APOPTOSIS AND EXPRESSION OF FAS (CD95) AND FAS LIGAND (CD95L) IN THE AIRWAYS OF SEVERE ASTHMA SUBJECTS

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Abstract

Airway epithelial denudation is characteristic of asthma and may contribute to non-specific bronchial hyperresponsiveness and airway remodeling. Apoptotic pathway in these cells is mediated by Fas-FasL interaction. Loss of the protective immune function by FasL could permit prolonged survival of inflammatory cells in the airway mucosa. We postulated that 1) the process of epithelial apoptosis is enhanced in asthma, 2) there is decreased epithelial expression of FasL which may contribute to the persistence of inflammatory cells in the airway walls of asthma subjects. We performed the TUNEL assay to detect the extent of apoptosis, and immunohistochemical staining to determine the expression of Fas and FasL in asthmatic airways. Formalin fixed paraffin embedded airway sections from 17 severe asthma subjects, 16 chronic asthma subjects and 18 control subjects were studied. Images of the conducting airways were captured using a Spot Cooled digital camera linked to a computer. Quantification for TUNEL and immunohistochemical staining was performed using Image Pro-Plus® software. Epithelial apoptosis was increased in the severe asthma versus chronic asthma and control groups (p = 0.0004 all airways; p = 0.01 cartilaginous airways; p = 0.004 membranous airways). Epithelial expression of Fas and FasL was increased in the severe asthma group compared to chronic asthma and control groups (p = 0.04 and 0.0004 respectively for all airways combined). There was no difference in epithelial Fas expression between the groups when cartilaginous and membranous airways were analyzed separately. Epithelial FasL expression was increased in the severe asthma group in both the cartilaginous and membranous airways compared to chronic asthma and control groups (p = 0.0004 and 0.0008 respectively). Fas expressing inflammatory cells in the wall of membranous airways were increased in the severe asthma versus chronic asthma and control groups. There was no statistically significant difference between the study groups in FasL expressing inflammatory cells in the airway wall compartments. We demonstrate that there is increased apoptosis and
epithelial expression of Fas and FasL in the airways of severe asthma group. Alteration in Fas or FasL expression does not contribute to persistence of asthma inflammation but could contribute to epithelial apoptosis.
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CHAPTER I

Introduction and Background

1.1 Asthma

Asthma is a complex inflammatory disease of the conducting airways that results from the interaction of several environmental and genetic factors (1). This disease is characterized physiologically by 1) variable airflow obstruction with spontaneous or pharmacologic reversibility and 2) increased airway responsiveness and clinically by a symptom complex of 1) attacks of dyspnoea of long and short duration, 2) associated with intermittent or persistent expiratory wheezing, and 3) productive or non-productive cough.

The pathologic features of asthma include 1) epithelial desquamation or denudation, 2) goblet cell hyperplasia, 3) increased deposition of interstitial collagens (types I, III and V), fibronectin and tenascin in the subepithelial connective tissue (increased fibrotic response), 4) smooth muscle hypertrophy and hyperplasia, 5) increased vascularity of the airway wall, 6) edema and marked inflammatory infiltration of the sub-mucosa particularly by eosinophils and lymphocytes, coupled with 7) hypertrophy of the sub-mucosal glands and 8) plugging of the airway lumen (2). These clinical, physiologic and pathologic features are the hallmarks of asthma.

Risk factors for the development of asthma

There are several risk factors for the development of asthma, one of them of notable importance is parental (particularly maternal) history of asthma and atopy (3). In subjects who have asthma and are between 20-44 years of age, early onset disease (i.e. before age 15 years) has a stronger association with parental history of asthma than with onset after age 15 years (4). However, airway hyper-responsiveness and symptoms of asthma were strongly associated with parental asthma for all ages (4).
Risk factors operating in utero and the neonatal period include younger maternal age (5), less weight gain during pregnancy (6) and low birth weight. It is an unexplained observation, in most childhood studies, that asthma and allergy are more prevalent in males than in females in Hispanic population (7). Both transient and persistent childhood wheezing have been associated with early childhood viral respiratory tract infections (8). The observations that there is a fifty percent higher prevalence of asthma in African-Americans (9) and a higher incidence of persistent wheezing in Hispanic children (8) demonstrate an influence of ethnic factors on disease production. Whether these ethnic differences are due to genetic factors or are related to socio-economic or cultural differences is unclear.

Atopy, as demonstrated by positive allergy skin tests or increased serum IgE levels is a very important risk factor for the development (7) and persistence of early childhood asthma (8).

Environmental risk factors such as increased environmental allergen exposure, moving from a rural to an urban environment (10), exposure to furry pets (11) or passive exposure to tobacco smoke during childhood (8) are also important risk factors for childhood and adult onset asthma.

Dietary factors play an important role in asthma. Reduction in the incidence of this disease has been seen in Asian children eating a fully Asian diet compared with those children eating a mixed Asian and English diet (12). Australian children who eat oily fish regularly have a four fold lower risk of developing asthma (13). Direct or indirect exposure to industrial or occupational airborne pollutants, especially substances with a low molecular weight are related to occupational sensitization and hence to asthma (14).

1.2 The airway epithelium: Morphology, function and pathophysiology

Morphology

The airway wall architecture is composed of the epithelium which rests upon a basement membrane, the lamina propria and the submucosa containing smooth muscle, glands and
cartilage (15). The airway epithelium forms an interface between the inspired air and the underlying subepithelial airway wall tissue. The epithelium maintains a physico-chemical and mechanical homeostasis necessary for its efficient and effective functioning. The airway epithelium consists of three major cell types, 1) ciliated cells 2) secretory cells and 3) basal cells which collectively form a pseudostratified ciliated lining layer (16).

1) Ciliated cells are the pre-dominant cell types and are the terminally differentiated cells in the upper airways (17). These ciliated cells are sealed together by apical tight junctions. This cohesive mechanism serves as a permeability barrier thereby protecting and restricting access of inhaled noxious agents to the underlying tissues (18). Their main function is mucociliary clearance of inhaled infectious, particulate and toxic materials in the respiratory system.

2) Secretory cells comprise 15-20% of the airway epithelium and are present in several forms. Mucous or goblet cells produce airway mucus in which the inhaled particles, including viruses and bacteria can get trapped (17). Clara cells produce the surfactant apoproteins A and B and secretory leukoprotease inhibitors. They are believed to detoxify inhaled materials and thus participate in the protection of the airways (19). Serous cells also produce anti-proteases (20), whereas neuroendocrine cells contain amines and peptide hormones (21). The mucous cell is the predominant secretory cell in the larger airways, whereas the Clara cell is predominant in the bronchioles (17).

3) Basal cells are considered to be the stem cells or the progenitor cells of the airway epithelium. These are pyramidal in shape and are integrally involved in the attachment of the tall columnar cells to the airway basement membrane, thus contributing to the pseudostratified appearance of the epithelium (17).

**Barrier functions**

**Role of airway epithelial integrity**
The airway epithelium plays a crucial role in initiating and augmenting the first line of host defence (as part of the non-specific immune system) against the entry of noxious substances. This physico-chemical homeostasis is mediated via the integrity of the epithelium which contributes to the physical barrier functions, secretory and ciliary function leading to effective mucociliary clearance and the secretion of protective mediators against injurious agents.

Intact airway epithelium forms a continuous layer by virtue of several adhesion mechanisms which are important in determination of epithelial architecture. Desmosomes, intermediate junctions and the gap junctions maintain cell-to-cell adhesion and communication (signaling) processes. The tight junction, which is a narrow belt like structure surrounding each cell at the apical pole provides a physical barrier. Contact with the basement membrane is maintained by hemidesmosomes, focal contacts and specific receptors for the components of the extracellular matrix (18).

**Mucociliary clearance**

Inhaled irritants and particles including bacteria and viruses are trapped in the mucus. They are cleared from the airways by the continuous and coordinated beating of the cilia which propels the tracheobronchial secretions towards the pharynx. The clearance of these particles is facilitated by the secretion of surfactant which lowers the surface tension, making the particles less sticky.

**Secretion of protective mediators**

The airway epithelium secretes a number of putative protective compounds that either have smooth muscle relaxing or chemorepellant properties. These compounds include antibacterial substances (lactoferrin and lysozyme), anti-proteases (secretory leukoprotease inhibitor, α₁-anti-chymotrypsin, tissue inhibitors of metalloproteases), anti-oxidants (glutathione redox cycle, superoxide dismutase and catalase) (22), components of the complement system which act as
opsonins allowing efficient phagocytosis by the macrophages (23) and secretory immunoglobulin A (sIgA) (24).

The airway epithelium also secretes compounds like prostaglandin (PG) E₂, prostacyclin, nitric oxide (NO) and epithelium derived relaxing factor (EpDRF) that suppress airway smooth muscle contraction and cause bronchodilatation (25). Airway epithelial cells contain neutral endopeptidase (NEP) which is involved in the metabolism of airway smooth muscle spasmogens like neuropeptides. Epithelial damage and loss of NEP activity may diminish peptide breakdown and thereby enhance bronchoconstriction (26). These broncho-relaxants play an important role in regulating bronchomotor tone.

Transforming growth factor β (TGF β) has been identified in airway epithelial cells. This cytokine has dual pro and anti-inflammatory properties. It is an important mediator in the regulation of cell growth, differentiation, signaling, repair and downregulation of local inflammatory events (27).

**Pathophysiology**

**Recruitment of inflammatory cells**

Airway epithelial cells can synthesize and secrete several chemokines (chemotactic cytokines) and pro-inflammatory cytokines responsible for the differentiation, recruitment (chemotaxis) and activation of inflammatory cells in the airways, thus amplifying the inflammatory response.

Chemotactic cytokines include lymphocyte chemo-attractant factor, granulocyte-macrophage colony-stimulating factor (GMCSF) and the members of the chemokine superfamily. GMCSF is a potent chemo-attractant mediator and activator for neutrophils and eosinophils, and potentiates the differentiation and survival of these cells by blocking their apoptotic pathways (28).

Interleukin 8 (IL 8) in the presence of GMCSF has been shown to be chemo-attractant for eosinophils. This cytokine is also a potent chemo-attractant for neutrophils and T lymphocytes (29). Airway epithelial cells can also release RANTES (regulated on activated, normal T cell
expressed and secreted) and monocyte chemotactic proteins (MCP 1, MCP 4) which are potent eosinophil and monocyte/basophil chemo-attractants respectively (30, 31).

Multi-functional cytokines like interleukin 1β (IL 1β), interleukin 6 (IL 6), interleukin 11 (IL 11), interleukin 16 (IL 16), tumor necrosis factor α (TNF α), interferon γ (IFN γ) and TGF β have proinflammatory and chemotactic effects on a variety of target cells, are involved in activation of B lymphocytes and monocytes and can induce acute-phase protein synthesis (28). TNF α, IFN γ and IL 1β cause swelling of the mitochondria, thus impairing their ability to produce adenosine triphosphate (ATP). This in turn decreases ciliary activity in airway epithelial cells and impairs transport of mucus (32).

Airway epithelial cells are also capable of secreting arachidonic acid metabolites like 12 and 15-hydroxyeicostetraenoic acid (12 HETE and 15 HETE), prostaglandin F₂α (PGF₂α) (33) platelet activating factor (PAF) (34) and possibly leukotriene B₄ (LTB₄) (35) which are potent chemo-attractants for eosinophils, neutrophils and monocytes, and also increase mucus production and secretion.

Cell-cell adhesion and communication (signaling)

Airway epithelial cells can express cell adhesion molecules like intercellular cell adhesion molecule-1 (ICAM 1), vascular cell adhesion molecule-1 (VCAM 1), E selectin, and lymphocyte function-associated antigen-3 (LFA 3). Increased expression, synthesis and release of these molecules during an inflammatory response may contribute to the adhesion and subsequent maturation and activation of leukocytes in the epithelial compartment (28).

Increased expression of CD44 has been observed following epithelial damage in vitro. CD44 interacts with the cytoskeletal actin modifying adhesive properties at a sub-cellular level (36).

The adhesion molecules E cadherins and integrins are also associated with the actin cytoskeleton through catenins (37) and α-actinin or talin (38), respectively. These molecules have been
suggested as markers of repair and alterations in their expression may be involved in the disruption of cellular architecture of asthmatic airways. (37).

1.3 Epithelial sloughing in asthma: - Loss of barrier functions

Epithelial damage and shedding is a characteristic and diagnostic feature of asthma and may correlate with airway hyperresponsiveness (39). There is still a debate about whether airway epithelial damage is artifactual and is caused by biopsy forceps used in fiberoptic bronchoscopy or whether the epithelium in asthma is actually fragile. The evidence of widening of intercellular spaces, epithelial swelling and shedding observed in bronchial biopsy specimens is supported by increased numbers of epithelial cells in the broncho-alveolar lavage fluid (BAL) and sputum of subjects with asthma (40). Sloughed epithelial cells in the form of clumps known as Creola bodies have been recognized in the sputum of subjects with asthma. The number of damaged and denuded cells increases substantially during an exacerbation of asthma (41).

The effects of epithelial damage can be structural, functional and metabolic. Damage may also result in an altered phenotype, with the epithelium becoming a significant source of autocoid mediators, cytokines, chemokines and growth factors with the potential to promote inflammation, fibroblast and smooth muscle proliferation and matrix deposition (42).

Environmental allergen induced inflammation can be persistent, and consequently may lead to epithelial denudation, resulting in sub-epithelial fibrosis and chronic airway remodeling (43). Exposure to environmental proteinases e.g. Der p 1 from fecal pellets of house dust mite attack epithelial integrity by cleaving the tight junction protein occludin. Tight junction breakdown increases epithelial permeability allowing the allergens to interact with the underlying dendritic antigen presenting cells. Opening of tight junctions by environmental proteinases may be the initial step in the development of asthma in response to a variety of allergens (44). Epithelial desquamation is a prominent and recognized feature in fatal asthma (45) and is also observed in
biopsy and BAL specimens of subjects with mild asthma or in those subjects who have been asymptomatic for long periods of time. The number of shed epithelial cells in bronchial washings is increased four-fold in subjects with asthma compared to normal controls (46). Thus, it is not clear whether the loss of epithelial integrity contributes to airway hyper-responsiveness or is a consequence of airway inflammation, damage and sloughing.

Airway hyperresponsiveness is defined as a 20% or greater fall in FEV\textsubscript{1} in response to a provoking agent at a concentration or level of exposure that causes no or minimal change in FEV\textsubscript{1} in subjects who do not suffer from asthma. The provoking agents can be histamine, methacholine, hypertonic saline and sometimes exercise and cold air hyperventilation (47) The possible mechanisms by which damaged airway epithelium may affect airway responsiveness are 1) enhanced penetration of inhaled allergens, irritant particles and noxious gases to the underlying mediator secreting cells and sub-mucosal tissue, 2) easier access of irritant factors to sensory nerves which are involved in neuropeptide release and induction of reflex bronchoconstriction, 3) lack of synthesis of neutral endopeptidase and cholinesterase thus increasing the tissue concentration of contractile agents, 4) decreased production of bronchorelaxants like epithelium derived relaxant factor (EpDRF) and prostaglandin E\textsubscript{2} (PGE\textsubscript{2}) which protect the airways from excessive bronchoconstriction (48,49).

