochondria into the cell cytosol, aiding the subsequent cleavage and activation of caspase 9 (Fig 3).

Mitochondria-facilitated apoptosis is regulated by several proteins, including Bcl-2 and Bcl-xL.50,51 We generated airway epithelial cell lines that stably overexpressed each of these regulators and treated these cells with 10 μmol/L dexamethasone for 24 h. The overexpression of either Bcl-2 or Bcl-xL prevented apoptotic cell death compared to cells transfected with an empty vector (Fig 4).

CONCLUSION

In summary, damage to the airway epithelium is an important pathologic event in patients with asthma, although demonstrating it by the currently available methods can be problematic. In addition to any derangements that may occur in the communication between the epithelium and underlying mesenchymal cells, and any damage that may be caused by inflammatory mediators and cells, we suggest that CSs may elicit apoptosis of the epithelium. Apoptosis is elicited in a concentration range similar to that attainable in vivo, is associated with the disruption of mitochondrial polarity and the activation of both caspase-9 and caspase-3, and can be blocked by overexpressing the apoptotic regulators Bcl-2 and Bcl-xL. Yet clear differences exist between CS-induced apoptosis in the cultured epithelium vs cultured hematopoietic cells. In the latter, apoptosis is rapid (ie, ≤12 h) and nearly complete (ie, >70% of cells), whereas in the former, apoptosis is slower and accounts for approximately 10 to 15% of cells.

Our data suggest that the use of CSs may be one factor in the airways remodeling and epithelial damage that are seen in many patients with chronic, persistent asthma. This may seem provocative but should be placed within the context of the asthma state in the airways. Data that may seem irreconcilable may in fact have a common theme. CS therapy in modest concentrations is useful in suppressing inflammation, and the absence of inflammation may allow a damaged epithelium to recover. In contrast, CS in higher concentrations may elicit damage that is independent of that caused by inflammatory mediators. This suggests that while CSs are and should remain the mainstay of controller therapy for asthma, combination therapy with other agents, such as long-acting β-adrenergic agonists or leukotriene receptor antagonists, may be preferable in attaining asthma control compared to the use of high concentrations of CSs. The long-term implications of therapy-induced airway damage will require further investigation.

ACKNOWLEDGMENTS: I thank Kimm Hamann, PhD, Marcus Peter, PhD, and Charles Rudin, MD, at the University of Chicago for their advice. I thank Bertha Marroquin and Kimberly Wojek for their many years of technical assistance.

Figure 4. Apoptosis after CS treatment in 1HAEo− airway epithelial cells stably overexpressing Bcl-2 or Bcl-xL by TUNEL staining. Each transfected cell line was treated with 10 μmol/L dexamethasone, budesonide, beclomethasone, or triamcinolone for 24 h and was processed for TUNEL staining. * = p = 0.05 (vs empty vector transfected cells within each group); † = p = 0.03 (vs empty vector transfected cells within each group); ‡ = p = 0.01 (vs empty vector transfected cells within each group); and § = p < 0.002 (vs empty vector transfected cells within each group). Bud = budesonide; Bec = beclomethasone; Tri = triamcinolone; EV = empty vector. See legend of Figure 3 for abbreviations not used in text. Reprinted with permission from Dorscheid et al.46

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Primary pulmonary hypertension (PPH) is a serious pulmonary vascular disease occurring mostly in adult women. Although its occurrence in families was reported within a few years after the original clinical report, PPH was formerly believed rarely to have a genetic basis. Recent progress has not only clarified a basic molecular mechanism for PPH in families, but has also identified mutations of the same gene in many sporadic PPH patients, suggesting that its basis is commonly genetic. Extensive investigations in many centers are now in progress to provide a complete dissection of all the pathogenetic mechanisms of PPH.

Key words: cell surface receptors; epidemiology; genetics; germ-line mutation; hemodynamics; linkage; protein-serine-threonine kinases; pulmonary hypertension

Genetics and Pulmonary Hypertension*

James E. Loyd, MD

Primary pulmonary hypertension (PPH) is a serious pulmonary vascular disease occurring mostly in adult women. Although its occurrence in families was reported within a few years after the original clinical report, PPH was formerly believed rarely to have a genetic basis. Recent progress has not only clarified a basic molecular mechanism for PPH in families, but has also identified mutations of the same gene in many sporadic PPH patients, suggesting that its basis is commonly genetic. Extensive investigations in many centers are now in progress to provide a complete dissection of all the pathogenetic mechanisms of PPH.

Key words: cell surface receptors; epidemiology; genetics; germ-line mutation; hemodynamics; linkage; protein-serine-threonine kinases; pulmonary hypertension

Abbreviations: BMPR2 = bone morphogenetic protein receptor type II; cM = centiMorgan; FPPH = familial primary pulmonary hypertension; LOD = logarithm of the odds; PPH = primary pulmonary hypertension; TGF = transforming growth factor

Primary pulmonary hypertension (PPH) is a lethal disease that has a mean patient survival time of < 3 years. It occurs at all ages and affects women twice as commonly as men. Despite many important advances in different aspects of PPH during the past decade, including progress in understanding biological mediators, genetic causes, and new therapies, the possibility of prevention and cure seems elusive until all of the basic mechanisms have been fully characterized.

Genetics

The prevalence of familial PPH (FPPH) is unknown, but only 6% of PPH patients reported a family history of PPH in the large National Institutes of Health prospective trial of 187 PPH patients. Penetrance is variable and quite low in some families. It seems possible that many PPH patients who appear to have acquired the disease may in fact have a familial basis that is unrecognized, in part due to low penetrance. The clinical and pathologic manifestations are identical whether the disease is familial or acquired. Transmission studies of families who were collected through our national FPPH registry revealed vertical transmission of the disease in up to five generations, which is highly indicative of a single dominant gene. Other unique features of the transmission of FPPH include variable age of onset, genetic anticipation, and incomplete penetrance. Our database contains records of 207 cases of FPPH that have occurred among > 2,000 individuals in these 101 families. Recently, we learned that five families with PPH in Tennessee have common ancestors and together comprised the largest reported kindred with PPH. This one family contains 18 PPH cases (16 women and 2 men) as well as 22 obligate heterozygotes (9 women and 13 men), who are individuals that have transmitted the disease but not developed it themselves.

Microsatellite Marker Search

A microsatellite marker search3 using a set of highly polymorphic, short, tandem-repeat markers was performed in 19 affected individuals from six families and detected evidence for linkage with chromosome 2q markers. We subsequently genotyped 19 additional markers spanning approximately 40 centiMorgans (cM) on the long arm of chromosome 2 and obtained a maximum two-point LOD score of 6.97 at a theta of 0 with the marker D2S389. A multipoint linkage analysis yielded a maximum LOD score of 7.56 with the marker D2S311. A haplotype analysis

*From the Department of Pulmonary and Critical Care Medicine, Vanderbilt Medical Center North, Nashville, TN. This work was supported by National Institutes of Health grant HL 48164 from the National Heart and Blood Institute.

Correspondence to: James E. Loyd, MD, Professor of Allergy, the Department of Pulmonary and Critical Care Medicine, Room T1219, Vanderbilt Medical Center North, Nashville, TN 37232; e-mail: Jim.Loyd@Vanderbilt.edu