

Asthmatic Bronchial Epithelium Is More Susceptible to Oxidant-Induced Apoptosis

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Abnormal apoptotic mechanisms are associated with disease pathogenesis. Because the asthmatic bronchial epithelium is characteristically damaged with loss of columnar epithelial cells, we postulated that this is due to unscheduled apoptosis. Using an antibody directed toward the caspase cleavage product of poly(ADP-ribose) polymerase, immunohistochemistry applied to endobronchial biopsies showed higher levels of staining in the bronchial epithelium of subjects with asthma as compared with normal control subjects (% epithelial staining [median (range) = 10.5 (1.4–24.5) versus 0.4 (0.0–9.7)]; $P < 0.001$). Because we were unable to determine whether this difference was due to ongoing inflammation *in vivo*, cultures of normal and asthmatic bronchial epithelial cells were used to study apoptosis *in vitro*. In complete growth medium, these cells showed no difference in their rate of proliferation or viability. However, cells from subjects with asthma were more susceptible to the apoptotic effects of H_2O_2 than cells from normal control subjects (% apoptotic cells = 32.2 [8.8–54.9] versus 14.3 [6.4–24.7]; $P < 0.05$), even though both were similarly affected by treatment with actinomycin D. These data indicate that the susceptibility of asthmatic bronchial epithelium to oxidants is greater than normal. This susceptibility may contribute to the rising trends in asthma associated with air pollution and diets low in antioxidants.

There has been a marked increase in prevalence of asthma over the last 30 yr. This is most likely due to changes in the environment acting on a susceptible genotype both in the induction and worsening of established disease (1). This proposal is supported by epidemiologic studies identifying multiple interacting risk factors, including inhaled pollutants (e.g., environmental tobacco smoke [ETS], particulate matter [PM_{10}], oxides of nitrogen, and ozone [O_3]), reduced exposure to bacterial products, and repeated respiratory virus exposure (2). Because these agents impact on the surface of the airway, their interaction with the bronchial epithelium may reflect the activity of those susceptibility genes that operate locally in the airways to lead to altered inflammation, injury, and repair responses.

The fully differentiated normal bronchial epithelium acts as a physical barrier to protect the internal milieu of the

conducting airways by secreting mucus and cytoprotective molecules, displaying ciliary activity, and interacting with cells of the innate and adaptive immune systems. In asthma, the bronchial epithelium shows evidence of damage with loss of columnar cells from their basal cell attachments (3, 4). Although it has been suggested that epithelial damage observed in asthmatic biopsies obtained by fiberoptic bronchoscopy may be artifactual (5), the enhanced expression of the epidermal growth factor receptor (EGFR, HER1, c-erbB1) (6) is evidence that injury has occurred *in vivo*. One consequence of epithelial injury in asthma is that the epithelium becomes an important source of autacoid mediators, chemokines, and growth factors that contribute to ongoing inflammation and myofibroblast activation, leading to tissue remodeling (7).

The extent of epithelial disruption seen in asthma is not observed in other inflammatory diseases such as cystic fibrosis (CF) and chronic obstructive pulmonary disease (COPD), even though these conditions are closely associated with microbial infections and high concentrations of irritant tobacco smoke, respectively. Although these differences may reflect the quality of inflammation, this cannot be the sole explanation, as airway eosinophilia can be observed in the absence of asthma or bronchial hyperreactivity (BHR) (8) and, as with COPD and CF, neutrophils may dominate inflammation in severe asthma. This raises the possibility that the extent of epithelial damage in asthma is due to increased susceptibility to a damaging agent and/or an inadequate repair response.

Apoptosis is a mechanism that is fundamental to tissue homeostasis. Failure to initiate apoptosis after sustaining severe DNA damage is known to contribute to the development of neoplasia (9), whereas unscheduled apoptosis contributes to neurodegenerative disorders such as Alzheimers disease (10) and autoimmune disease including Hashimoto's thyroiditis. Apoptosis has been observed in alveolar epithelial cells in pulmonary fibrosis (11) and in keratinocytes in atopic dermatitis (12), a condition that predisposes to severe asthma. Although epithelial apoptosis is increased in fatal asthma (13), it has been reported that there is no difference between the number of TUNEL-positive, late apoptotic cells in epithelium of bronchial biopsies from normal subjects versus those with asthma (14). Because large numbers of asthmatic bronchial epithelial cells are characteristically shed into the airways lining fluid (3), we considered the possibility that late apoptotic cells are rapidly lost from biopsies before or during the bronchoscopy procedure. Therefore, we have analyzed bronchial epithelium for evidence of early apoptosis by examining the presence of p85, a peptide fragment which is

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Abbreviations: bronchoalveolar lavage, BAL; bronchial epithelial growth medium, BEGM; bronchial hyperresponsiveness, BHR; cystic fibrosis, CF; cytokeratin 18, CK18; chronic obstructive pulmonary disease, COPD; environmental tobacco smoke, ETS; fetal bovine serum, FBS; glycol methacrylate, GMA; monoclonal antibody, mAb; poly(ADP-ribose) polymerase, PARP; phosphate-buffered saline, PBS; particulate matter, PM_{10} ; transforming growth factor- β , TGF- β ; tumor necrosis factor- α , TNF- α .

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generated by cleavage of poly(ADP-ribose) polymerase (PARP) by caspase-3 (15), a cysteine-aspartate protease that is involved in initiation of the apoptotic process. Furthermore, to determine whether there is a fundamental difference in epithelial susceptibility in asthma, we have established primary cultures of bronchial epithelial cells from normal subjects and subjects with asthma to compare apoptotic responses under identical conditions *in vitro*.