This epithelial damage may be a consequence of the stimulatory and cytotoxic effects of mediators such as major basic protein (MBP), eosinophil cationic protein (ECP) and eosinophil peroxidase (EPO) released from activated eosinophils which are capable of epithelial damage \textit{in vitro}. Elevated levels of these mediators have been detected in sputum of subjects who have asthma and around areas of epithelial damage in subjects who died of asthma (50).

Matrix metalloproteinases e.g. MMP 9 (gelatinase B) and MMP 2 also contribute to weakening of the epithelium through their capacity to cleave adhesion molecules, degrade basement membrane collagen and proteoglycans, and may lead to both epithelial disruption and airway
wall matrix turnover (51). The epithelial damage in asthma is variable and patchy, and is followed by repair and restitution mechanisms with proliferation of basal cells after injury. (52). Airway epithelium is involved in perception of broncho-constriction due to the presence of intra-epithelial sensory nerves. Epithelial damage and desquamation may cause poor perception of the severity of broncho-constriction in asthma and may lead to a delay in starting appropriate treatment which is possibly one of the factors contributing to death from asthma (53).

1.4 Apoptosis

Mechanism

Apoptosis, or programmed cell death, is a genetically determined physiological phenomenon responsible for the elimination of unnecessary, damaged and potentially harmful cells from the surrounding healthy cells to ensure structural and functional tissue homeostasis.

This mechanism is characterized morphologically by cell shrinkage and blebbing of the cell membrane, condensation of cytoplasm with nuclear chromatin becoming pyknotic and compacted, followed by fragmentation of the nucleus. The fragmentation of nuclear chromatin into histone associated 180-200 base pair oligonucleosomes is a biochemical hallmark of apoptosis. A ‘DNA ladder’ pattern of inter-nucleosomal DNA fragmentation is also characteristic of this process. The cell becomes convoluted and breaks into several membrane bound vesicles containing a variety of intact organelles and nuclear fragments. These ‘apoptotic bodies’ are then engulfed by macrophages or neighboring phagocytic cells. The histological hallmark of apoptosis is the absence of associated tissue inflammation and damage to the surrounding host tissue (54, 55).

Apoptosis versus necrosis

Apoptosis and necrosis are two distinct and basic forms of cell death. It is usually possible to distinguish between the mechanisms of apoptosis and necrosis based on the morphological and
biochemical characteristics. Current understanding of the differences between apoptosis and necrosis are summarized in table 1.

**Molecules**

**Fas receptor** *(Fas, CD 95; APO 1)* is a 48 kD type 1 membrane protein and is a member of the tumor necrosis factor/nerve growth factor receptor super family (56). Fas is ubiquitously expressed in various tissues with abundant expression in thymus, liver, heart and kidney. This protein shows a moderate level of expression in lung, skin and activated T lymphocytes, eosinophils and natural killer (NK) cells (57).

**Fas Ligand** *(FasL, CD 95L; APO 1L)* is a 40 kD type II trans-membrane protein belonging to the tumor necrosis factor family of ligands. This protein is expressed on activated T lymphocytes and in various tissues including small intestine epithelium, kidney, spleen, thymus, ovary, testis, uterus, prostate and lung (58). The membrane bound FasL undergoes metalloproteinase mediated proteolytic cleavage to generate soluble functionally active cytokine. This indicates that FasL may be working locally via cell-cell interactions, under physiological or pathological conditions to induce apoptosis (59) (figure 1).

**Bcl-2 family** of proteins consists of both inhibitors (anti-apoptotic) and promoters (pro-apoptotic) of apoptosis. The anti-apoptotic proteins include Bcl-xL, Mcl 1, A1, Bcl w and the proapoptotic proteins are Bax, Bcl-xS, Bad, Bid and Bak. Many of these proteins interact with each other through a complex network of homo- and hetero dimers. It is thought that the ratio of anti-apoptotic versus proapoptotic dimers is important in determining the resistance of a cell to apoptosis (60). These proteins are located in the mitochondrion, which through the protein-protein interaction of the family members regulate the release of cytochrome c. Bcl 2 family members may regulate apoptosis at multiple steps in the cell death pathway. The initial step that results from multiple apoptotic stimuli is swelling of the mitochondria, rupture of the outer
mitochondrial membrane and the release of mitochondrial proteins such as cytochrome c and AIF (apoptosis inducing factor).

Anti-apoptotic Bcl-2 family members, such as Bcl-xL are able to prevent these characteristic mitochondrial changes, whereas pro-apoptotic Bcl-2 family members, such as Bax, are able to promote these changes. Bcl-xL and Bax may independently regulate mitochondrial physiology through the formation of ion channels and/or through the regulation of each other's function by heterodimerization. The release of mitochondrial proteins, such as cytochrome c results in the activation of Apaf 1 (apoptosis activating factor-1). Apaf-1 binds to downstream caspases and processes them into proteolytically active forms which trigger the caspase cascade ultimately resulting in the death of the cell. Bcl-xL may bind to Apaf 1 and prevent this conversion, whereas Bax may complex with Bcl-xL and may prevent it from binding to Apaf-1 (61).

Caspases belong to the interleukin-1β converting enzyme (ICE) family of cysteine proteases. These enzymes are present in the cytoplasm as pro forms and require proteolytic processing by other proteases, often another caspase, or by autocatalytic cleavage to produce the active form. Different triggering stimuli initiate a stereotyped process of apoptosis regardless of the reason for the initiation of apoptosis (62).

**Process (regulation)**

The apoptosis cascade can be broadly sub-divided into three stages: initiation, effector and degradation (63, 64). The 'initiation' phase can be triggered by extra-cellular or intra-cellular, pharmacological and physiological stimuli (e.g. chemotherapy, radiation, Fas ligation). These stimuli may be seemingly unrelated in nature however, they share common downstream pathways. The 'effector' phase involves detection and transduction of the triggering signal. This phase also involves the activation of proteases as well as their positive and negative regulators. The signal transduction pathways send this message to the death effector machinery. The molecules involved in the effector stage can be either modulators or effectors. The modulators
are the pro and anti-apoptotic Bcl-2 family of proteins, apoptosis activating factors (Apafs) and apoptosis inhibitory proteins (AIPs). The effector molecules are caspases 1-13. The 'degradation' phase shows characteristic structural lesions of apoptosis i.e. cytoplasmic and chromatin condensation, nuclear DNA fragmentation, production and activation of oxygen radicals, elevation of cytosolic calcium and cytosolic acidification.

**Fas receptor signaling**

The molecular pathways involved in the initiation of apoptosis and the activation of the caspase cascade have been diagrammatically summarized in figure 2.

Extracellular ligation of Fas receptor results in the recruitment of adaptor proteins to form a death inducing signaling complex (DISC) (65). The protein tyrosine phosphatase, Fas associated phosphatase-1 (FAP-1) may also have a role in DISC formation (66). The ability of the Fas receptor to trigger apoptosis is dependent on an intra-cytoplasmic, eighty amino acid conserved common death domain (DD) which mediates the ligand dependent recruitment of other death domain containing proteins such as FADD (Fas associated protein with death domain). Binding of trimeric FasL induces trimerization of Fas and FADD. In turn, FADD associates with the pro-enzyme form of caspase-8 through dimerization of a domain known as the death effector domain (DED). Formation of DISC results in dimerization and activation of caspase-8. Caspase-8 triggers a cascade of events, either through Bcl-2 family members or by activating caspase-3.

Caspase-3 has been shown to cleave and inactivate many cytoplasmic and nuclear proteins including functional and structural proteins like PARP (poly ADP ribose polymerase). Cleavage of PARP can be used as a convenient marker of apoptosis. Caspase-3 can also activate other caspases like caspase-6 which cleaves lamins, a major constituent of the nuclear envelope proteins. Caspase-3 is also directly linked to the onset of DNA fragmentation and cell death via the cleavage and activation of DFF (DNA Fragmentation Factor) (65). Cleavage of DFF allows the translocation of CAD (caspase-activated deoxyribonuclease) into the nucleus and degradation
of chromosomal DNA. In granzyme B (GraB) mediated apoptosis, GraB cleaves caspase 10 which in turn activates caspase 7, caspase 3 and other downstream caspases. Release of cytochrome c and adenosine triphosphate (ATP) from the mitochondria promotes Apaf-1 mediated activation of caspase 9 which then cleaves caspase 3 (65).

1.5 Apoptosis in asthma

In the context of this study, the characteristic airway inflammation of asthma may be associated with:

1) excessive apoptosis of airway epithelium

2) delayed or diminished apoptosis of inflammatory cells in the airway wall leading to their enhanced survival and chronicity of an inflammatory process.

Damage to the airway epithelium in asthma can be attributed either to the direct damaging effects of an insult to the cells or to the indirect effects of an acute systemic inflammatory response. Persistence of inflammation may be due to failure of clearing mechanisms or apoptosis of these inflammatory cells which results in release of their toxic contents, thus perpetuating and amplifying the inflammatory process. The presence of large numbers of inflammatory cells, especially eosinophils, has been related to the clinical severity of asthma (67).

The co-expression of Fas and FasL in the airway epithelium (both basal cells and columnar epithelium) suggests that the physiological turnover of this tissue is regulated by the Fas/FasL mediated apoptotic pathway (68). Induction of apoptosis through an interaction of Fas with its ligand is a well recognized phenomenon in these tissues and may be mediated through an autocrine, paracrine or a juxtacrine loop causing self or adjacent cell death (figure 1). The estimated rate of turnover of the normal airway epithelium is one percent per day, suggesting a basal regulation of the Fas/FasL interaction that prevents excessive apoptosis in these tissues (69). Although apoptosis is part of a physiological process of epithelial renewal, it can be
pathological if it occurs in excess. It has been suggested that increased epithelial damage and turnover in asthma may be due to excessive and accelerated apoptosis, and may be regulated and mediated by the interaction of apoptotic molecules like Fas and FasL (70). Fas receptor expression appears to be greater in areas of epithelial injury and may play a role in epithelial integrity in asthma (unpublished observation cited in 71). There is *in vivo* evidence of upregulation of Fas and FasL in idiopathic pulmonary fibrosis (IPF) suggesting a role of these molecules in airway tissue remodeling (72). It is possible that the apoptosis of the epithelial cells induced by epithelial injury as seen in asthma may lead to fibrosis and structural changes and ultimately cause irreversible obstruction of the airways. However, over-expression of Fas and FasL may participate in tissue destruction by inducing excessive apoptosis. It has been suggested that stimulation of the Fas/FasL pathway not only mediates apoptosis in the airway epithelium, it may also play a pro-inflammatory role by inducing IL 8 production which may amplify the inflammatory response. Induction of IL 8 secretion by Fas ligation may be regulated by NF κB activation (73).

Due to its capacity to induce apoptosis, FasL has been suggested to be involved in the phenomenon of immune privilege or apparent defect in the function of the immune system (74). Immunologically privileged organs like eye, brain, testis, adrenal gland and placenta have shown strong FasL expression. This expression may contribute to the immune privileged status of these organs by preventing dangerously destructive immune responses. Since the inflammatory cells like neutrophils, eosinophils and lymphocytes are Fas bearing, we can speculate that altered regulation or function of FasL derived from the airway epithelium may disturb the protective function of FasL. It is quite likely that disruption of a cyto-protective function due to downregulation of FasL may be a contributing factor in the persistence of airway inflammation, accumulation of immune effector cells and delay in initiation of the repair process of the airways.
1.61 **Objectives**

1) Investigate the role played by Fas and FasL interaction on airway epithelial damage and shedding.

2) Determine the effects of loss of airway epithelial FasL on the persistence of inflammation in the airway wall.

1.62 **Hypotheses**

1) The process of apoptosis in the airway epithelium is enhanced in asthma.

2) There is decreased epithelial expression of FasL which may contribute to the persistence of inflammatory cells in the airway wall of asthma subjects.

1.63 **Specific aims**

1) To investigate the degree of airway epithelial cell apoptosis in subjects suffering from asthma of varying degrees of clinical severity.

2) To demonstrate the increased or decreased expression of Fas and/or FasL in the airway epithelium and the inflammatory cells in the airway wall of asthma subjects.

3) To determine the statistical relationship between the clinical severity of asthma, increased or decreased expression of Fas and/or FasL and the degree of airway epithelial cell apoptosis.

To test these hypotheses, we examined airway tissue obtained from subjects who had died suddenly but had a history suggestive of acute asthmatic attacks, subjects who had chronic asthma and had undergone lung resection for a pulmonary neoplasm, and subjects who did not suffer from asthma (control subjects). Airway tissue was either obtained at autopsy (from subjects who had severe asthma), or from surgical specimens (from subjects who had chronic asthma and from control subjects). Three specimens in the group of chronic asthma were
obtained at autopsy from those subjects who had died of causes other than asthma. All the tissues were examined for the degree of apoptosis and expression of Fas and FasL.
2.1 Study design

Study population

The study population comprised three groups; subjects who had severe asthma, subjects who had chronic asthma and those subjects who did not have asthma (control subjects). The subjects who had chronic asthma and the control subjects were part of an ongoing study to investigate the relationship between the lung structure and function being conducted at the University of British Columbia McDonald Research Laboratories and iCAPTURE Centre, St. Paul’s Hospital. The study of lung structure and function is based on a prospective study of pulmonary function in patients requiring lung resection for the treatment of carcinoma or any other localized neoplastic or non-neoplastic lesion of the lung. A modified Medical Research Council questionnaire was administered to the study subjects for evaluation of their symptoms, smoking history and occupational exposures to potential carcinogens.

The subjects who had chronic asthma and the non-asthmatic controls had their pulmonary function tests done in the week prior to surgical resection of lung. Their lung function tests were done in the clinical pulmonary function laboratory in compliance with the American Thoracic Society recommendations. The percent predicted of normal lung function were calculated according to the formulas of Crapo and coworkers (76).

The subjects in the study groups were matched as closely as possible for age and sex. Ethics approval for the use of human tissue was obtained from the University of British Columbia and St. Paul’s Hospital Institutional Review Boards.

Subject phenotype

Severe asthma — this group consisted of seventeen subjects. These subjects had died during an acute attack of asthma or suddenly due to unknown causes and had history suggestive of acute or
severe asthma. On autopsy examination, the histopathological findings in their lung tissue were consistent with severe asthma. The tissue specimens were obtained at coroner’s autopsy and kindly provided by Dr. B. Lifschultz, Medical Examiner, Cook county, Illinois. Demographic information for this group of subjects is presented in table 2. However, detailed demographic information on these subjects was unavailable due to the nature of their terminal event. All the subjects with severe asthma were of African-American origin and belonged to the inner city population of Chicago.

**Chronic asthma** – lung tissue specimens from sixteen subjects with chronic asthma were obtained. These subjects had undergone pulmonary resection for a peripheral lung tumor. The majority of them were diagnosed on histopathology examination as bronchogenic carcinoma. The clinical criteria used to establish the diagnosis of asthma included prior physician diagnosis and treatment for asthma. The clinical diagnosis of asthma was made on the basis of an evaluation of the patients’ history and medical files by a respiratory physician. The tissue samples from subjects who had chronic asthma also showed histopathological evidence of the inflammation and structural changes characteristic of asthma as assessed by a pathologist specializing in pulmonary pathology. Subject demographics and lung function data for this group are described in table 3 and table 5, respectively. Tissue specimens from three subjects in this group (subject # 5, 14 and 15) were obtained at autopsy and therefore, limited clinical data is available on these subjects. Death in these three subjects was due to causes other than asthma.

**Control subjects** – this group comprised eighteen subjects. These individuals had surgical resection of a lung lobe for indications unrelated to asthma. Blocks of tissue containing airways were examined from all subjects. The demographic and lung function information for this group of subjects is presented in table 4 and table 6, respectively.

### 2.2 Research methodology

Two methodologies were used in this study:
1) Terminal deoxynucleotidyl transferase (TdT) mediated dUTP Nick End Labeling (TUNEL) assay was used for the detection of apoptosis.

2) Immunohistochemical staining methods using specific antibodies directed against human Fas and FasL were performed to study the expression of these protein molecules in the airway epithelium and the inflammatory cells in the airway wall.

**Preparation of tissue specimens**

Since the patient population was being studied retrospectively, lung tissue specimens had already been fixed in 10% neutral buffered formalin and tissue blocks were prepared by embedding in paraffin. Five μm thick cross sections of the tissue specimens were cut using a tissue sectioning microtome, the sections were placed on silanized slides and dried overnight at 37°C. The slides were further baked at 65°C for 1 hour for the tissue sections to be fixed on to the slide, prior to TUNEL or immunohistochemical staining.

**Detection of apoptosis in airway epithelium**

*In situ* Terminal deoxynucleotidyl transferase (TdT) mediated dUTP Nick End Labeling (TUNEL)

This is a popular method to detect apoptosis *in situ* in formalin fixed paraffin embedded tissues. DNA modification is not the only indicator of apoptotic cell death. There are well characterized morphological changes in individual cells that can be identified in histological sections. Detection of apoptosis by TUNEL method allows simultaneous evaluation of the cell morphology and the nuclear staining and permits differentiation between the normal and apoptotic cells. However, it is important to mention that normal cells in S phase of cell cycle may stain positive by TUNEL assay but do not demonstrate the morphological characteristics of apoptosis. A non-isotopic system (TdT-FragEL™ Cat. # QIA33 Oncogene™ Research Products Boston, MA) was used for the detection and labeling of DNA breaks in apoptotic nuclei in
airway tissue sections. The manufacturer’s protocol for TUNEL assay was slightly modified for this study. However, the principle of the assay remains the same.

**Principle of TUNEL assay**

The principle of TUNEL assay is relatively simple and is illustrated in figure 3. TdT binds to exposed 3'-hydroxyl ends of DNA fragments which are generated in response to apoptotic signals. The TdT enzyme catalyzes the incorporation of biotin labeled and un-labeled deoxynucleotides (dNTP’s). Biotinylated nucleotides are detected by the avidin-biotin-alkaline phosphatase complex (ABComplex/AP). Avidin has four binding sites for biotin. The strong affinity of avidin for biotin makes this method of detection more sensitive than other available methods of detection. The biotinylated alkaline phosphatase in the ABComplex/AP binds with the substrate-chromogen solution which consists of a combination of Naphthol-ASBI phosphate and New Fuschin. The chromogen, New Fuschin reacts with the labeled sample to generate an insoluble colored (deep pink-red) precipitate at the site of DNA fragmentation.

**Procedure**

The optimized manufacturer’s protocol included proteinase K titration and incubation of tissue sections at 37°C for 1 hour. Incubation periods in the other steps of the manufacturer’s protocol were also modified and optimized for the study samples. The labeled nucleotides were detected by ABComplex/AP reagent (code # K 0376) obtained from DAKO Diagnostics Canada Inc (Mississauga, Ontario) Naphthol-ASBI phosphate and New Fuschin were purchased from Sigma Inc. (St. Louis, MO).

**Negative and positive control**

Human airway tissue sections prepared in the same manner as described above were included in each batch of TUNEL staining as negative and positive controls. Positive control was generated by pre-treating the tissue with DNAse in the presence of Mg++ for 30 minutes at room temperature. Negative control was generated by omitting the TdT enzyme and substituting it
with distilled water (dH2O). A set of two control slides was provided by the manufacturer. Each slide had two sections on it, one section was obtained from a cell block of HL60 cells which were induced to undergo apoptosis with 0.5 µg/ml of actinomycin and the other section was obtained from untreated HL60 cells. These control slides were also included with each batch of TUNEL staining and were treated as test samples.

**Outline of TUNEL assay protocol (for details refer to appendix V, V.1 TUNEL assay)**

Deparaffinization / Rehydration → Permeabilization with proteinase K → Equilibration →

Generation of Positive Control

DNA Fragment Labeling with TdT → Termination of Labeling Reaction → Detection of

Generation of Negative Control

Labeled DNA → Substrate / Chromogen Reaction → Microscopic Examination → Quantification

**Detection of Fas and FasL expression by immunohistochemistry**

Immunohistochemical staining methods were used to detect the expression of Fas and FasL in the airway epithelium and the inflammatory cells in the airway wall.

**Immunohistochemical staining for FasL**

The optimization of the protocol for immunohistochemical staining with FasL antibody was performed manually. The optimized protocol was used to stain the controls and the study samples using a DAKO® Auto-stainer. The steps of deparaffinization, rehydration, and epitope retrieval were performed manually on all the control and the study samples.

**Principle of the assay**

In this method, the un-labeled primary antibody binds to the antigen. A biotin labeled secondary antibody, sometimes also known as the link antibody, is applied to the tissue sections. The secondary antibody must be directed against the primary antibody. If the primary antibody is
made in rabbit, mouse or goat, the secondary antibody must be directed against rabbit, mouse or goat immunoglobulins, respectively. The antigen-antibody complex was detected by the avidin-biotin-alkaline phosphatase complex (ABComplex/AP). The four open sites on avidin bind to the biotin on the link antibody. The substrate chromogen which forms an insoluble (deep pink-red) colored precipitate with the enzyme localizes the antigen defined by the above antigen-antibody binding reactions.

**Heat-induced epitope retrieval**

Formalin fixation followed by paraffin embedding of the tissue specimens causes alteration in the three dimensional structure of the tissue proteins and forms cross linking bridges. These protein cross links and conformational changes may destroy the cell surface epitopes (antigens) or alter their reactivity with the antibody. This altered state of immunological reactivity may influence the sensitivity of the technique of immunohistochemistry and diminish specific staining. The process of epitope retrieval restores the conformation of the epitope and breaks down cross linkages. There are several methods available for demasking of epitopes on formalin fixed paraffin embedded tissue.

In this study, a heat-induced method of epitope retrieval was used. A water bath at temperatures between 95-98°C was used as a source of heat. The slides were placed in a pre-heated solution of 10 mM Na citrate (code # S 279 Fisher Scientific Company New Jersey) pH 6.0 and heated for 30 minutes. The target retrieval process was stopped by allowing the slides to cool at room temperature outside the water bath. Immunohistochemical staining was performed according to the protocol.

**Blocking**

Endogenous biotin is widespread in a variety of tissues and often causes non-specific staining by binding to avidin. This binding activity was overcome by inserting an avidin-biotin blocking
(Biotin Blocking system code # X0590, DAKO® Diagnostics Inc., Mississauga, Ontario) step to block the endogenous biotin.

To prevent non-specific binding of antibodies, the tissue sections were pre-treated with Universal blocking solution (cat. # CD 310082 DAKO® Diagnostics Canada Inc., Mississauga, Ontario). Pre-treatment of tissues with the universal blocker does not interfere with the reactivity of primary antibody and can be used with antibodies produced in any species of animals.

**Antibodies**

Primary antibody – Polyclonal goat anti-human IgG FasL (N-20) G code # sc-834-G (Santa Cruz Biotechnology Inc. Santa Cruz, CA).

Secondary antibody – Biotinylated rabbit anti-goat IgG code # E 0466 (DAKO® Diagnostics Inc., Mississauga, Ontario).

Negative control - Rabbit immunoglobulin fraction (normal) negative control (code # X 0903 DAKO® Diagnostics Inc., Mississauga, Ontario).

**Gold standards**

Staining for positive control was performed on sections from testis, uterus, prostate and lung tissue using the same concentration of the primary antibody. Staining for negative control was also performed on these tissues using a non-specific antibody of the same isotype. These tissues have been reported to show high to moderate levels of FasL expression (58). The control tissues underwent the same steps of tissue pretreatment and processing as the study samples.

**Outline of immunohistochemistry assay protocol for FasL (for details refer to appendix V, V.2 immunohistochemical staining for FasL)**

Deparaffinization / Rehydration → Epitope retrieval → Non-specific Protein Blocking → Biotin Blocking → Primary Antibody → Biotinylated Secondary Antibody → Detection
(ABComplex/AP) → Substrate-Chromogen (Naphthol-ASBI phosphate/New Fuschin) → Microscopic Examination → Quantification

**Immunohistochemical staining for Fas**

The immunohistochemistry procedure performed for the detection of Fas was based on a signal amplification method. This procedure results in the amplification of the signal, allowing detection of extremely small quantities of target antigen. The techniques used in this system were based on avidin-biotin and peroxidase methodologies. In this technique the peroxidase catalyzes the deposition of a biotinylated phenolic compound followed by secondary reaction with streptavidin peroxidase. This system is called the Catalyzed Signal Amplification (CSA) System (DAKO® code # K 1500).

**Principle of the assay** (figure 4)

The first incubation was with hydrogen peroxide to inhibit the endogenous peroxidase activity and was followed by incubation with biotin blocking reagents to inhibit binding to endogenous biotin. Subsequently the tissue specimens were incubated with a protein block to suppress non-specific binding to the tissues of reagents that would follow in the subsequent steps. In this assay a mouse monoclonal primary antibody or a negative control reagent is bound by a biotinylated secondary antibody. The streptavidin-biotin-peroxidase complex binds to the biotinylated secondary antibody via its streptavidin. The bound peroxidase catalyzes the precipitation of biotinylated phenol molecules onto the specimen which amplifies the number of biotin molecules available to bind to streptavidin peroxidase. Staining was completed by incubating with diaminobenzadine/hydrogen peroxide as chromogen/substrate which results in a brown colored precipitate at the antigen site.

**Heat induced epitope retrieval**
DAKO® target retrieval solution 10X concentrate code # S 1699 in a 1:10 dilution was used at pH 6-6.2. The heating conditions for the process of epitope retrieval were the same as described previously.

**Blocking**

Endogenous peroxidase activity in the tissue specimens was inhibited by incubating the sections with 3% hydrogen peroxide in methanol prior to the application of the primary antibody. Blocking for endogenous avidin-biotin and non-specific proteins was performed by the methods described in appendix V, V.3.

**Antibodies and reagent**

Primary - Monoclonal mouse anti-human IgG CD95, APO-1/Fas, DX2 code # M 3554 DAKO® Diagnostics Inc., Mississauga, Ontario).

Negative control – Purified mouse IgG κ isotype (cat. # 03021 D 0.5 mg/ml Pharmingen).

Antibody dilution was prepared in antibody diluting buffer cat. # CD 200082, DAKO® Diagnostics Inc., Mississauga, Ontario).

**Gold standards**

Fas receptor has been described to be widely distributed in lymphoid tissue, liver and lung (58). These tissues were prepared identically to the study samples. Sections obtained from these tissues were included in each batch and staining for negative and positive control was performed on them.

**Outline of immunohistochemistry protocol for Fas (for details refer to appendix V, V.3 immunohistochemical staining for Fas)**

Deparaffinization / Rehydration → Epitope Retrieval → Peroxidase Block → Avidin-Biotin Block → Protein Block → Primary Antibody or Negative Control → Link Antibody →
Streptavidin-Biotin Block → Amplification Reagent → Streptavidin Peroxidase → Substrate-Chromogen solution → Microscopic examination → Quantification.

2.3 Strategies for performing morphometric measurements and quantification of staining

All the non-branching cartilaginous and membranous airways that fulfilled the criteria of inclusion were selected for analysis. The respiratory bronchioles were excluded from the analysis. Those airways that fulfilled the criteria of inclusion were selected for analyses and quantification from each of the stains was performed.

Criteria for airway selection (figure 5)

Integrity of the basement membrane – Presence of an intact well defined boundary of the basement membrane for at least eighty percent of the circumference of the airway lumen was of foremost importance for including the airways for analysis.

Presence and distribution of the smooth muscle layer – The presence of a well defined continuous smooth muscle boundary of approximately constant thickness around at least eighty percent of the airway wall.

Long to short diameter ratio of < 3.3 – The longest luminal diameter and the widest diameter perpendicular to this diameter were measured at the smooth muscle layer and a ratio between these two diameters (aspect ratio) was calculated. Airways with aspect ratio greater than 3.3 were considered to be cut tangentially and were excluded from analysis.

The above criteria for the airway selection have been used in several previous morphometric studies that were conducted at our laboratory (77).

Digital image capture for quantitative analysis

Digital photomicrographs of the selected airways from the tissue sections were acquired by a Spot Camera (Diagnostic Instrument Inc., USA) coupled to Nikon Light Microscope (Nikon
Microphot, Tokyo, Japan). This setup was linked to a computer. The digital images were archived on compact discs (CD’s).

Depending upon the size of the airways, images were captured at magnifications between x20 to x400. For the morphometric measurements of the structural components of the airway wall, images were captured at low magnifications between x20 to x100. However, for quantifying TUNEL and immunohistochemistry staining, the images were captured at higher magnifications between x120 to x400. Larger airways were arbitrarily divided into four fields of view. An image of each field of view was captured at high magnification.

**Stereologic analysis for morphometric measurements and quantification of staining**

Image Pro-Plus® analysis software (Media Cybernetics Silver Spring, MD) was used to process, analyze and archive digital image information while integrating text, data and graphics. This software was used to determine the geometric measurements of the perimeters of each of the four layers of the airway wall by manual single line continuous tracing of the photomicrographs. Morphometric measurements were made on slides stained for FasL analysis. Dimensions of the individual airway wall components and compartments were measured using appropriate spatial calibrations (figure 6). Quantification of TUNEL positive airway epithelial cells and immunohistochemical staining for Fas and FasL positive airway epithelial and the inflammatory cells in the airway wall, was performed by point counting method. The absolute cell count of positively stained airway epithelial cells for three subjects, one from each subject group, was also performed for the TUNEL assay. The point counting method determined the fractional area (Aa) of the airway wall occupied by cells positive for TUNEL, Fas and FasL immunohistochemical staining. In this method, a grid mask containing 6956 points (probe) was superimposed onto the image (section profile). The points falling on positively stained airway epithelium and inflammatory cells in the airway wall were manually tagged by different classes of tag points (figure 7). By this method, the points of intersection between the probe and the section profile
were tabulated. The numerical data for morphometric measurements and point counting was exported to Excel spreadsheets for analysis. The morphometric measurements and point counting were performed in a blinded fashion.

**Structural dimensions of the airway wall**

All the airway wall dimensions were expressed either as millimeters (perimeter) or square millimeters (area) of the airway wall. Standardized nomenclature (15) was adopted with some modifications to define the sub-divisions of the airway wall.