Materials and Methods

Subjects

Anonymous archival glycol methacrylate (GMA) embedded bronchial biopsies, taken from 9 normal subjects and 13 subjects with asthma were used for the immunohistochemical study. The asthmatic group consisted of six males and seven females, with a mean (\pm SD) age of 37.0 ± 15.2 yr (range, 20–67), and a mean FEV₁ of 68% predicted (range, 39.3–106%). The normal group was comprised of four males and five females, with a mean age of 20.9 ± 1.3 yr (range, 20–22) and a mean FEV₁ of $104 \pm 7.5\%$ of predicted. The subjects with asthma were classified according to the Global Strategy for Asthma Management and Prevention (GINA) guidelines (16), with those subjects with mild asthma ($n = 7$) receiving only the β_2 -agonist albuterol and those with severe asthma ($n = 6$) being treated with a regular mean inhaled steroid dose of 950 $\mu\text{g}/\text{d}$ (range, 200–2,000) and a regular oral corticosteroid at a mean of 36 mg/d (range, 10–60).

For bronchial brushing, nine nonatopic, nonasthmatic control subjects (five male) with a mean (\pm SD) age of 22.7 ± 4.9 yr (range, 19–33); and 11 atopic asthmatic subjects (10 male) with a mean (\pm SD) age of 26.0 ± 7.1 yr (range, 19–37) were characterized according to symptoms, pulmonary function, and medication. The control group had a mean FEV₁ of 98% predicted (range, 78–110) and a PC₂₀ histamine > 8 mg/ml, whereas the atopic asthmatic group had a mean FEV₁ of 90% predicted (range, 78–100) and a PC₂₀ histamine of 2.7 (geometric mean) (range, 0.3–16) mg/ml. Subjects with mild asthma ($n = 9$) were receiving albuterol only, whereas the two subjects with moderately severe asthma were on a mean inhaled steroid dose of 1,000 $\mu\text{g}/\text{d}$. These two subjects withheld this medication for a minimum of 1 wk before bronchoscopy. All subjects were nonsmokers and had been free from respiratory tract infections for a minimum of 4 wk before inclusion in the study. Written informed consent was obtained from all volunteers before participation, and ethical approval was obtained from the Joint Ethics Committee of Southampton University Hospital Trust.

Fiberoptic Bronchoscopy

Epithelial brushings were obtained by bronchoscopy using a fiberoptic bronchoscope (FB-20D; Olympus, Tokyo, Japan) in accordance with standard published guidelines (17). Bronchial epithelial cells were obtained using a standard sterile single-sheathed nylon cytology brush (BC 9C-26101; Olympus). On average, five to six consecutive brushings were sampled from the bronchial mucosa of the second and third generation bronchi, and for large scale culture a maximum of 12–14 brushings were performed. Cells were harvested into 5 ml sterile phosphate-buffered saline (PBS) after each brushing. At the completion of the procedure, 5 ml RPMI with 10% fetal bovine serum (FBS) was added and the sample centrifuged at $150 \times g$ for 5 min to harvest the cells. Epithelial cell purity was assessed by performing differential cell counts of the harvested cell suspension.

To obtain luminal cells, bronchoalveolar lavage (BAL) was performed according to standard protocols (17). Cells were harvested by centrifugation and then processed for cytochemical analysis by cytocentrifugation. Differential cell counts confirmed

the presence of epithelial cells before immunostaining for p85 PARP was undertaken.

Biopsy Processing and Immunohistochemistry

Processing of biopsies into GMA and the immunohistochemical method applied to this material have been described in detail (18). Briefly, 2- μm sections were processed for immunohistochemistry using the streptavidin-biotin-peroxidase detection system. The sections were incubated with either a rabbit anti-p85 PARP antibody (Promega, Southampton, UK) at 1:250 or a monoclonal antibody (mAb) to cytokeratin 18 (CK18; Sigma Chemicals, Poole, Dorset, UK) at 1:1,000 overnight at 4°C or room temperature, respectively. In each case, negative controls comprising matched immunoglobulins and buffer alone were included. After washing, anti-rabbit or anti-mouse biotinylated IgG Fab fragments at 1:600 or 1:300, respectively (Dako Ltd, Wycombe, UK) were applied to the sections for 2 h. This was followed by a 2-h incubation with streptavidin-biotin horseradish-peroxidase complex at 1:200 (Dako Ltd, Wycombe, UK) before visualization with aminoethylcarbazine as chromogen. Sections were counterstained with Mayer's hematoxylin and mounted in DPX.

Epithelial expression of immunoreactive p85 PARP in GMA sections was quantified by computer-assisted image analysis (Colovision 1.7.6; Improvision, Coventry, UK). For each biopsy specimen, the entire intact epithelium in two nonserial sections was systematically assessed based on red, blue, and green (RGB) color balance. At the beginning of each session, the image analysis system was standardized using the same section of bronchial mucosa stained for p85 PARP, to ensure reproducibility of analysis (6). Each slide was coded and measurement of p85 PARP expression was performed by an observer who was unaware of the clinical group from which the biopsy specimen was derived.

Analysis of PARP Cleavage by SDS-PAGE and Western Blotting

H292 bronchial epithelial cells were seeded into 24-well dishes in RPMI 1640/10% fetal calf serum and grown to 70–80% confluence. After serum starvation for 24 h, the cells were treated in serum-free medium with or without 10 ng/ml tumor necrosis factor (TNF)- α in the absence or presence of H₂O₂ (50–400 μM) for 1, 3, or 4 h. At the end of each time point, cells were lysed into SDS sample buffer and analyzed by SDS-PAGE and Western blotting with p85 PARP antibody and a pan PARP antibody, using previously described protocols (6).

Epithelial Cultures

Primary cultures were established by seeding freshly brushed bronchial epithelial cells into culture dishes containing 3 ml of serum-free hormonally-supplemented Bronchial Epithelium Growth Medium (BEGM; Clonetics, San Diego, CA) containing with 50 U/ml penicillin and 50 $\mu\text{g}/\text{ml}$ streptomycin. When confluent, the cells were passaged (p1) using trypsin and were allowed to further expand until used for experimentation at passage 2 or 3; control experiments confirmed that there was no significant difference between the responses of the cells at p2 or p3. Viability was assessed by exclusion of trypan blue dye and the epithelial nature of cells assessed by immunocytochemistry using a pan-CK antibody and antibodies specific for CK13 and CK18.