The following morphometric measurements were performed to determine the structural dimensions of the airway wall compartments:

- $P_i$ – internal perimeter (luminal border of the airway epithelium)
- $P_{bm}$ – perimeter of the basement membrane
- $P_{mi}$ – perimeter of the inner border of the smooth muscle layer
- $P_{mo}$ – perimeter of the outer border of the smooth muscle
- $P_{sl}$ – measured length of denuded basement membrane

$P_i$ was measured only in those airways where more than 50% of basement membrane was covered with intact epithelium. In specimens with missing epithelium, $P_i$ was equal to $P_{bm}$.

- $WA$ – the airway wall area which was calculated by the differences between areas bound by the different perimeters
- $A_i$ – the internal or luminal area
- $WA_e$ – the area occupied by the airway epithelium and was enclosed by $P_i$ and $P_{bm}$
- $WA_{subepith}$ – the subepithelial area which was enclosed by $P_{bm}$ and $P_{mi}$
- $WA_{sm}$ – the smooth muscle area which was enclosed by $P_{mi}$ and $P_{mo}$
- $WA_{sub}$ – the submucosal area of the airway wall bounded by the $P_{bm}$ on the inner side and the $P_{mo}$ on the outer side of the airway lumen
- $WA_{tot}$ – the total airway wall area which was calculated ($WA_e + WA_{sub}$)
$A_{bm}$ – measured luminal or internal area, same as $A_i$.

$A_{bm}^*$ - calculated area of an idealized fully relaxed dilated airway lumen by assuming the $P_{bm}$ to be circular. $A_{bm}^* = P_{bm}^2 / 4 \pi$ where the measured luminal perimeter equals the circumference of the calculated airway lumen.

$A_{bm} / A_{bm}^*$ - ratio of the measured luminal area to the calculated luminal area. This ratio represents the degree to which the actual caliber of a contracted airway deviates from the caliber that it would have had in a fully relaxed and dilated state. $A_{bm}/A_{bm}^*$ is an indicator of airway narrowing in the resected specimen (78).

2.4 **Statistical analyses**

Data were expressed as the mean ± standard error of the mean. The airways in each subject group were sub-divided into cartilaginous and membranous categories. The frequency distribution of the $P_{bm}$ as a determinant of airway size in the study groups was compared using the Analysis of Variance (ANOVA) with airways nested within subject. Since the number and size of the measured airways differed among subjects and the airway wall measurements are dependent on the size of the airway, the area of airway compartment was converted to their square root to linearize the relationship to perimeter of basement membrane. The restricted maximal likelihood (REML) method of Feldman (79) was used to examine the relationship between the airway wall tissue compartments and the perimeter of basement membrane as a determinant of airway size. The slopes and intercepts of perimeter of basement membrane (Pbm) versus epithelial, sub-epithelial, smooth muscle and total airway wall area were determined for cartilaginous and membranous airways of the subject groups.

The mean area fraction ($A_a$) of positively stained cells by TUNEL assay in the subject groups was compared using single factor ANOVA. Appropriate adjustments for multiple comparisons for the analysis were made using Least Significant Difference (LSD) method. The Results were
confirmed by Student-Keul's and Tukey's methods. Both these methods yielded similar results. Due to the non-normal distribution of data for Fas and FasL, Kruskall-Wallis (K-W) test was used in place of standard ANOVA to perform these analyses (80). A non-parametric method for multiple comparisons called the Steel-Dwass-Critchlow-Fligner method (which is based on pairwise Wilcoxon tests on the group medians) was used to determine which groups differ from one another (81). The K-W test is the non-parametric alternative to ANOVA that does not require the assumption of normality (80).

Expression of Fas and FasL in the airways was quantified and evaluated in the airway epithelium and the inflammatory cells in the airway wall compartments i.e. the epithelium, sub-epithelium, smooth muscle, sub-mucosal layer (sub-epithelial and smooth muscle layer combined) and the entire airway wall. The cartilaginous and membranous airways were analyzed combined and then separately to examine the expression of these apoptotic molecules in the airway epithelium and the inflammatory cells in the airway wall compartments. The positive staining epithelial cells were not sub-divided into columnar cells and basal cells, neither was the intensity of staining graded.

For the membranous airways, the ratio for the measured luminal area and the idealized luminal area \((\text{Abm}/\text{Abm}^*)\) was compared between the study groups using ANOVA method with individual airways nested within subject. An unpaired Student's t-test was used to determine significant differences between the observers for inter-observer and intra-observer variability for point counting and morphometric measurements. Differences were considered to be significant when probability \((p)\) value was < 0.05.

The inter-observer variability was assessed by examining seven randomly selected sections from cartilaginous and membranous airways. The morphometric measurements of structural dimensions of the airway wall compartments and point counting for positively stained cells were performed by two observers on the same airway sections. The scoring was performed in a
blinded fashion by the two observers. The measurements were plotted on a graph (% difference vs. mean) and compared for the presence of statistical significance using paired Student's t-test. The intra-observer variability was determined for a single observer evaluating the same airway sections on two separate occasions. Ten random sections from cartilaginous and membranous airways were selected and duplicate morphometric measurements for structural dimensions of airway wall components and point counting for positively stained cells were performed on the same airway sections. The measurements were plotted on a graph and compared for statistical significance between the two scores using Student's t-test.
CHAPTER III

Sub-study

3.1 Effects of postmortem delay time intervals and temperature conditions on TUNEL staining

Introduction

Fragmentation of DNA during the process of apoptosis is a prerequisite for detection in the TUNEL procedure. This technique relies upon the incorporation of labeled nucleotides at the 3'-hydroxyl ends of double stranded DNA breaks by the TdT enzyme. In apoptosis as well as in necrosis, the nuclear DNA is fragmented leaving behind free 3'-hydroxyl ends. It is still controversial as to whether other mechanisms of cell changes that induce DNA fragmentation such as necrosis and autolysis also show positive staining with the TUNEL procedure (75). Postmortem temperature conditions, postmortem time intervals and the method of fixation of tissue can greatly vary and may alter the staining pattern of TUNEL. These factors could also affect the recognition of typical tissue morphology of apoptosis.

Objective

We conducted a sub-study to investigate the effects of postmortem delay at different temperatures on airway epithelial and smooth muscle TUNEL staining.

The objectives of the study were:

1. To confirm the reliability of detecting apoptosis by the TUNEL technique in tissues obtained at autopsy, as optimal preservation until fixation of these tissues is not always guaranteed.

2. To investigate the influence of autolytic changes that might interfere with TUNEL staining in autopsy material leading to false positive or false negative results.

Procedure
In this study, eight defined postmortem time intervals were combined with three temperature conditions to simulate different situations (morgue conditions) concerning postmortem delay for autopsy material.

Lung tissue specimen that was to be used was obtained from the operating room immediately after surgical resection. Prior to inflating the lung with optimum cutting temperature solution (OCT), the lung tissue was dissected and sectioned into airway tissue containing slices which were approximately 3mm in thickness. These slices were stored for different postmortem time intervals (0, 6, 12, 18, 24, 36, 48 and 72 hours) at three defined temperatures i.e. 37°C = normal body temperature, 4°C = refrigerator temperature and 19-21°C = room temperature. The slices of lung tissue were completely covered and stored in 300 mls of 1X phosphate buffered saline (1X PBS) pH 7.2.

After the defined time intervals of postmortem delay, from 0 up to 72 hours, the lung tissue was immersion fixed in 10% neutral buffered formalin at room temperature for 24-36 hours and embedded in paraffin. Four μm thick sections were cut and placed on polylysine coated microscopic slides, dried and fixed overnight at 37°C. TUNEL staining procedure was carried out using the protocol described previously. A section of lung tissue that was to be used as a positive control for TUNEL method was pretreated with DNAse prior to TdT reaction. Negative control consisted of omission of TdT from the incubation buffer. For qualitative and semi-quantitative analysis, tissue sections were examined using a light microscope at x250 magnification.

From each tissue section for a defined postmortem delay time intervals at a defined temperature, three fields of view were randomly selected. Semi-quantitative analysis for TUNEL staining was performed by counting one hundred airway epithelial cells in each field of view at x250 magnification. Since the size of a smooth muscle cell is relatively larger than the epithelial cell, fewer smooth muscle cells were counted at the same magnification.
For airway epithelial cells, counts were expressed as number of cells staining positive for TUNEL per one hundred airway epithelial cells counted. For smooth muscle cells, counts were expressed as number of cells staining positive per high power field (x250).

**Results**

Table 7 shows an overview of the TUNEL staining pattern of the airway tissue sections after defined intervals of postmortem delay and temperature conditions. It was observed that between 0-36 hours of postmortem delay and at all temperature conditions, there were 1-4 stained epithelial cells per section. Sections of lung tissue stored for 12-24 hours of postmortem delay at 37°C showed slightly more frequent positive nuclear TUNEL staining than at RT or at 4°C. A similar pattern of staining was seen in smooth muscle cells. However, there was a dramatic increase in the number and intensity of nuclear labeling signal in the tissue stored for 36-48 hours and 48-72 hours at all temperature conditions (graph 1 and 2) for epithelial as well as the smooth muscle cells. This dramatic increase in number and intensity of TUNEL positive cells was less pronounced in tissue stored at 4°C. It was observed that not only the nuclei, but also the cytoplasm showed diffuse staining of the cells and there was enhanced background staining (Figure 8, panels A, B,C and D).

**Conclusion**

These results clearly demonstrate that staining of airway tissue by TUNEL technique is valid with post mortem delay of up to 36 hours at all temperature conditions especially 4°C, which is the usual morgue refrigeration temperature.
CHAPTER IV

Results

4.1 Subject demographics

Subject demographics for subjects in the severe asthma, chronic asthma and the control groups are shown in table 2, 3 and 4. The subjects who had chronic asthma and the subjects in the control group were matched as closely as possible for age and sex. The subjects who had severe asthma were younger and ranged in age from 8 to 63 years (32 ± 12 years). The subjects who had chronic asthma ranged in age from 32 to 72 years (56 ± 13 years) whereas, the control subjects were between 18 and 80 years of age (49 ± 17 years). The subjects in the chronic asthma group smoked more (38 ± 27 packyears) than the control subjects (13 ± 15 packyears). The subject group who had chronic asthma showed reduced lung function whereas the control subjects had normal lung function. The pre-bronchodilator (salbutamol) FEV$_1$ % predicted and the FEV$_1$/FVC ratio in the subject group who had chronic asthma was 82 ± 21 and 0.73 ± 0.08 respectively. The FEV$_1$ % predicted and the FEV$_1$/FVC ratio in the control subjects was 97 ± 10 and 0.77 ± 0.042, respectively. The reduction in lung function in the chronic asthma group can be attributed in part to the effect of packyears of smoking. Two subjects who had chronic asthma (subject # 6 and 7) and one subject in the control group (subject # 9) had pre-bronchodilator FEV$_1$ % predicted higher than post bronchodilator FEV$_1$ % predicted. The lung function data for subjects who had chronic asthma and the control subjects are described in table 5 and table 6 respectively.

4.2 Pathologic examination of tissue sections

Airway tissue samples from seventeen subjects who had severe asthma, sixteen subjects who had chronic asthma and eighteen control subjects were analyzed for staining by TUNEL assay, Fas and FasL immunohistochemical staining. The microscopic appearance of the tissue sections
varied in each group. The airway epithelium from tissue sections obtained from subjects who had severe asthma showed extensive damage. The basement membrane was either completely or partially denuded of epithelium leaving behind the basal cell layer. The columnar epithelial cells showed patchy loss of cilia in some sections. Marked goblet cell hyperplasia was observed in these tissue sections. Some airways showed complete occlusion of the lumen by mucous plugging (figure 9). The basement membrane was prominent and apparently thickened in the tissue sections from these subjects. The mucosa and the submucosa of the airway wall showed marked infiltration with inflammatory cells, one of the hallmarks of asthma. The demonstration of goblet cell hyperplasia, epithelial damage (figure 10A), subepithelial fibrosis and the presence of an inflammatory infiltrate in the airway wall were only consistent with the characteristic features of asthma. In tissue sections obtained from subjects who had chronic asthma, there were less extensive areas of epithelial shedding, goblet cell hyperplasia and mucus plugging of the airway lumen (figure 10B). In contrast, airway sections from control subjects were unremarkable (figure 10C). There was very little or no epithelial damage with very few goblet cells or inflammatory cells. The basement membrane was of normal thickness (observation).

4.3 Airway wall dimensions

Table 8 summarizes the data on the type and number of airways examined. A total of 311 cartilaginous and membranous airways obtained from 51 subjects were included and measured to determine airway wall dimensions. The number of airways examined per subject ranged from one to twenty-two. There were one hundred and seven cartilaginous (34%) and two hundred and four (66%) membranous airways. The dimensions of the airway wall components and compartments that were measured are shown in figure 6. Table 9 shows the morphometric measurements (mean ± SEM) of the perimeters of airway wall compartments of the cartilaginous and membranous airways. To test whether a similar range of airway sizes was being compared in
the three subject groups, the frequency distribution of the perimeters of the basement membrane ($P_{bm}$) for the airways were compared using Analysis of Variance (ANOVA) with the airways nested in the subject. Graph 3 shows the frequency distribution curve for membranous airways in the study groups. The frequency distribution of membranous airway size was similar in all three subject groups ($p = 0.85$ ANOVA). The cartilaginous airways of the subject groups were statistically different ($p = 0.038$ ANOVA) as shown by the frequency distribution curve (graph 4). Basement membrane denudation (% mean ± SEM) was considerably higher in subjects who had severe asthma than in subjects who had chronic asthma or control subjects (table 10).

The slopes and intercepts of the relationships for the square root of the epithelial area, subepithelial area, smooth muscle area and the total airway wall area versus basement membrane perimeter for cartilaginous and membranous airways determined by the REML method for each subject group are summarized in table 11 and 12 (a). Graphs 5, 6, 7 and 8 show the relationship between the square root of the area of individual airway wall compartments and the basement membrane perimeter for cartilaginous airways. Similar relationships of the individual airway wall compartments to basement membrane perimeter for membranous airways are demonstrated in graphs 9, 10, 11 and 12. The regression lines (thick line = severe asthma, thin line = chronic asthma and dotted line = control subjects) were obtained using the values for slopes and intercepts.

The degree of airway narrowing expressed as the ratio of the measured luminal area ($A_{bm}$) to the idealized luminal area ($A_{bm}^*$) was calculated for membranous airways of the subject groups. The mean ± SEM for measured airway luminal area / idealized luminal area for the subjects who had severe asthma, subjects who had chronic asthma and the control subjects were $0.52 ± 0.038$, $0.62 ± 0.034$ and $0.66 ± 0.028$, respectively. The ratio was statistically significantly different between the subjects who had severe asthma compared to the subjects who had chronic asthma and the control groups ($F = 4.56$, $p = 0.011$ ANOVA airways nested in subject). A pairwise comparison
was done between the subject groups using an un-paired student's t-test. The airway narrowing was not significantly different between the subjects in severe asthma versus chronic asthma or chronic asthma versus control groups (p = 0.063 and 0.41 respectively). The severe asthma group showed significantly increased airway narrowing compared to the control group (p = 0.0063). Graph 13, 14 and 15 demonstrate the degree of airway narrowing in subjects who had severe asthma, subjects who had chronic asthma and the control subjects. The distance between the regression line of $P_{bm}$ versus $A_{bm*}$ and the data points is an indicator of airway narrowing.

4.4 Evaluation of airway epithelial cell apoptosis

Figure 11 (panels A and B) show staining of positive and negative control airway tissue sections for TUNEL assay. Positive control was generated by treating the tissue section with DNase whereas, negative control was generated by the omission of TdT enzyme. Figure 12 (panels A, B and C) demonstrates intranuclear TUNEL staining of fragmented DNA in the airway epithelial cells in the tissue sections obtained from study subjects. Positive TUNEL staining was identified predominantly in cells which showed nuclear pyknosis reflecting apoptotic cell death. To determine whether there was a statistically significant difference between the study groups for the presence of apoptosis in the airway epithelium, the area fraction occupied by TUNEL positive cells across the three subject groups i.e. severe asthma, chronic asthma and the controls was compared by single factor ANOVA. The LSD method was used for multiple comparisons. Consistency of the results was confirmed by Tukey's and Newman-Keul's methods.

Table 13 (a) shows the area fraction (Aa) of apoptotic airway epithelial cells that stained positive by the TUNEL method. The airway sections from the subject group who had severe asthma showed a significantly greater area fraction (mean ± SEM) of apoptotic airway epithelial cells as compared to chronic asthma and control subjects (F test = 9.17, p = 0.0004). As shown in graph 16 there was significantly increased apoptosis in the airway epithelium of the severe asthma
group versus the chronic asthma and control groups. However, the chronic asthma and the control groups did not differ significantly from each other. The area fraction (mean ± SEM) occupied by TUNEL positive epithelial cells in the three subjects from among the chronic asthma group whose lung tissues were obtained at the time of autopsy was 0.059 ± 0.034. The area fraction for this sub-group was not different than the area fraction calculated for the entire chronic asthma group (0.061 ± 0.019).

In addition, we counted absolute numbers of TUNEL positive cells in a sub-group of study subjects. Table 13 (b) shows the fraction of apoptotic cells that was determined by assessing TUNEL staining in airway sections of three subjects, one from each group. The subjects were selected randomly from the study groups. Since there is increased sloughing of airway epithelium in the severe asthma group (9.3%), the increased area fraction of TUNEL positive cells could have been simply a reflection of a decreased denominator i.e. the epithelial area rather than an increase in the apoptotic cells. Table 13 (b) shows that this is not the case. Despite increased epithelial shedding in the severe asthma group there are more TUNEL positive cells.

Based on the presence or absence of cartilage in the airway section, the airways in the subject groups were subdivided into cartilaginous and membranous airways. For cartilaginous airways, the fractional area (mean ± SEM) of apoptotic airway epithelial cells as demonstrated by TUNEL positivity was significantly increased in the severe asthma group as compared to the chronic asthma and the control groups (F test = 5.21, p = 0.01) (table 14). The subjects who had chronic asthma and the control subjects did not show any statistically significant difference for the presence of apoptosis in the cartilaginous airways. For membranous airways, the area fraction of apoptotic cells was not statistically different between the severe asthma and the control group. However, for these airways the severe asthma group showed significantly greater airway epithelial cell apoptosis than the chronic asthma group (F test = 6.32, p = 0.004) (table 14 and graph 17).
4.5 Evaluation of Fas expression in the airway

Figure 13 demonstrates the cell surface expression of Fas in tissue sections obtained from human liver, prostate gland and uterus. These tissue sections were considered the gold standards for the assay. Panels A, C and E show positive staining of tissue sections stained by mouse monoclonal Fas antibody while panels B, D and F are the negative controls stained with mouse isotype matched IgG for the purpose of comparison. Figure 15 (panels A, B and C) demonstrates cell surface expression of Fas in epithelial and inflammatory cells in the airway tissue obtained from subjects who had severe asthma, subjects who had chronic asthma and the control group respectively.

As summarized in table 15 and graph 18, Fas expression in the epithelium of cartilaginous and membranous airways combined was significantly increased in the severe asthma group compared to the chronic asthma and the control groups (F-test from the ANOVA = 3.27, p = 0.04 for all the airways combined). However, when the cartilaginous and the membranous airways were analyzed separately, there was no statistical difference between the study groups (F-test from ANOVA = 1.87, p = 0.17 for cartilaginous airways; F-test from ANOVA = 1.55 p = 0.22 for membranous airways) (table 16 and graph 19).

Table 17 and graph 20 summarizes the data for the area fraction of Fas expressing inflammatory cells in the airway wall compartments of cartilaginous and membranous airways. The area fractions of Fas positive inflammatory cells were statistically significantly different between the subject groups. The airway tissue which was obtained from the group of subjects who suffered from severe disease showed increased Fas expressing inflammatory cells in the sub-epithelium (p = 0.0001 K-W test), smooth muscle (p = 0.005 K-W test), sub-mucosal layer (p = 0.0002 K-W test) and the entire airway wall (p = 0.0003 K-W test) compared to the subjects who had chronic asthma and the control subjects. However, there was no evidence of a statistically significant
increase in the number of Fas expressing inflammatory cells in the epithelial layer (p = 0.2 K-W test) in any of the subject groups.

The cartilaginous airways were also evaluated separately for Fas expressing inflammatory cells in the airway wall compartments (table 18 and graph 21). The p values were determined for these analyses by using the K-W test. The number of Fas positive inflammatory cells, as estimated by the fractional area (Aa) of the airway wall compartments occupied by these cells, was not significantly different between the subject groups except for the sub-epithelial layer. The p values were > 0.05 for the presence of Fas expressing inflammatory cells in the epithelium, smooth muscle, submucosa and the entire airway wall. Fas expressing inflammatory cells in the sub-epithelial layer of the cartilaginous airway wall were significantly increased in the severe asthma group compared to the chronic asthma group (p = 0.008 K-W test). For the membranous airways (table 19 and graph 22), the severe asthma group demonstrated increased numbers of Fas expressing inflammatory cells in the sub-epithelium (p = 0.0008 K-W test), smooth muscle (p = 0.002 K-W test), sub-mucosal layer (p = 0.0002 K-W test) and the entire airway wall (p = 0.001 K-W test) compared to the chronic asthma and the control groups. Fas expressing inflammatory cells in the epithelial layer (p = 0.1 K-W test) were not significantly different between the severe asthma and the control group.

4.6 Evaluation of FasL expression in the airway

Figure 14 demonstrates the cell surface expression of FasL in tissue sections obtained from human testis, prostate gland and uterus. These tissue sections were considered as gold standards for the assay. Panels A, C and E show positive staining for FasL in these tissue sections while panels B, D and E are the corresponding negative controls for comparison. Figure 16 shows immunohistochemical staining of the airway tissue sections for FasL in the subject groups. Panels A, B and C are tissue sections obtained from subjects who had severe asthma, subjects
who had chronic asthma and the control subjects, respectively. FasL expression was demonstrated in the airway epithelium as well as the inflammatory cells in these tissue sections.

Table 20 and graph 23 summarizes the results of FasL expression in the epithelium of cartilaginous and membranous airways. The expression of FasL was greater in subjects who had severe asthma and chronic asthma than in control subjects (F-test from the ANOVA = 9.18, p = 0.0004). FasL expression was significantly increased in the epithelium of cartilaginous airways of subjects who had severe asthma compared to the subjects who had chronic asthma and the control group (F-test from ANOVA = 10.12, p = 0.0004) (table 21 and graph 24). As shown in table 21 and graph 24, the membranous airways of subjects who had severe or chronic asthma also demonstrated increased epithelial expression of FasL compared to the control subjects (F-test from ANOVA = 8.4, p = 0.0008).

There was no increase in FasL expressing inflammatory cells in the airway wall compartments of any subject groups. Table 22 and graph 25 shows that there was no evidence of a statistical difference between the study groups for the presence of FasL expressing inflammatory cells in the wall compartments of the combined cartilaginous and membranous airways. The cartilaginous airways, when evaluated separately, showed a borderline increase in FasL expressing inflammatory cells only in the smooth muscle layer of chronic asthma group compared to severe asthma and the control groups (p = 0.04 K-W test) (table 23 and graph 26). The number of FasL expressing inflammatory cells in the membranous airways of the study subject groups was not statistically different from one another for all the wall layers (table 24 and graph 27).

A ratio between the area fraction for FasL and Fas expression in the airway epithelium was determined. The mean ± SEM for subjects who had severe asthma, subjects who had chronic asthma and the control subjects were 0.84 ± 0.14, 0.87 ± 0.19 and 0.33 ± 0.1 respectively (p =
0.0071 severe asthma vs. controls, \( p = 0.024 \) chronic asthma vs. controls and \( p = 0.92 \) severe asthma vs. chronic asthma). Two-tail \( p \) values were determined by paired Student's t-test.

### 4.7 Inter-observer variability

The percent difference (mean ± SEM) for the inter-observer variability between the two observers for morphometric measurements of structural dimensions of the airway wall components i.e. \( P_i \), \( P_{bm} \), \( P_{mi} \) and \( P_{mo} \) was \( 12.7 ± 3.5 \), \( 3 ± 0.9 \), \( 1.7 ± 0.6 \) and \( 4 ± 1.2 \) respectively. Differences between the 2 observers were tested using paired Student's t-test. \( p \) values for morphometric measurements were as follows: \( P_i = 0.72 \), \( P_{bm} = 0.2 \), \( P_{su} = 0.11 \) and \( P_{sm} = 0.1 \). For point counting of TUNEL, Fas and FasL positive epithelial and inflammatory cells in the airway wall compartments, the percent difference between the two observers ranged from 2-15\%. Graph 28 and 29 show plots of % difference against mean (mm) for morphometric measurements and point counting of cells (mean positive points) respectively. Most of the points (with the exception of 2 outliers) fall close to the line of best fit demonstrating an agreement between the two observers. \( p \) value for the differences in point counting between the two observers was 0.42. There were no statistically significant differences in the variability between the two observers for either morphometric measurements or point counting of cells.

### 4.8 Intra-observer variability

The percent difference (mean ± SEM) between the two measurements for each of the perimeters was as follows: \( P_i 3.1 ± 2.7 \), \( P_{bm} 1.03 ± 0.9 \), \( P_{mi} 2.9 ± 1.3 \), \( P_{mo} 2.9 ± 1.5 \). The overall percent difference for point counting positive cells for TUNEL staining ranged from 2-12\%. Graph 30 and 31 show the plots for intra-observer variability for morphometric measurements and point counting. The \( p \) values were determined by paired Student's t-test for the difference in morphometric measurements and were as follows: \( P_i = 0.4 \), \( P_{bm} = 0.6 \), \( P_{su} = 0.1 \) and \( P_{sm} = 0.1 \).
For point counting, the p value for the difference between the two counts was 1. There were no statistically significant differences between two ratings for either morphometric measurements or point counting. The above results demonstrate a high degree of agreement between the two observers and repeatability of the quantification methods that were used in this study.
CHAPTER V

Discussion

The airway epithelium is fragile and sheds easily in subjects who have asthma. This has several structural and functional consequences. First, it permits unopposed influx of luminal contents containing environmental agents capable of causing tissue injury. Damaged epithelium may become a significant source of autocoid mediators, cytokines, chemokines and growth factors and could allow the spread of substances from the submucosa over the luminal surface. These potential tissue damaging agents could induce a local repair response involving restructuring and remodeling of the airway wall (82). A potential role of the airway epithelium is to act as a functional protective barrier to prevent the migration and survival of inflammatory cells into the mucosa. Due to its ability to express cell death inducing molecules like Fas and FasL, alteration or loss of this functional protective barrier may diminish or delay appropriate signals necessary to induce apoptosis and could permit enhanced migration and prolonged survival of the inflammatory cells in the mucosa. Physical detachment and disruption of the airway epithelium changes the epithelial permeability non-selectively or opens ‘gaps’ and thus facilitates the transepithelial migration of inflammatory cells. It has been demonstrated in a recent study that there is enhanced survival of inflammatory cells in the airway mucosa of subjects who have asthma compared to the airways of subjects who have chronic bronchitis (83). The mechanisms involved in the persistence of these inflammatory cells in the airways of subjects who have asthma, and whether this persistence is due to increased recruitment and / or delayed or diminished apoptosis is still unclear. Thus, it is important to address the question whether there is variable expression of death inducing molecules i.e. Fas and FasL in the airways of subjects who have severe asthma and chronic asthma versus controls. This study attempts to investigate for the first time, whether there is an association between the extent of airway epithelial cell apoptosis
and the expression of Fas and FasL in the airways of subjects who have asthma of varying clinical severity.

The implications of apoptosis in asthma are:

- Regulation and resolution of inflammation by induction of apoptosis in the inflammatory cells
- Regulation of tissue regeneration and repair process by limiting fibroblast activity and minimizing airway tissue remodeling

A persistent state of inflammation and mesenchymal activation could dysregulate or delay epithelial repair and consequently prolong the action of bioactive substances and the secretion of growth factors that sustain inflammation and remodeling respectively. Failure to clear unwanted inflammatory cells and mesenchymal cells may indicate malfunction of the apoptosis mechanism. Fas/FasL interaction may participate in tissue remodeling by removing irreparable epithelial cells and inflammatory cells. However, over-exuberance of these interactions could participate in tissue destruction and persistence of the inflammatory state by modulation of inflammatory mediators.

In this study, we used the TUNEL technique to detect apoptosis. This technique has been used and validated in several previous studies. It offers the advantage of revealing the DNA breaks which occur during apoptosis and quantifying apoptosis phenotypically within a heterogeneous population of cells. To complement the TUNEL technique and to investigate the possible mechanisms involved in apoptosis in the airways, we also evaluated the expression of Fas and its corresponding ligand, FasL, molecules that play an important role in the regulation of this phenomenon.

5.1 Subject demographics
In this study, the subjects in the severe asthma group were younger and belonged to the African-American inner-city population of Chicago. The lung tissue in this group of subjects was obtained at autopsy. Due to this limitation, demographic and lung function data on this group of subjects was not available. We could not determine the duration and severity of asthma, history of cigarette smoking and steroid use by these subjects. It is difficult to define severe asthma because the markers of severity not only reflect inherent pathophysiologic abnormalities but are also affected by such factors as the appropriateness of medical care and patient compliance. It could be speculated that genetic factors could influence the increase in severity of asthma in these subjects. Airway tissue samples from three subjects who had chronic asthma were also obtained at autopsy. These subjects had died of causes unrelated to asthma and therefore, clinical data on these subjects was not available. There were two subjects in the chronic asthma group and one subject in the control group who demonstrated a decrease in FEV₁ following aerosol inhalation of a bronchodilator. There were two subjects in the chronic asthma group who had a history of beta-agonist and corticosteroid (inhaled and oral) use for the purpose of asthma therapy. Two subjects in this group used a combination of beta-agonist and inhaled corticosteroids. Medication history on rest of the study subjects was not available.