Analysis of Apoptosis by Flow Cytometry

Apoptosis was measured according to the technique of Vermes and coworkers (19), in which binding of annexinV (AxV) was used to detect phosphatidylserine, which is externalized on the outer leaflet of the plasma membrane of apoptotic cells. Briefly, primary bronchial epithelial cells ($5 \times 10^4/\text{well}$) were grown to 80–90% confluence in 24-well plates (Nunc, Fisher Scientific, Lough-

borough, UK). The BEGM medium was then replaced with basal medium (BEBM; Clonetics) containing insulin, transferrin and sodium selenite supplement (Sigma), and the cells rendered quiescent for 24 h before exposure to H₂O₂, actinomycin D or TNF- α (Peptotech, London, UK) for up to 24 h as detailed in Results. Adherent cells were harvested with trypsin in Ca²⁺ and Mg²⁺-free Hanks' balanced salt solution and combined with nonadherent cells for analysis. After washing twice in cold PBS, the cells were resuspended at a density of 1×10^5 cells/100 μ l of binding buffer (10 mM HEPES pH 7.4, 140 mM NaCl, 2.5 mM CaCl₂) in 5 ml propylene FACS tubes. AxV-FITC (1 μ g/ml) and propidium iodide (PI) (2.5 μ g/ml) were added to the tubes and incubated in the dark for 15 min, after which 400 μ l of cold binding buffer were added and cells analyzed using a FACScan flow cytometer (Becton and Dickinson, Oxford, UK). In the case of H292 cells, PI was replaced with 7-aminoactinomycin D (7-AAD). Control tubes lacking AxV-FITC, PI, or both were included for the acquisition. Analysis of dotplots of FL1(AxV-FITC) versus FL2 (PI) was performed using WinMDI 2.8. The degree of apoptosis was expressed as the number of AxV⁺/PI⁻ cells shown as a percentage of total cells.

Cytokine Measurement by Enzyme-Linked Immunosorbent Assay

Release of IL-8 into culture supernatants of bronchial epithelial cells was measured using enzyme-linked immunosorbent assay kits for IL-8 according to the manufacturer's instructions (R&D Systems, Abingdon, UK).

Statistical Analysis

Data was analyzed by the Mann-Whitney U test or the Wilcoxon rank-sum test for paired samples. A *p* value < 0.05 was considered significant.

Results

Detection of Apoptosis in Bronchial Biopsies of Normal Subjects and Subjects with Asthma

To detect evidence of early apoptosis in bronchial biopsies, immunohistochemical analysis was undertaken using an antibody specific for p85, the caspase cleavage product of PARP. The specificity of this antibody was demonstrated using H292 bronchial epithelial cells exposed to H₂O₂ and TNF- α , which together caused an increase in apoptosis as measured by FACS analysis (%AxV⁺/7AAD⁻ cells = 18.4 ± 8.4 versus 2.9 ± 0.5 ; *P* < 0.01). As shown in Figure 1, exposure of H292 cells to increasing doses of H₂O₂ in the presence of TNF- α caused a dose-dependent increase in PARP cleavage. By comparison with a pan p85 antibody, which recognized full-length and cleaved PARP, the p85 antibody recognized only cleaved PARP.

When the p85-specific antibody was applied to bronchial biopsies, staining for p85 PARP in either the bronchial epithelium or submucosa of normal subjects was minimal and tended to be slightly higher than that seen in tissue sections stained with an irrelevant control immunoglobulin (Figure 2A versus 2C). In contrast, using biopsies from subjects with asthma of varying severity, there was marked staining for p85 PARP in the epithelium (Figure 2B), as well as in a limited number of inflammatory cells in the submucosa (Figure 2D). In the asthmatic bronchial epithelium, staining was frequently patchy (Figure 2E) and usually occurred in columnar cells showing both a nuclear

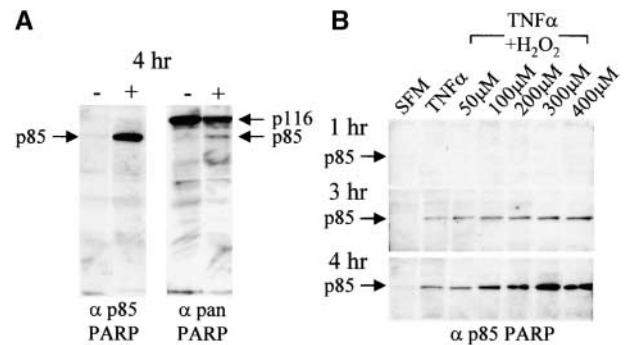


Figure 1. Detection of PARP Cleavage in Bronchial Epithelial Cells. H292 bronchial epithelial cells were exposed in SFM alone or with 10 ng/ml TNF- α in the absence or presence of H₂O₂ (50–400 μ M) for 1, 3, and 4 h and the cell lysates analyzed by SDS PAGE and Western blotting. **A** shows lysates from cells treated in the absence (–) or presence (+) of 10 ng/ml TNF- α and 300 μ M H₂O₂ for 4 h and probed with a pan PARP antibody or the p85 PARP antibody. **B** shows the time and dose dependence of the appearance of p85 PARP immunoreactivity following exposure to pro-apoptotic stimuli. Data are representative of three independent observations.

and cytoplasmic distribution. Furthermore, the majority of epithelial cells present in asthmatic BAL fluid also showed strong immunostaining for p85 PARP (Figures 2F–2H). Quantitation of staining using computerized image analysis confirmed that epithelial p85 PARP staining was significantly increased in the asthmatic biopsies when compared with those from normal control subjects (% epithelial staining [median (range) = 10.5 (1.4–24.5) versus 0.4 (0.0–9.7)]; *P* < 0.001) (Figure 3). As a control for the analysis of p85 PARP staining, we also stained cells with an antibody to CK18. In this case, staining was uniform within the epithelium and there was no significant difference in the extent of staining observed in asthmatic (% epithelial staining median [range] = 42.6 [17.3–49.8], *n* = 13) and normal (36.9 [30.2–53.5], *n* = 9; *P* > 0.05) bronchial epithelium.