5.2 Airway wall dimensions

The structural dimensions of the wall compartments of membranous and cartilaginous airways were examined in the subject groups. For the purpose of analyses, the airway wall compartments were defined as epithelial area (\(WA_e\)), sub-epithelial area (\(WA_{sub-epith}\)), smooth muscle area (\(WA_{sm}\)) and total airway wall area (\(WA_{tot}\)). A previous study was conducted at our laboratory by Kuwano et al. (84) used airway tissue from similar groups of subjects who had severe asthma and chronic asthma. This group of investigators compared the wall dimensions of membranous airways of the severe asthma group to those of subjects who had chronic obstructive pulmonary
disease (COPD) and of control subjects. They reported the sub-divisions of airway wall differently than we did (84). They defined the airway wall dimensions as (15) inner wall area (WA_i = A_e - A_{bm}) which is the area of tissue between the basement membrane and the outermost layer of smooth muscle, outer wall area (WA_o = A_o - A_e) which is the area from the outermost layer of the smooth muscle to the surrounding parenchyma and the smooth muscle area which is the area between the outermost and the innermost border of the smooth muscle (tissue between P_{mi} and P_{mo}). Their submucosal area represents the tissue between the basement membrane and the innermost border of smooth muscle. Thus the only comparable areas are the sub-epithelial area which conforms to their "sub-mucosal area" and the smooth muscle area. The slopes (S) and intercepts (I) that were determined by restricted maximal likelihood (REML) method (79) for the membranous airways for our study and the study conducted by Kuwano et al. (84) have been summarized in table 12 (a) and table 12 (b), respectively. The slope for the smooth muscle area appears to be greater in control subjects in our study. The slopes and intercepts for sub-epithelial and "submucosal area" in the control group appear to be similar although the slope in the present study group appears to be somewhat less steeper. The relative differences between the subject groups are similar in both studies, except the smooth muscle area in our chronic asthma is larger than in the control groups while this is reversed in the chronic asthma compared to the controls in the study by Kuwano et al. (84). However, it is noteworthy that the intercept for the smooth muscle area in the chronic asthma group in our study was higher than in the other two groups or any of the groups in the study by Kuwano et al. The comparability of these results between studies supports the observations of airway wall remodeling in severe asthma. As shown in graphs 9, 10, 11 and 12, the data points show close fit around the regression lines for all airway wall compartments.

5.3 Effects of postmortem autolysis on TUNEL staining
The aim of this study was to determine whether postmortem autolysis interferes with the TUNEL method to detect apoptosis. Recent studies (85) have suggested that the TUNEL assay may not always be specific for detecting apoptosis, especially in autopsy material. However, the content of free 3'-hydroxyl termini in apoptotic cells is usually higher than in necrotic cells. This may be the reason why the TUNEL assay preferentially stains apoptotic nuclei compared to nuclei in areas of necrosis, as seen in several other physiological and pathological conditions (86). This matter was of particular importance for our study, as all the airway tissue from subjects who had severe asthma and three subjects from the group of chronic asthma was obtained at autopsy. Temperature conditions and time intervals until autopsy can be highly variable. In our study, we systematically simulated postmortem delay up to 72 hours and combined this with 3 defined temperatures during this delay. Our results clearly demonstrate that TUNEL is a valid technique when postmortem time intervals are less than 36 hours at 4°C, the usual temperature of morgue refrigerators. The airway epithelial cells showed intense TUNEL staining of the nuclei accompanied by typical apoptotic morphology. Some autolytic changes were seen in lung tissue stored at room temperature for 24-72 hours. In these tissue sections, even the cytoplasm showed positive staining and there were focal areas of background staining. Many of these cells did not show morphological changes typical of apoptosis. Since the refrigeration temperature in the morgue is maintained at 4°C and the time interval between death and autopsy is invariably less than 36 hours, we believe that the effects of postmortem autolysis would be minimal or negligible in the tissues obtained at autopsy for this study.

5.4 Apoptosis in the airway epithelium

Our data demonstrate clearly that there is enhanced apoptosis of epithelial cells in the airways of subjects who have severe asthma. As demonstrated by the TUNEL assay, the extent of apoptosis was increased both in the cartilaginous as well as the membranous airways in this group of
subjects. These results correlate well with the characteristic finding of basement membrane
denudation and sloughing that is observed in mucosal biopsy specimens obtained from
symptomatic or asymptomatic subjects who have asthma (41). The airway epithelial cells that
were stained by TUNEL assay showed the characteristic histological findings of apoptosis. The
results of our sub-study to examine the effects of autolysis on TUNEL staining have clearly
demonstrated that there is no effect on the staining of airway tissues for up to 36 hours
postmortem at room temperature, 37°C or 4°C. Therefore, we do not believe that postmortem
autolysis is the explanation for the TUNEL positivity.

Increased airway epithelial apoptosis could result in the epithelial denudation and sloughing
which is a characteristic feature of asthmatic airways. However, our study does not elucidate the
mechanism of this process. In this study, we cannot demonstrate a cause and effect relationship
between enhanced airway epithelial apoptosis and sloughing. However, these findings are
consistent with our hypothesis.

In support of the increased susceptibility of the fragile and loose asthmatic epithelium to undergo
apoptosis, it has been demonstrated (87) that cell-cell aggregation is associated with the
preservation of pro-survival bcl-2 protein levels. Another study (88) has demonstrated the role of
α3β1 binding to matrix fibronectin in the upregulation of bcl-2 expression. Dissolution of integrin
mediated adhesion must occur when epithelial cells detach from the underlying basement
membrane. Signal transduction induced by cell-cell adhesion/aggregation preserves bcl-2
expression. Impaired adhesiveness of the epithelial cells due to gaps and disrupted tight (44)
junctions may promote apoptosis by disturbing the binding of integrin to matrix fibronectin.

These findings taken together suggest that the bcl-2 pathway may represent a mechanism by
which both cell-matrix and cell-cell interactions through integrins can prevent apoptosis. It
would be interesting to examine the differences in composition and expression of basement
membrane matrix proteins in the airways from the three subject groups.
5.5 Expression of FasL in the airway

In this study, we have investigated the expression of Fas and FasL in the airway tissue obtained from groups of subjects suffering from asthma of varying clinical severity. The results of this study are consistent with recent studies showing constitutive co-expression of Fas and FasL in human airway epithelium (71). Our results show that the expression of FasL was not decreased in the airway epithelial cells of subjects who had severe asthma or subjects who had chronic asthma compared to control subjects. On the contrary, in our study, the expression of FasL was increased in the epithelium of subjects who had severe asthma as well as subjects who had chronic asthma compared to the control subjects.

The increased expression of FasL in the present study was demonstrated in the epithelium of cartilaginous as well as the membranous airways. It is possible to speculate that increased expression of FasL is a compensatory mechanism to the degree of epithelial damage or a protective response to the presence of inflammatory cells in the mucosa. Despite the increased expression, the continued presence and accumulation of inflammatory cells in the airways of subjects who have asthma can be attributed in part to insufficient expression or functionally defective action of FasL to protect the asthmatic airways. There might be a very fine balance in the regulation of Fas/FasL interaction. The basal expression of FasL may be low enough to prevent autocrine or paracrine death of self or adjacent cells, at the same time sufficient to destroy or prevent the infiltration of Fas bearing inflammatory cells which might cause extensive damage of the surrounding airway wall tissue. Our data suggest that while FasL may be removed by shedding and damage to the epithelium, the loss of this death ligand is more than compensated for by the remaining adherent and intact epithelial cells. It is conceivable that the enhanced Fas/FasL interaction in the airway epithelium may initiate the necessary apoptotic signals and / or modulate the epithelial sloughing which is a characteristic feature of asthma.
A significantly increase in the area fraction of FasL expressing inflammatory cells was found in the cartilaginous airways of chronic asthma group. There was no difference between the subject groups in the expression of this molecule in the inflammatory cells in the wall of membranous airways. It is conceivable that the persistence inflammatory cells is due to the effects of over-expression of pro-inflammatory and pro-survival cytokines in the airways of subjects suffering from asthma. Among the possible inflammatory cell survival factors are GM-CSF and IL-5. In a recent study (83), a significant correlation was shown between the expression of GM-CSF and the number of non-apoptotic eosinophils and macrophages in the submucosa of bronchial biopsy specimens taken from subjects who had asthma. A crucial role is played by the balance between pro-survival genes such as Bcl-2 and tumor suppressor genes like p53. Bcl-2 is capable of inhibiting apoptosis by altering cell cycle rates, whereas p53 is involved in the induction of this phenomenon by blocking the cells in the G2 phase of the cell cycle. Vignola et al (83) have reported that the bronchial biopsy specimens obtained from subjects who had asthma showed increased infiltration by cells expressing Bcl-2 compared to those from subjects who had chronic bronchitis and control subjects. The expression of this pro-survival molecule has also been shown to be increased in sputum eosinophils in subjects who had severe asthma compared with those who had mild asthma (89) suggesting their prolonged survival and decreased apoptosis in the asthma airways.

Another recent study showed that besides being an apoptotic molecule, FasL also has pro-inflammatory effects since it can stimulate recruitment of polymorphonuclear neutrophils (PMN's) by inducing IL-8 secretion in airway epithelial cells (73). IL-8 is a potent chemoattractant for neutrophils and lymphocytes. PMN's express both Fas and FasL (90) and can serve as a source of soluble Fas receptor and FasL, which may function in a paracrine pathway to mediate epithelial apoptosis. It is possible that PMN's that undergo spontaneous apoptosis through an autocrine pathway, could also induce apoptotic cell death in co-existent
epithelial cells and that this effect may be exerted by PMN's release of soluble FasL. PMN's are the major source of DNA in airway secretions and may contribute to the tenacious mucus plugs that sometimes completely occlude the lumen of asthmatic airways. It is possible that the abundantly expressed FasL in the airway epithelial cells of subjects who have severe asthma may be either functionally defective or have inefficient action when it binds to its corresponding Fas receptor. A report by Kayagaki et al (91) has demonstrated the existence of a loss of function mutation in the murine FasL gene that affects its biological activity. The presence of this polymorphism results in the expression of a dysfunctional protein which affects the receptor ligand binding due to loss of the correct conformation of the receptor binding site. Such polymorphisms could predispose susceptible individuals to persistent inflammation due to the absence or altered expression of FasL. When mice, that expressed mutant FasL were sensitized and challenged with ovalbumin, they demonstrated markedly reduced FasL mRNA and protein expression on the airway epithelium as compared to non-challenged mice (92). This suggests either increased cleavage of FasL from the epithelial cell membrane which may be due to increased matrix metalloproteinase activity in the airways (93) or alterations in the expression and presentation at the cell surface due to distortion of its molecular structure. The alterations in the balance of FasL availability relative to the pace of inflammatory cell accumulation may contribute to the establishment of an inflammatory state. It can be speculated that the constitutive, functional expression of FasL represents the basal or homeostatic state of airway epithelium. It would be reasonable to suggest that subjects who have severe asthma could have a polymorphic FasL gene which might result in the expression of a weaker genetic variant of FasL. As a result, this may breakdown the functional protective barrier of FasL and may predispose the airway epithelium to chronic inflammation. It is interesting to suggest that the process of cell shedding may partly result from persistent expression of FasL and upregulation of Fas expression on the airway epithelial cells and thus result in apoptosis. The regions of denuded
epithelium which are now deficient in the FasL barrier may permit the entry of inflammatory cells in the airway wall.

5.6 Expression of Fas in the airways

Our data demonstrate a borderline increase in the expression of Fas in the airway epithelium of asthma subjects. The increase in expression was not different between the subject groups when the cartilaginous and the membranous airways were analyzed separately. The inflammatory cells in the airway wall showed increased expression of Fas in subjects who had severe asthma compared to those who had chronic asthma and the control subjects. This expression was more pronounced in all the compartments of the membranous airways of subjects who had severe asthma.

There are several studies which suggest that there might be changes in the function of Fas receptor in inflammatory cells in asthma. One such study (94) suggests that CD4⁺ T lymphocytes from asthmatic airways demonstrate resistance to Fas-dependent apoptosis due to a subtle alteration in lymphocyte’s ability to activate and initiate Fas-dependent signaling. Another study (95) explains the persistence of inflammatory cellular infiltrates by demonstrating a locally defective surface expression of Fas on pulmonary T lymphocytes in broncho-alveolar lavage fluid collected from subjects who had mild asthma. Additional studies show delayed apoptosis in peripheral blood eosinophils of subjects who had inhalant allergy and atopic dermatitis (96). The data of these previous studies explain the probable reasons for persistence of airway inflammation. It is possible that there might be other regulatory factors which may influence the persistence of these inflammatory cells in the airway wall.

A deletion mutation in the Fas gene has been described (97) to be associated with lymphoproliferative syndrome in humans. This deletion in the Fas gene results in either an unstable protein or the retention of the protein inside the cell. Several other mutations in the
intra-cellular and the extra-cellular domain of Fas have been described (98) that may result in failure of extracellular expression of mutant protein or impaired binding to FasL. Such alterations in the responsiveness to Fas ligation could create a population of death-resisting inflammatory cells, even if Fas or FasL expression is normal or, as seen in our study, increased in the airway epithelium.

The propensity to apoptosis via the Fas/FasL pathway might be more dependent on the ratio of expression of these apoptotic proteins rather than the absolute expression of either of them. Therefore, we examined the ratio between epithelial expression of FasL and Fas. We found a significant association between the ratio of FasL to Fas and severe asthma. This ratio might be important in maintaining a balance between Fas/FasL interaction in the asthmatic airways.

The death domain has a tendency to self-aggregate and during this process, both membrane bound Fas and FasL undergo metalloproteinase-mediated proteolytic cleavage resulting in generation of soluble Fas (sFas) and soluble FasL (sFasL) (99). There is evidence that these apoptotic molecules work locally through close cell-cell interactions and the purpose of shedding soluble forms is to attenuate the apoptotic process. However, it has been shown that membrane bound forms of these molecules are equally or more active than the soluble forms (99). The role of sFas has been studied by Kato et al (100). Soluble Fas is also generated by the alternative splicing of mRNA and has been identified in the supernatants of activated human lymphocytes and several tumor cell lines (101). The report of Kato et al suggests that serum sFas levels might be upregulated during the chronic inflammation phase of asthma irrespective of the atopic status of the subject.

5.7 Possible effects of asthma therapy

Corticosteroids have a prominent place in the treatment of asthma as they reduce airway inflammation by several different mechanisms, one of which is the inhibition or suppression of
synthesis of pro-inflammatory cytokines from various cell types particularly T lymphocytes (102). It is important to note that these drugs directly promote in vivo apoptosis of eosinophils and of activated T lymphocytes (103). A recent study that was conducted using bronchial biopsy specimens from subjects who had asthma suggested that steroids exerted beneficial effects in these subjects by facilitating resolution of eosinophilic inflammation and by promoting airway epithelial cell survival and proliferation, possibly through increased expression of bcl-2 and proliferating cell nuclear antigen (PCNA) (104). These results have been substantiated by a study on A549 cells demonstrating the inhibitory effects of dexamethasone on IFN-γ and Fas-induced apoptosis (105). These studies suggest a potential role of corticosteroids to protect against epithelial lung injury. The effects of corticosteroids on airway epithelial cell apoptosis have also been studied by Dorscheid et al (106). This group has challenged the findings of previous groups and has suggested the possibility that the enhanced epithelial apoptosis in the airways of subjects who have severe asthma may be related to corticosteroid use for therapeutic purposes in these subjects (106). Due to unavailability of demographic data on subjects in the severe asthma group, we cannot link our findings of enhanced airway epithelial apoptosis to steroid use. However, it is probable that many of the subjects in this group were receiving corticosteroids since inhaled or oral corticosteroids are widely prescribed to patients who have asthma. On the other hand, another group of investigators have shown the beneficial effects of corticosteroids on the structural integrity of the epithelium (107). This group assessed bronchial biopsy specimens taken from patients before and after budesonide treatment. The pretreatment biopsy specimens showed severely damaged epithelium in subjects who had asthma compared to normal control subjects. The histologic appearance of the bronchial epithelium improved in this group of subjects who had asthma after three months of budesonide treatment. However, this possible beneficial effect of corticosteroids to restore airway epithelial integrity was not examined at the molecular level in this study. The expression of FasL has been seen to be markedly increased in
the epithelium of steroid treated subjects who have asthma (104), indicating that it may exhibit the ability to trigger the apoptotic death of Fas bearing self or adjacent cells. Theophylline has been shown to downregulate the expression of bcl-2 protein in eosinophils and may induce their apoptosis (108).