Analysis of Apoptosis in Bronchial Epithelial Cells *In Vitro*

Although immunochemical analysis suggested that there was increased apoptosis in the asthmatic bronchial epithelium, we could not distinguish whether this was a consequence of the ongoing inflammation in the bronchial tissue. Therefore, we established primary cultures of normal and asthmatic bronchial epithelial cells to measure apoptosis under controlled conditions *in vitro*. However, because there was no information on the properties of normal and asthmatic epithelial cell cultures established from bronchial brushings, it was first necessary to characterize the primary cultures to ensure that any differences in apoptotic behavior were not a consequence of differing viability or growth patterns.

Characterization of Bronchial Brushings and Epithelial Cultures. Cytologic analyses of samples of bronchial brushings showed that the yield of epithelial cells obtained by brushing either normal or asthmatic airways was > 90%, with the remaining cells being neutrophils or eosinophils. By immunocytochemistry, the majority of the epithelial

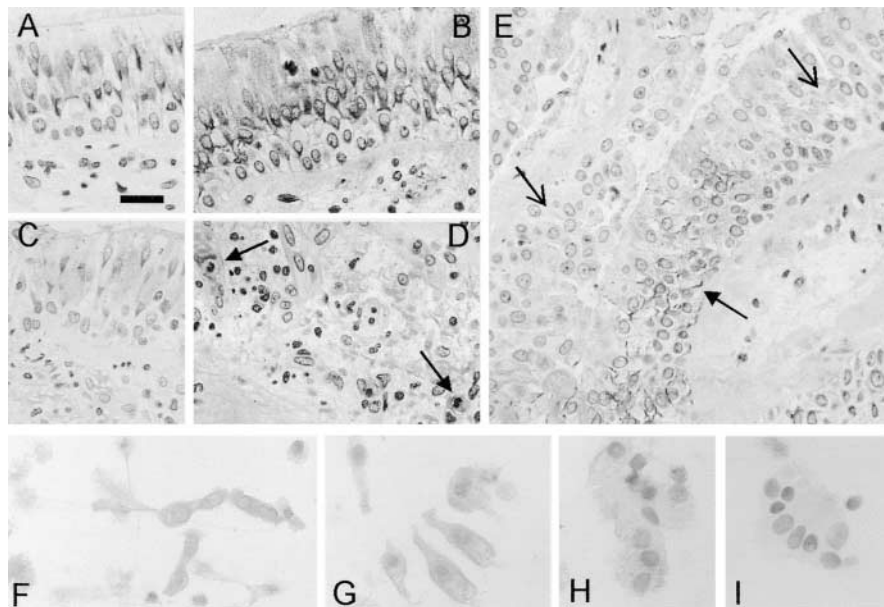


Figure 2. Bronchial biopsies from normal subjects and subjects with asthma were stained by immunohistochemistry for the presence of p85 PARP. The plates show typical epithelial staining patterns observed in normal subjects (A) or subjects with asthma (B). C shows staining obtained with control rabbit immunoglobulin. D shows detail of p85 PARP staining in the submucosa where the arrows indicate p85 PARP immunoreactivity in a subset of the submucosal cells. E shows the patchy nature of the p85 staining in the asthmatic bronchial epithelium. F–H show three fields from cytopreparations of BAL cells demonstrating p85 PARP-positive staining of shed columnar epithelial cells and a field from a matched negative control cytopreparation (I). Bar = 20 μ m.

cells were of a columnar phenotype staining positively for CK18 and negatively for CK13; in each case, a small percentage (< 10%) of the cells were of a basal cell phenotype expressing both CK18 and CK13. Primary epithelial cultures could be established in BEGM, with a frequency of 9/10 and 11/16 for normal and asthmatic epithelial cells, respectively. Failure to establish a culture invariably resulted from mycotic infection rather than from inability of the cells to proliferate. Infection usually occurred if brushings were heavily contaminated with blood, hence extreme care was taken during the bronchoscopy procedure to minimize trauma to the mucosal surface. No gross morphologic differences were evident between the normal and asthmatic cultures and no difference was observed in their mean generation time (Figure 4), with cell yields from 5–6 brushings being in the range ($1.5\text{--}4.0 \times 10^6$) and ($2.0\text{--}4.5 \times 10^6$) at the end of p1 and viabilities of 98% and 95% for normal

and asthmatic cultures, respectively. Cultures usually expanded through p2 and p3, but thereafter became quiescent. For large-scale cultures, up to a maximum of 12–14 brushings have been collected, and this was well tolerated by most patients; these more extensive brushings produced a corresponding increase in cell yield. Immunocytochemical analysis of the monolayer cultures showed that all cells were CK18⁺ and CK13⁺, suggesting that these proliferating cells resembled basal cells (data not shown).

Susceptibility to oxidant-induced apoptosis. Having established that the *in vitro* growth properties of the epithelial cultures were similar, they were rendered quiescent for 24 h to compare their sensitivity to oxidant stress as determined by induction of apoptosis. Initial experiments confirmed that increasing concentrations of H₂O₂ over the range 50–500 μ M caused a dose-dependent increase in apoptotic (i.e., AxV⁺/PI⁻) cells; however, as the dose increased, there was a sharp increase in the number of ne-

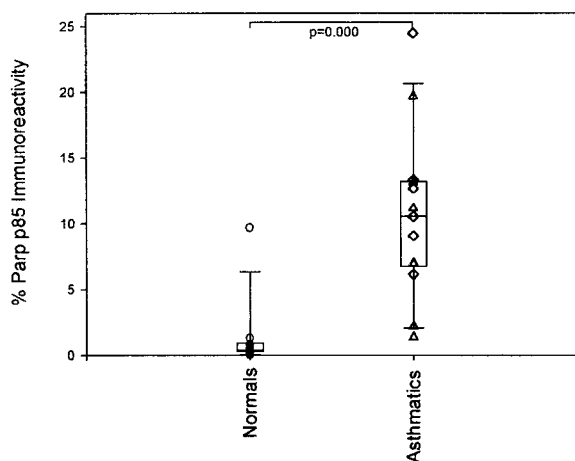


Figure 3. Quantitative analysis of p85 PARP staining in normal and asthmatic bronchial biopsies. Data were analyzed using Mann-Whitney U test.

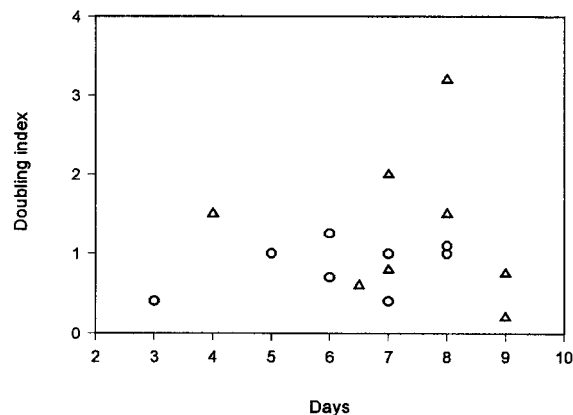


Figure 4. Characterization of the growth properties of normal and asthmatic bronchial epithelial cultures. The mean generation time of normal (circles) and asthmatic (triangles) epithelial cultures was calculated from cell yield en passage.

crotic (ie. AxV⁺/PI⁺) cells (data not shown), therefore a working dose of 300 μ M was selected for subsequent studies. Using these conditions, the asthmatic epithelial cell cultures were found to be significantly more sensitive to H₂O₂, with the percentage of AxV⁺/PI⁻ cells (median [range]) being 32.2% (8.8–54.9%) in the subjects with asthma and 14.3% (6.4–24.7%) in the normal subjects ($P < 0.05$) (Figure 5A). In contrast, there was no significant difference in the response of asthmatic or normal epithelial cell cultures to the pro-apoptotic effects of the DNA and RNA synthesis inhibitor, Actinomycin D (34.4 [13.5–44.2] versus 24.1 [7.1–54.6]; $P = 0.34$).

Neither normal nor asthmatic epithelial cultures showed any increase in apoptosis in the presence of TNF- α (Figure 5B), and no additional effect of TNF- α was observed over that obtained with H₂O₂ alone. This was not due to lack of TNF- α receptors, as IL-8 release was increased by TNF- α (IL-8 release [median (range)]) in the absence and presence

of TNF- α was 2.5 [0.1–8.2] and 14.3 [3.2–103.6] ng/10⁶ cells [$P < 0.01$] for normal epithelial cultures and 2.5 [0.1–14.5] versus 16.2 [1.8–49.6] ng/10⁶ cells [$P < 0.01$] for asthmatic cultures).

Discussion

Disruption of the bronchial epithelium with shedding of the columnar cells as Creola bodies is a characteristic finding in asthma (3), correlating both with BHR (4) and epithelial permeability (20). Even though there is substantial evidence of epithelial damage, it is surprising that a previous study failed to reveal any difference in the levels of apoptosis in asthmatic bronchial epithelium using the TUNEL technique (14), which identifies cells undergoing DNA fragmentation at a relatively late stage of apoptosis. In contrast with that report, in the present study we have found a significant increase in early apoptotic cells in asthmatic bronchial epithelium through the use of an antibody that detects PARP cleavage by caspase-3. Our findings are also consistent with a recent report by Benayoun and co-workers who found that the presence of the active, cleaved form of caspase-3 was increased in the bronchial epithelium of untreated subjects with asthma when compared with nonasthmatic control subjects (21). Close examination of the pattern of p85 staining in the asthmatic biopsies revealed patchy areas of strong immunoreactivity, whereas areas of adjacent epithelium were only weakly stained. The appearance of the epithelial staining suggested that those areas of positive immunoreactivity might be destined to be shed as Creola bodies. This suggestion is supported by our finding that detached epithelial cells found in BAL fluid also stained positively for p85 PARP. Because caspases cleave a variety of structural proteins such as cytokeratin (22), we postulate that disruption of the cytoskeleton leads to loss of apoptotic cells from the epithelium before DNA fragmentation occurs. Consistent with this proposal, we found that pro-apoptotic stimuli caused detachment of bronchial epithelial cells from their culture dishes from ~ 4 h of treatment (data not shown). Similarly, caspase activation and initiation of apoptosis has been observed in bronchial epithelial cells following detachment caused by disruption of the actin cytoskeleton (23). These effects on cell adhesion may explain the discrepancy between the current study and the previous study that used TUNEL.

In addition to epithelial staining, there was also an increase in apoptotic cells in the submucosa which could be clearly distinguished from p85 PARP negative cells; however, we have not attempted to characterize the nature of these cells. The apoptosis did not appear to be due to the use of corticosteroids, which are known to promote eosinophil apoptosis (24), because apoptosis was evident in submucosal cells of patients using bronchodilators only. Because the use of TUNEL to demonstrate the occurrence of apoptotic eosinophils in airway tissue is contentious (25), further studies using double staining to identify eosinophils and p85 PARP and other markers of caspase activation are warranted.