In our study, due to limited availability of clinical data on subjects who had either severe asthma or chronic asthma, the influence of different asthma therapies could not be tested. It is likely that some of these subjects were treated with oral or inhaled corticosteroids, beta-adrenergic agonists and theophylline for varying periods of time. It is possible that these therapies alone or in combination, may alter the expression or function of both Fas and FasL.

5.8 Role of Fas/FasL interaction in autoimmune and other diseases

The role of Fas/FasL interaction has been extensively studied in autoimmune and other disease states (109). These interactions negatively regulate the immune system but can also contribute to immunopathology, as occurs in Fas-mediated damage of target tissues in hepatitis and other organ specific auto-immune diseases. Altered Fas or FasL function may contribute to autoimmune, malignant and infectious diseases. Overall, it appears that the knowledge of these interactions can prove beneficial for designing novel therapeutic strategies.

5.9 Limitations of the study

It is important to consider the limitations of this study. Almost nothing is known about different patterns of Fas and FasL expression in people from different racial groups. Our study group of severe asthma consisted of an urban population of African American subjects who had died suddenly and had a history suggestive of acute asthma attacks. It is possible that African Americans are more genetically susceptible to develop severe asthma and that this genetic susceptibility manifests itself through greater expression of Fas and FasL and greater epithelial
cell apoptosis. Although this is unlikely there could also be differences in environmental factors and therapeutic regimes based on ethnicity. Such factors, which could contribute to disease severity include access to appropriate treatment and compliance to medications. One of the suspected factors for the increased asthma mortality is poor access to adequate treatment.

The asthma subjects in the study group may have been treated with several therapies including inhaled and oral corticosteroids and inhaled beta adrenergic agonists for varying periods of time and with varying intensity of treatment. It is possible that these therapies, alone or in combination may alter the expression and/or function of both Fas and FasL. The effects of different asthma treatments could not be studied in the present study due to unavailability of complete demographic data.
CHAPTER VI

Summary and Conclusions

6.1 Summary

We demonstrate that:

- The degree of airway narrowing was more in the subjects in severe asthma group.
- There was enhanced epithelial apoptosis in both the membranous and the cartilaginous airways of severe asthma subjects.
- Fas and FasL were co-expressed in the airway epithelium of all the subject groups.
- There was an increased epithelial expression of Fas in the severe asthma group.
- Epithelial expression of FasL was increased in the severe asthma and the chronic asthma groups.
- There was an increase in the number of Fas expressing inflammatory cells in the airway wall compartments of severe asthma subjects.
- The increase in Fas expressing inflammatory cells was more pronounced in the membranous airways.
- FasL expressing inflammatory cells were not significantly increased in the airways of any of the subject groups except in the smooth muscle compartment of cartilaginous airways of chronic asthma subjects.
6.2 Conclusions

Continuous epithelial cell apoptosis, caused by persistently upregulated expression of Fas and FasL may result in excessive death of the epithelial cells which may overwhelm the mechanisms of clearance by macrophages, necessary to maintain homeostasis. These processes of enhanced airway epithelial apoptosis accompanied by steadily upregulated Fas/FasL interactions may be related to asthma severity. The causal relationship of these processes to asthma severity still needs to be determined.

A prolonged state of inflammation may interfere with the process of re-epithelialization and thus result in the overgrowth of mesenchymal cells. This phenomenon could provide one possible explanation for the increased susceptibility of the airways of subjects who have asthma, to environmental insults. Limiting the exposure of immune effector cells to environmental proteinase attacks may directly or indirectly diminish allergic sensitization and point toward new approaches for asthma therapy.

The intricacies in the control of inflammatory cell longevity and the exquisite balance between pro-survival and pro-apoptotic signaling are fundamental and critical mechanisms involved in the pathogenesis of asthma. Understanding the mechanisms of how apoptosis in these inflammatory cells is regulated may help in the development of new therapeutic strategies. Induction of apoptosis in eosinophils is beneficial in the suppression of allergic inflammation and is one of the modes of action of corticosteroids in the treatment of asthma and atopic diseases is to induce apoptosis in eosinophils and, presumably in lymphocytes.

Identification of candidate function altering polymorphisms in human Fas and FasL genes may provide valuable understanding in predicting the genetic predisposition associated with susceptibility and clinical course of asthma. Presence of these polymorphisms could lead to abnormal functional and cellular responses to triggering factors. It would be important to trace changes in epithelial cell gene expression in the earlier stages of asthma.
An improvement in future knowledge to understand the multiple routes and the intrinsic mechanisms leading to epithelial injury may provide a better insight into the development of therapeutic strategies to specifically block the deleterious or excessive immune mediated effects (cytoprotective effect) while facilitating the elimination of pro-inflammatory cells (cytotoxic effects).

It would be an ideal design to study these apoptotic molecules at the mRNA level and also to investigate the role of function altering polymorphisms. The balance in the regulatory mechanisms between other pro-survival and pro-apoptotic molecules in the inflammatory cells and epithelial cells needs further research.
APPENDIX I

Table 1

Summary of differences between necrosis and apoptosis

**Necrosis versus Apoptosis**

<table>
<thead>
<tr>
<th>Features</th>
<th>Necrosis</th>
<th>Apoptosis</th>
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<tr>
<td><strong>tissue distribution</strong></td>
<td>groups of cells</td>
<td>isolated cells</td>
</tr>
<tr>
<td><strong>tissue reaction</strong></td>
<td>lysis and release of cellular contents resulting in inflammation</td>
<td>phagocytosis of membrane bound vesicles by macrophages or neighboring phagocytic cells, little or no inflammation</td>
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<tr>
<td><strong>Morphology</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>cell</strong></td>
<td>swelling</td>
<td>cell shrinkage, loss of membrane contact with neighboring cells</td>
</tr>
<tr>
<td><strong>plasma membrane</strong></td>
<td>loss of integrity, enhanced permeability</td>
<td>blebbing, formation of apoptotic bodies</td>
</tr>
<tr>
<td><strong>organelles</strong></td>
<td>damaged</td>
<td>intact</td>
</tr>
<tr>
<td><strong>nucleus</strong></td>
<td>disintegrated</td>
<td>condensed then fragmented</td>
</tr>
<tr>
<td><strong>lysosomes</strong></td>
<td>ruptured</td>
<td>intact</td>
</tr>
<tr>
<td><strong>mitochondria</strong></td>
<td>defective, ATP depleted, swollen, ruptured</td>
<td>swollen, permeability changes, may rupture, cytochrome c release</td>
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<tr>
<td><strong>Biochemistry</strong></td>
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<td></td>
</tr>
<tr>
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<td><strong>protein</strong></td>
<td>non-specific degradation</td>
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<td><strong>anti-death molecules</strong></td>
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<td><strong>Adenosine triphosphate requirement</strong></td>
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Table 2.

Demographic characteristics of subjects who had severe asthma

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Table 3.

Demographic characteristics of subjects who had chronic asthma

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Chronic asthma
Table 4

Demographic characteristics of control subjects

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<th>Subject</th>
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<td>n</td>
<td>n</td>
<td>n</td>
<td>n</td>
</tr>
<tr>
<td>11</td>
<td>41</td>
<td>167</td>
<td>59</td>
<td>M</td>
<td>20</td>
<td>n</td>
<td>n</td>
<td>n</td>
<td>n</td>
</tr>
<tr>
<td>12</td>
<td>49</td>
<td>154</td>
<td>55</td>
<td>F</td>
<td>33</td>
<td>n</td>
<td>n</td>
<td>n</td>
<td>n</td>
</tr>
<tr>
<td>13</td>
<td>32</td>
<td>172</td>
<td>69</td>
<td>M</td>
<td>14</td>
<td>n</td>
<td>n</td>
<td>n</td>
<td>n</td>
</tr>
<tr>
<td>14</td>
<td>80</td>
<td>173</td>
<td>54</td>
<td>M</td>
<td>47</td>
<td>n</td>
<td>n</td>
<td>n</td>
<td>n</td>
</tr>
<tr>
<td>15</td>
<td>61</td>
<td>176</td>
<td>69</td>
<td>F</td>
<td>0.3</td>
<td>n</td>
<td>n</td>
<td>n</td>
<td>n</td>
</tr>
<tr>
<td>16</td>
<td>68</td>
<td>171</td>
<td>73</td>
<td>M</td>
<td>0</td>
<td>n</td>
<td>n</td>
<td>n</td>
<td>n</td>
</tr>
<tr>
<td>17</td>
<td>37</td>
<td>169</td>
<td>59</td>
<td>M</td>
<td>0</td>
<td>n</td>
<td>n</td>
<td>n</td>
<td>n</td>
</tr>
<tr>
<td>18</td>
<td>18</td>
<td>185</td>
<td>70</td>
<td>M</td>
<td>0</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>mean±SD</td>
<td>49±17</td>
<td>171±7</td>
<td>65±12</td>
<td>11:7</td>
<td>13±15</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Age at pulmonary resection

** OCS = oral corticosteroids

# ICS = inhaled corticosteroids
+ tissue obtained at autopsy therefore, limited clinical data available

NA = history not available

y = use of medication

n = no use of medication

M = male   F = female
Table 5
Lung function data on subjects who had chronic asthma

<table>
<thead>
<tr>
<th>Subjects</th>
<th>Pre-bronchodilator</th>
<th>Post-bronchodilator</th>
<th>Bronchodilator response %</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>FEV$_1$(pp)</td>
<td>FEV$_1$/FVC</td>
<td>FEV$_1$(pp)</td>
</tr>
<tr>
<td>1</td>
<td>83</td>
<td>0.77</td>
<td>88</td>
</tr>
<tr>
<td>2</td>
<td>44</td>
<td>0.68</td>
<td>60</td>
</tr>
<tr>
<td>3</td>
<td>79</td>
<td>0.79</td>
<td>84</td>
</tr>
<tr>
<td>4</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>5*</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>6*</td>
<td>91*</td>
<td>0.71</td>
<td>88*</td>
</tr>
<tr>
<td>7*</td>
<td>107*</td>
<td>0.74</td>
<td>106*</td>
</tr>
<tr>
<td>8</td>
<td>NA</td>
<td>NA</td>
<td>57</td>
</tr>
<tr>
<td>9</td>
<td>52</td>
<td>0.56</td>
<td>61</td>
</tr>
<tr>
<td>10</td>
<td>67</td>
<td>0.59</td>
<td>77</td>
</tr>
<tr>
<td>11</td>
<td>81</td>
<td>0.67</td>
<td>84</td>
</tr>
<tr>
<td>12</td>
<td>81</td>
<td>0.68</td>
<td>91</td>
</tr>
<tr>
<td>13</td>
<td>113</td>
<td>0.82</td>
<td>114</td>
</tr>
<tr>
<td>14*</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>15*</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>16</td>
<td>101</td>
<td>0.73</td>
<td>102</td>
</tr>
<tr>
<td><strong>mean ± SD</strong></td>
<td><strong>82 ± 21</strong></td>
<td><strong>0.73 ± 0.08</strong></td>
<td><strong>92 ± 18</strong></td>
</tr>
</tbody>
</table>

NA = history not available

FEV$_1$ = forced expiratory volume in one second

FVC = forced vital capacity

Bronchodilator response = post-bronchodilator FEV$_1$ – pre-bronchodilator FEV$_1$ / pre-bronchodilator FEV$_1$
● tissue obtained at autopsy therefore, limited clinical data available

● prebronchodilator FEV₁ (pp) higher than postbronchodilator FEV₁ (pp)
Table 6.

Lung function data on control subjects

<table>
<thead>
<tr>
<th>Subjects</th>
<th>Pre-bronchodilator</th>
<th>Post-bronchodilator</th>
<th>Bronchodilator response %</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>FEV₁(pp)</td>
<td>FEV₁/FVC</td>
<td>FEV₁(pp)</td>
</tr>
<tr>
<td>1</td>
<td>77</td>
<td>0.79</td>
<td>79</td>
</tr>
<tr>
<td>2</td>
<td>99</td>
<td>0.79</td>
<td>100</td>
</tr>
<tr>
<td>3</td>
<td>94</td>
<td>0.77</td>
<td>97</td>
</tr>
<tr>
<td>4</td>
<td>113</td>
<td>0.84</td>
<td>117</td>
</tr>
<tr>
<td>5</td>
<td>98</td>
<td>0.79</td>
<td>101</td>
</tr>
<tr>
<td>6</td>
<td>103</td>
<td>0.72</td>
<td>NA</td>
</tr>
<tr>
<td>7</td>
<td>100</td>
<td>0.83</td>
<td>103</td>
</tr>
<tr>
<td>8</td>
<td>112</td>
<td>0.82</td>
<td>NA</td>
</tr>
<tr>
<td>9</td>
<td>92</td>
<td>0.77</td>
<td>90</td>
</tr>
<tr>
<td>10</td>
<td>103</td>
<td>0.72</td>
<td>109</td>
</tr>
<tr>
<td>11</td>
<td>98</td>
<td>0.71</td>
<td>99</td>
</tr>
<tr>
<td>12</td>
<td>108</td>
<td>0.75</td>
<td>149</td>
</tr>
<tr>
<td>13</td>
<td>83</td>
<td>0.69</td>
<td>99</td>
</tr>
<tr>
<td>14</td>
<td>103</td>
<td>0.81</td>
<td>105</td>
</tr>
<tr>
<td>15</td>
<td>92</td>
<td>0.77</td>
<td>95</td>
</tr>
<tr>
<td>16</td>
<td>88</td>
<td>0.77</td>
<td>91</td>
</tr>
<tr>
<td>17</td>
<td>103</td>
<td>0.79</td>
<td>107</td>
</tr>
<tr>
<td>18</td>
<td>78</td>
<td>0.75</td>
<td>87</td>
</tr>
<tr>
<td>mean ± SD</td>
<td>97 ± 10</td>
<td>0.77 ± 0.042</td>
<td>102 ± 16</td>
</tr>
</tbody>
</table>

FEV₁ = forced expiratory volume in one second

FVC = forced vital capacity

Bronchodilator response = post-bronchodilator FEV₁ - pre-bronchodilator FEV₁/

pre-bronchodilator FEV₁

▲ prebronchodilator FEV₁ (pp) higher than postbronchodilator FEV₁ (pp)
Table 7.