As we were unable to differentiate whether the increased level of epithelial apoptosis in asthmatic biopsies

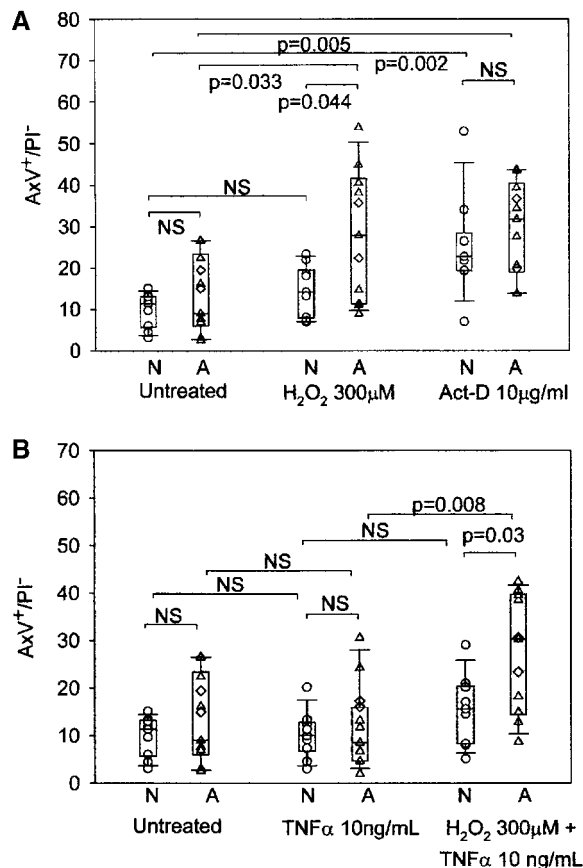


Figure 5. The susceptibility of normal and asthmatic bronchial epithelial cells to oxidant induced apoptosis. Primary cultures of quiescent normal (circles) ($n = 9$) or asthmatic (triangles and diamonds denote subjects with mild and moderate asthma, respectively) ($n = 11$) bronchial epithelial cells were untreated or exposed to (A) 300 μ M H₂O₂, or to 10 μ g/ml actinomycin D or (B) TNF- α (10 ng/ml) in the absence or presence of 300 μ M H₂O₂ for 24 h. Cells were then harvested and apoptosis was measured by FACS analysis using AxV and PI. The figure shows % early apoptotic cells (AxV⁺/PI⁻) where each data point is a mean of duplicate observations obtained for each individual donor.

was merely a consequence of ongoing inflammation, we extended our studies to analyze apoptosis *in vitro* using primary cultures established from bronchial brushings. The respiratory epithelium is in direct contact with the environment and is a major target for oxidative injury from inhaled oxidants (O_3 , ETS, nitrogen and sulfur dioxides, PM_{10}). Because inflammatory cells such as eosinophils and neutrophils also generate endogenous oxidants together with $TNF-\alpha$, a known pro-apoptotic stimulus (26), we studied a model of oxidant injury with or without $TNF-\alpha$. It has been estimated that local concentrations of around $100 \mu M H_2O_2$ can easily be generated by activated granulocytes (27); therefore, the amounts of oxidant used in the present study might be expected to fall within physiologic or pathophysiologic ranges. The finding that asthmatic bronchial epithelial cells were more susceptible to oxidant-induced apoptosis *in vitro* is consistent with our observation of increased caspase activation *in vivo*. As our *in vitro* studies were performed with monolayer cultures of undifferentiated cells, this suggests that the susceptibility of asthmatic bronchial epithelial cells to oxidants is not dependent on their differentiation state; whether this is due to an inherited or acquired defect remains to be determined. Significantly, neither normal nor asthmatic bronchial epithelial cells were caused to undergo apoptosis by $TNF-\alpha$. Because $TNF-\alpha$ caused IL-8 release, our data indicate that $TNF-\alpha$ has proinflammatory effects on normal and asthmatic epithelium which are distinct from survival or apoptotic mechanisms.

A number of studies have indicated roles for reactive oxygen (ROS) and reactive nitrogen (RNS) species in the pathology of asthma both in terms of increased burden and decreased antioxidant defenses (28). Airway responses have been shown to correlate with oxidant generation by eosinophils after antigen challenge *in vivo* (29) and neutrophil superoxide generation correlates with BHR (30). Airway lining fluid from subjects with asthma has a lower antioxidant capacity than fluid from normal subjects (31), and intracellular Cu,Zn superoxide dismutase (Cu,Zn-SOD) activity is decreased in asthma, but could be normalized upon corticosteroid treatment (32). In a separate study, reduced SOD activity was found in brushed bronchial epithelial cells from patients with asthma and was shown to strongly correlate with BHR (33). Although these studies have demonstrated altered oxidant defenses in asthma, most appear to be a consequence of the inflammatory process. In contrast, in the present study, the primary epithelial cultures were taken through several generations *in vitro* to allow recovery from any changes that had occurred *in vivo* as consequence of airway inflammation. Thus, our finding that asthmatic bronchial epithelial cells retained a higher susceptibility to oxidant injury suggests a different underlying cause than in the previous studies. This susceptibility may explain the extent of epithelial fragility in asthma as compared with other inflammatory conditions in the airways.

In asthma, inflammatory products have been proposed to cause tissue injury and cytokine release, leading to a self-perpetuating cycle of injury and inflammation. However, this does not explain how the appropriate microenvironment is created in the airway to establish and maintain

this inflammation, nor does it account for the increased sensitivity of subjects with asthma to components of the inhaled environment. Epidemiologic studies have demonstrated important links between air pollutants such as diesel exhaust particles (DEP), O_3 , and ETS in asthma pathogenesis and exacerbation (34), and others have shown a strong link between diets low in antioxidants and asthma (35). Our studies help explain these phenomena because the sensitivity of asthmatic epithelial cells to oxidant-induced apoptosis may be a key triggering mechanism that facilitates the induction and establishment of chronic inflammatory responses. This proposal is consistent with a previous report in which asthmatic bronchial epithelial cells were found to be more sensitive to the effects of DEP with respect to induction of cytokine release and inhibition of ciliary beat activity (36). Together, these observations could account for the involvement of environmental pollutants in asthma exacerbations and provide a link to the rising trends in asthma observed over the last few decades.

Increased apoptosis has also been reported to occur in keratinocytes of subjects with atopic dermatitis (12), suggesting that epithelial susceptibility to apoptosis may contribute to the organ-specific expression of some allergic diseases. Because atopic dermatitis is a condition that predisposes to severe asthma, it is possible that skin and airway epithelial cells may express common susceptibility genes in addition to those for atopy. These considerations have important implications for our understanding of asthma pathogenesis and treatment and wider implications for mechanisms of allergic diseases.

Apoptotic mechanisms play a key role following tissue injury by enabling disposal of a dying cell without induction of a proinflammatory response, as occurs after necrosis. One of these disposal mechanisms involves the recognition of phosphatidylserine on the outer leaflet of the plasma membrane by specific receptors on macrophages. Recognition and engulfment of the apoptotic cell leads to increased expression and release of $TGF-\beta$ by the macrophages (37) to limit further inflammation. Because $TGF-\beta$ has been found to decrease expression of Cu,Zn-SOD in rat hepatocytes (38), this may contribute to the low levels of this enzyme found in bronchial epithelial cells (32). Furthermore, as $TGF-\beta$ is also a profibrogenic mediator, high levels of epithelial apoptosis may be responsible for contributing to subepithelial fibrosis, a characteristic feature of the remodeled airways in asthma (7). Whether subepithelial myofibroblasts also contribute to ongoing epithelial apoptosis, as has been demonstrated in pulmonary fibrosis (11), remains to be determined. Such interactions between epithelial cells and immune cells and/or fibroblasts illustrate the complexity of the tissue microenvironment and its potential to influence disease phenotype.

In conclusion, we have provided evidence for increased apoptosis in the bronchial epithelium in asthma and, by using primary cultures, have shown an increase in the susceptibility of the asthmatic bronchial epithelium to the effects of oxidants. In being preserved through several generations *in vitro*, this susceptibility is unlikely to be due to the effects of airway inflammation and may help to explain gene and environment interactions in asthma. The avail-

ability of a reliable method for the growth of primary cultures from normal and asthmatic bronchial epithelium should now pave the way toward dissection of the underlying mechanisms that control the altered susceptibility and function of the epithelium in asthma.

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References

- Holgate, S. T. 1999. The epidemic of allergy and asthma. *Nature* 402:B2-B4.
- Martinez, F. D., and P. G. Holt. 1999. Role of microbial burden in aetiology of allergy and asthma. *Lancet* 354:SI112-SI115.
- Montefort, S., J. A. Roberts, R. Beasley, S. T. Holgate, and W. R. Roche. 1992. The site of disruption of the bronchial epithelium in asthmatic and non-asthmatic subjects. *Thorax* 47:499-503.
- Jeffery, P. K., A. J. Wardlaw, F. C. Nelson, J. V. Collins, and A. B. Kay. 1989. Bronchial biopsies in asthma: an ultrastructural, quantitative study and correlation with hyperreactivity. *Am. Rev. Respir. Dis.* 140:1745-1753.
- Ordenez, C., R. Ferrando, D. M. Hyde, H. H. Wong, and J. V. Fahy. 2000. Epithelial desquamation in asthma: artifact Or pathology? *Am. J. Respir. Crit. Care Med.* 162:2324-2329.
- Puddicombe, S. M., R. Polosa, A. Richter, M. T. Krishna, P. H. Howarth, S. T. Holgate, and D. E. Davies. 2000. The involvement of the epidermal growth factor receptor in epithelial repair in asthma. *FASEB J.* 14:1362-1374.
- Holgate, S. T., D. E. Davies, P. M. Lackie, S. J. Wilson, S. M. Puddicombe, and J. L. Lordan. 2000. Epithelial-mesenchymal interactions in the pathogenesis of asthma. *J. Allergy Clin. Immunol.* 105:193-204.
- Wark, P. A., P. G. Gibson, and K. Fakes. 2000. Induced sputum eosinophils in the assessment of asthma and chronic cough. *Respirology* 5:51-57.
- Schutte, B., and F. C. Ramaekers. 2000. Molecular switches that govern the balance between proliferation and apoptosis. *Prog. Cell Cycle Res.* 4:207-217.
- Wellington, C. L., and M. R. Hayden. 2000. Caspases and neurodegeneration: on the cutting edge of new therapeutic approaches. *Clin. Genet.* 57:1-10.
- Uhal, B. D., I. Joshi, W. F. Hughes, C. Ramos, A. Pardo, and M. Selman. 1998. Alveolar epithelial cell death adjacent to underlying myofibroblasts in advanced fibrotic human lung. *Am. J. Physiol.* 275:L1192-L1199.
- Trautmann, A., M. Akdis, D. Kleemann, F. Altnauer, H. U. Simon, T. Graeve, M. Noll, E. B. Brocker, K. Blaser, and C. A. Akdis. 2000. T cell-mediated Fas-induced keratinocyte apoptosis plays a key pathogenetic role in eczematous dermatitis. *J. Clin. Invest.* 106:25-35.
- Chagani, T., D. Ionescu, W. M. Elliott, B. Lifschultz, and P. D. Paré. 2001. Fas (CD95) and FasL (CD95L) expression in airway epithelium of subjects with fatal and non-fatal asthma. *Am. J. Respir. Crit. Care Med.* 163:A739 (Abstr.)
- Druilhe, A., B. Wallaert, A. Tscopoulos, J. R. Silva, I. Tillie-Leblond, A. B. Tonnel, and M. Pretolani. 1998. Apoptosis, proliferation, and expression of Bcl-2, Fas, and Fas ligand in bronchial biopsies from asthmatics. *Am. J. Respir. Cell Mol. Biol.* 19:747-757.
- Knaapen, M., M. De Bie, J. Muhring, and M. Kocks. 1999. Cleaved PARP as a marker for apoptosis in tissue sections. *Promega Notes* 72:7-9.
- WHO/NHLBI Workshop Report. 1995. Global strategy for asthma management and prevention. National Institute of Health, National Heart, Lung and Blood Institute. Bethesda, MD; NIH publication number 96-3659A.
- Hurd, S. Z. 1991. Workshop summary and guidelines: investigative use of bronchoscopy, lavage, and bronchial biopsies in asthma and other airway diseases. *J. Allergy Clin. Immunol.* 88:808-814.
- Britten, K. M., P. H. Howarth, and W. R. Roche. 1993. Immunohistochemistry on resin sections: a comparison of resin embedding techniques for small mucosal biopsies. *Biotech. Histochem.* 68:271-280.
- Vermes, I., C. Haanen, H. Steffens-Nakken, and C. Reutelingsperger. 1995. A novel assay for apoptosis: flow cytometric detection of phosphatidylserine expression on early apoptotic cells using fluorescein labelled Annexin V. *J. Immunol. Methods* 184:39-51.
- Ilowite, J. S., W. D. Bennett, M. S. Sheetz, M. L. Groth, and D. M. Nierman. 1989. Permeability of the bronchial mucosa to ^{99m}Tc-DTPA in asthma. *Am. Rev. Respir. Dis.* 139:1139-1143.
- Benayoun, L., S. Letuve, A. Druilhe, J. Boczkowski, M. C. Dombret, P. Mechighel, J. Megret, G. Leseche, M. Aubier, and M. Pretolani. 2001. Regulation of peroxisome proliferator-activated receptor gamma expression in human asthmatic airways: relationship with proliferation, apoptosis, and airway remodeling. *Am. J. Respir. Crit. Care Med.* 164:1487-1494.
- Caulin, C., G. S. Salvesen, and R. G. Oshima. 1997. Caspase cleavage of keratin 18 and reorganization of intermediate filaments during epithelial cell apoptosis. *J. Cell Biol.* 138:1379-1394.
- White, S. R., P. Williams, K. R. Wojcik, S. Sun, P. S. Hiemstra, K. F. Rabe, and D. R. Dorscheid. 2001. Initiation of apoptosis by actin cytoskeletal derangement in human airway epithelial cells. *Am. J. Respir. Cell Mol. Biol.* 24:282-294.
- Woolley, K. L., P. G. Gibson, K. Carty, A. J. Wilson, S. H. Twaddell, and M. J. Woolley. 1996. Eosinophil apoptosis and the resolution of airway inflammation in asthma. *Am. J. Respir. Crit. Care Med.* 154:237-243.
- Erjefalt, J. S., and C. G. Persson. 2000. New aspects of degranulation and fates of airway mucosal eosinophils. *Am. J. Respir. Crit. Care Med.* 161:2074-2085.
- Wallach, D., E. E. Varfolomeev, N. L. Malinin, Y. V. Goltsev, A. V. Kovalenko, and M. P. Boldin. 1999. Tumor necrosis factor receptor and Fas signaling mechanisms. *Annu. Rev. Immunol.* 17:331-367.
- Schraufstatter, I., P. A. Hyslop, J. H. Jackson, and C. G. Cochrane. 1988. Oxidant-induced DNA damage of target cells. *J. Clin. Invest.* 82:1040-1050.
- Morcillo, E. J., J. Estrela, and J. Cortijo. 1999. Oxidative stress and pulmonary inflammation: pharmacological intervention with antioxidants. *Pharmacol. Res.* 40:393-404.
- Sedgwick, J. B., W. J. Calhoun, R. F. Vrtis, M. E. Bates, P. K. McAllister, and W. W. Busse. 1992. Comparison of airway and blood eosinophil function after in vivo antigen challenge. *J. Immunol.* 149:3710-3718.
- Postma, D. S., T. E. Renkema, J. A. Noordhoek, H. Faber, H. J. Sluiter, and H. Kauffman. 1988. Association between nonspecific bronchial hyperreactivity and superoxide anion production by polymorphonuclear leukocytes in chronic air-flow obstruction. *Am. Rev. Respir. Dis.* 137:57-61.
- Kelly, F. J., I. Mudway, A. Blomberg, A. Frew, and T. Sandstrom. 1999. Altered lung antioxidant status in patients with mild asthma. *Lancet* 354:482-483.
- De Raeve, H. R., F. B. Thunnissen, F. T. Kaneko, F. H. Guo, M. Lewis, M. S. Kavuru, M. Secic, M. J. Thomassen, and S. C. Erzurum. 1997. Decreased Cu,Zn-SOD activity in asthmatic airway epithelium: correction by inhaled corticosteroid in vivo. *Am. J. Physiol.* 272:L148-L154.
- Smith, L. J., M. Shamsuddin, P. H. Sporn, M. Denenberg, and J. Anderson. 1997. Reduced superoxide dismutase in lung cells of patients with asthma. *Free Radic. Biol. Med.* 22:1301-1307.
- Hiltermann, T. J., J. Stolk, S. C. van der Zee, B. Brunekreef, C. R. de Bruijne, P. H. Fischer, C. B. Ameling, P. J. Sterk, P. S. Hiemstra, and L. van Bree. 1998. Asthma severity and susceptibility to air pollution. *Eur. Respir. J.* 11:686-693.
- Hatch, G. E. 1995. Asthma, inhaled oxidants, and dietary antioxidants. *Am. J. Clin. Nutr.* 61:625S-630S.
- Devalia, J. L., H. Bayram, M. M. Abdelaziz, R. J. Sapsford, and R. J. Davies. 1999. Differences between cytokine release from bronchial epithelial cells of asthmatic patients and non-asthmatic subjects: effect of exposure to diesel exhaust particles. *Int. Arch. Allergy Immunol.* 118:437-439.
- McDonald, P. P., V. A. Fadok, D. Bratton, and P. M. Henson. 1999. Transcriptional and translational regulation of inflammatory mediator production by endogenous TGF-beta in macrophages that have ingested apoptotic cells. *J. Immunol.* 163:6164-6172.
- Kayanoki, Y., J. Fujii, K. Suzuki, S. Kawata, Y. Matsuzawa, and N. Taniguchi. 1994. Suppression of antioxidative enzyme expression by transforming growth factor-beta 1 in rat hepatocytes. *J. Biol. Chem.* 269:15488-15492.