TUNEL staining of airway epithelium and smooth muscle cells at different postmortem time intervals and temperature conditions

<table>
<thead>
<tr>
<th>PMD (hrs)</th>
<th>Airway epithelium</th>
<th>Smooth muscle</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>RT</td>
<td>37°C</td>
</tr>
<tr>
<td>0</td>
<td>1.67 ± 0.88</td>
<td>-</td>
</tr>
<tr>
<td>6</td>
<td>0.33 ± 0.33</td>
<td>1 ± 0.58</td>
</tr>
<tr>
<td>12</td>
<td>1.67 ± 0.33</td>
<td>1 ± 0.58</td>
</tr>
<tr>
<td>18</td>
<td>1.3 ± 0.88</td>
<td>0.33 ± 0.33</td>
</tr>
<tr>
<td>24</td>
<td>1 ± 0.58</td>
<td>3 ± 0.58</td>
</tr>
<tr>
<td>36</td>
<td>0.33 ± 0.33</td>
<td>1 ± 0.58</td>
</tr>
<tr>
<td>48</td>
<td>94.33 ± 3.48</td>
<td>91.67 ± 3.33</td>
</tr>
<tr>
<td>72</td>
<td>100 ± 0</td>
<td>100 ± 0</td>
</tr>
</tbody>
</table>
Table 8

Type and number of airways examined in the tissue sections

<table>
<thead>
<tr>
<th>Subject groups</th>
<th>Total # of AW's</th>
<th>Range of AW's/subject</th>
<th>C / M</th>
</tr>
</thead>
<tbody>
<tr>
<td>Severe asthma</td>
<td>123</td>
<td>1 – 22</td>
<td>54C / 69M</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>44 / 56</td>
</tr>
<tr>
<td>Chronic asthma</td>
<td>62</td>
<td>1 – 12</td>
<td>18C / 44M</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>29 / 71</td>
</tr>
<tr>
<td>Control subjects</td>
<td>126</td>
<td>1 – 21</td>
<td>35C / 91M</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>28 / 72</td>
</tr>
<tr>
<td>All subjects</td>
<td>311</td>
<td>1 – 22</td>
<td>107C / 204M</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>34 / 66</td>
</tr>
</tbody>
</table>

C = cartilaginous airways, M = membranous airways
AW = airway
Table 9

Morphometric measurements of perimeter of the airway wall compartments (mean ± SEM) in the subject groups

**Perimeter of airway wall compartments (mean ± SEM) (mm)**

<table>
<thead>
<tr>
<th>Perimeter (mm)</th>
<th>Severe asthma</th>
<th>Chronic asthma</th>
<th>Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>C</td>
<td>M</td>
<td>C</td>
</tr>
<tr>
<td>P₁</td>
<td>8.54 ± 0.99</td>
<td>3.25 ± 0.38</td>
<td>5.18 ± 0.41</td>
</tr>
<tr>
<td>P₉₈</td>
<td>8.88 ± 1</td>
<td>3.34 ± 0.36</td>
<td>5.43 ± 0.54</td>
</tr>
<tr>
<td>P₉₁</td>
<td>8.21 ± 1.062</td>
<td>3.21 ± 0.38</td>
<td>4.93 ± 0.33</td>
</tr>
<tr>
<td>P₉₂</td>
<td>8.82 ± 1.13</td>
<td>3.53 ± 0.39</td>
<td>5.28 ± 0.38</td>
</tr>
</tbody>
</table>

C = cartilaginous airways, M = membranous airways

Definition of abbreviations: see materials and methods
Table 10

Percent denudation of basement membrane of airway sections collected from study subjects

<table>
<thead>
<tr>
<th>Subject groups</th>
<th>% denudation (mean ± SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Severe asthma</td>
<td>46.12 ± 10.22</td>
</tr>
<tr>
<td>Chronic asthma</td>
<td>1.36 ± 1.25</td>
</tr>
<tr>
<td>Controls</td>
<td>0.45 ± 0.26</td>
</tr>
</tbody>
</table>
Table 11

Slopes (S) and intercepts (I) of regression analyses determined by the restricted maximum likelihood (REML) method for cartilaginous airways

<table>
<thead>
<tr>
<th></th>
<th>Severe asthma n = 17</th>
<th>Chronic asthma n = 16</th>
<th>Controls n = 18</th>
</tr>
</thead>
<tbody>
<tr>
<td>( p_{bm} )</td>
<td>8.88 ± 1</td>
<td>5.43 ± 0.54</td>
<td>7.51 ± 0.7</td>
</tr>
<tr>
<td><strong>Epithelial area</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>0.301</td>
<td>0.0775</td>
<td>0.127</td>
</tr>
<tr>
<td>S</td>
<td>0.0158</td>
<td>0.0706</td>
<td>0.0511</td>
</tr>
<tr>
<td>( p ) value</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Severe asthma vs. controls = 0.0052</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Sub-epithelial area</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>0.0170</td>
<td>-0.0223</td>
<td>0.117</td>
</tr>
<tr>
<td>S</td>
<td>0.107</td>
<td>0.0994</td>
<td>0.0576</td>
</tr>
<tr>
<td>( p ) value</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Severe asthma vs. controls &lt; 0.00001</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chronic asthma vs. controls = 0.041</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Smooth muscle area</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>0.121</td>
<td>-0.0715</td>
<td>0.0119</td>
</tr>
<tr>
<td>S</td>
<td>0.0938</td>
<td>0.118</td>
<td>0.0778</td>
</tr>
<tr>
<td>( p ) value</td>
<td></td>
<td></td>
<td>NS</td>
</tr>
<tr>
<td><strong>Total airway wall area</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>0.236</td>
<td>-0.00971</td>
<td>0.141</td>
</tr>
<tr>
<td>S</td>
<td>0.138</td>
<td>0.168</td>
<td>0.110</td>
</tr>
<tr>
<td>( p ) value</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Severe asthma vs. controls = 0.016</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chronic asthma vs. controls 0.032</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 12 (a)
Slopes (S) and intercepts (I) of regression analyses determined by the restricted maximum likelihood (REML) method for membranous airways

### Slopes (S) and intercepts (I) of regression analysis

<table>
<thead>
<tr>
<th></th>
<th>Severe asthma</th>
<th>Chronic asthma</th>
<th>Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n = 17</td>
<td>n = 16</td>
<td>n = 18</td>
</tr>
<tr>
<td>$P_{bm}$</td>
<td>3.34 ± 0.36</td>
<td>3.29 ± 0.26</td>
<td>2.88 ± 0.23</td>
</tr>
<tr>
<td><strong>Epithelial area</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>0.110</td>
<td>0.0824</td>
<td>0.0827</td>
</tr>
<tr>
<td>S</td>
<td>0.0513</td>
<td>0.0616</td>
<td>0.0495</td>
</tr>
<tr>
<td><strong>Epithelial area</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>0.0187</td>
<td>0.0700</td>
<td>0.0472</td>
</tr>
<tr>
<td>S</td>
<td>0.0981</td>
<td>0.0511</td>
<td>0.0469</td>
</tr>
<tr>
<td><strong>Sub-epithelial area</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>Severe asthma vs. controls &lt; 0.000001</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Severe asthma vs. chronic asthma &lt; 0.00001</td>
<td></td>
<td></td>
</tr>
<tr>
<td>S</td>
<td>0.116</td>
<td>0.0531</td>
<td>0.0569</td>
</tr>
<tr>
<td><strong>Smooth muscle area</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>0.00993</td>
<td>0.120</td>
<td>0.0518</td>
</tr>
<tr>
<td>S</td>
<td>0.116</td>
<td>0.0531</td>
<td>0.0569</td>
</tr>
<tr>
<td><strong>Smooth muscle area</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>0.0804</td>
<td>0.170</td>
<td>0.105</td>
</tr>
<tr>
<td>S</td>
<td>0.160</td>
<td>0.0947</td>
<td>0.0903</td>
</tr>
<tr>
<td><strong>Total airway wall area</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>Severe asthma vs. controls = 0.000077</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Severe asthma vs. chronic asthma = 0.00011</td>
<td></td>
<td></td>
</tr>
<tr>
<td>S</td>
<td>0.0903</td>
<td>0.0947</td>
<td>0.0903</td>
</tr>
<tr>
<td><strong>Total airway wall area</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>0.0804</td>
<td>0.170</td>
<td>0.105</td>
</tr>
<tr>
<td>S</td>
<td>0.160</td>
<td>0.0947</td>
<td>0.0903</td>
</tr>
<tr>
<td><strong>Total airway wall area</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 12 (b)

Slopes (S) and intercepts (I) of regression analyses determined by the restricted maximum likelihood (REML) method for membranous airways (84)

<table>
<thead>
<tr>
<th></th>
<th>Fatal asthma n = 8</th>
<th>Non-fatal asthma n = 7</th>
<th>Controls n = 15</th>
</tr>
</thead>
<tbody>
<tr>
<td>$P_{bm}$</td>
<td>2.82 ± 1.71</td>
<td>2.82 ± 1.71</td>
<td>2.64 ± 1.46</td>
</tr>
<tr>
<td>Outer wall area</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>0.070</td>
<td>0.047</td>
<td>0.116</td>
</tr>
<tr>
<td>S</td>
<td>0.167</td>
<td>0.116</td>
<td>0.079</td>
</tr>
<tr>
<td>Inner wall area</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>0.007</td>
<td>0.012</td>
<td>0.083</td>
</tr>
<tr>
<td>S</td>
<td>0.130</td>
<td>0.103</td>
<td>0.057</td>
</tr>
<tr>
<td>Sub-mucosa</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>0.015</td>
<td>-0.0004</td>
<td>0.067</td>
</tr>
<tr>
<td>S</td>
<td>0.106</td>
<td>0.092</td>
<td>0.052</td>
</tr>
<tr>
<td>Smooth muscle</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>0.005</td>
<td>0.026</td>
<td>0.056</td>
</tr>
<tr>
<td>S</td>
<td>0.068</td>
<td>0.044</td>
<td>0.020</td>
</tr>
</tbody>
</table>
Table 13 (a)

Apoptosis in the airway epithelium as demonstrated by TUNEL positive cells in cartilaginous and membranous airways combined. Values are expressed as area fraction (Aa) mean ± SEM

**Area fraction (Aa) of TUNEL positive cells (mean ± SEM)**

<table>
<thead>
<tr>
<th>Subject groups</th>
<th>Aa (mean ± SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Severe asthma</td>
<td>0.26 ± 0.044*</td>
</tr>
<tr>
<td>Chronic asthma</td>
<td>0.061 ± 0.019</td>
</tr>
<tr>
<td>Controls</td>
<td>0.15 ± 0.031</td>
</tr>
</tbody>
</table>

*p = 0.0004 Severe asthma significantly different from chronic asthma and control groups

(p value for significant difference between the subject groups determined by single factor ANOVA followed by Least Significant Difference (LSD) method for multiple comparisons)

Table 13 (b)

TUNEL positive cells in a sub-group of study subjects

**Absolute count of TUNEL positive cells**

<table>
<thead>
<tr>
<th>Subject group</th>
<th># of airways</th>
<th>C / M</th>
<th>% BM denudation</th>
<th>Point counting</th>
<th>Absolute cell count</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Mean Aa (SEM)</td>
<td>Fraction (SEM)</td>
</tr>
<tr>
<td>Severe asthma</td>
<td>6</td>
<td>4C/2M</td>
<td>9.3</td>
<td>0.054 (0.12)</td>
<td>0.878 (0.062)</td>
</tr>
<tr>
<td>Chronic asthma</td>
<td>6</td>
<td>2C/4M</td>
<td>0</td>
<td>0.22 (0.09)</td>
<td>0.63 (0.058)</td>
</tr>
<tr>
<td>Controls</td>
<td>6</td>
<td>1C/5M</td>
<td>0</td>
<td>0.043 (0.034)</td>
<td>0.111 (0.059)</td>
</tr>
</tbody>
</table>
Table 14

Distribution of TUNEL staining in the airway epithelium of cartilaginous and membranous airways when analyzed separately. Results are expressed as area fraction (Aa) mean ± SEM.

*Area fraction (Aa) of TUNEL positive cells (mean ± SEM)*

<table>
<thead>
<tr>
<th>Subject groups</th>
<th>Cartilaginous airways</th>
<th>Membranous airways</th>
</tr>
</thead>
<tbody>
<tr>
<td>Severe asthma</td>
<td>0.33 ± 0.052*</td>
<td>0.24 ± 0.055**</td>
</tr>
<tr>
<td>Chronic asthma</td>
<td>0.13 ± 0.069</td>
<td>0.057 ± 0.018</td>
</tr>
<tr>
<td>Controls</td>
<td>0.14 ± 0.042</td>
<td>0.15 ± 0.029</td>
</tr>
</tbody>
</table>

*p = 0.01 Severe asthma significantly different from chronic asthma and controls

**p = 0.004 Severe asthma significantly different from chronic asthma

(p value for significant difference between the subject groups determined by single factor ANOVA followed by Least Significant Difference (LSD) method for multiple comparisons)
Table 15

Expression of Fas in the epithelium of cartilaginous and membranous airways combined.

Data are presented as area fraction (Aa) mean ± SEM.

<table>
<thead>
<tr>
<th>Subject groups</th>
<th>Aa (mean ± SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Severe asthma</td>
<td>0.26 ± 0.036*</td>
</tr>
<tr>
<td>Chronic asthma</td>
<td>0.16 ± 0.021</td>
</tr>
<tr>
<td>Controls</td>
<td>0.18 ± 0.028</td>
</tr>
</tbody>
</table>

*p = 0.04 statistically significant difference between severe asthma, chronic asthma and control groups.

(p value for significant difference between the subject groups determined by single factor ANOVA followed by Least Significant Difference (LSD) method for multiple comparisons)
Table 16

Expression of Fas in the epithelium of cartilaginous and membranous airways when analyzed separately. Data are presented as area fraction (Aa) mean ± SEM.

<table>
<thead>
<tr>
<th>Subject groups</th>
<th>Aa mean ± SEM C</th>
<th>M</th>
</tr>
</thead>
<tbody>
<tr>
<td>Severe asthma</td>
<td>0.29 ± 0.033</td>
<td>0.22 ± 0.027</td>
</tr>
<tr>
<td>Chronic asthma</td>
<td>0.17 ± 0.021</td>
<td>0.15 ± 0.016</td>
</tr>
<tr>
<td>Controls</td>
<td>0.22 ± 0.043</td>
<td>0.17 ± 0.024</td>
</tr>
</tbody>
</table>

p = 0.17 for cartilaginous airways, there is no statistically significant difference between the subject groups.

p = 0.22 for membranous airways, there is no statistically significant difference between the subject groups.

(p value for significant difference between the subject groups determined by single factor ANOVA followed by Least Significant Difference (LSD) method for multiple comparisons)
Table 17

Expression of Fas in the inflammatory cells in the airway wall compartments of cartilaginous and membranous airways. Results presented as area fraction (Aa) mean ± SEM

Area fraction (Aa) of Fas positive inflammatory cells in the airway wall compartments (mean ± SEM)

<table>
<thead>
<tr>
<th>Airway wall area</th>
<th>Subject groups</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Severe asthma</td>
<td>Chronic asthma</td>
<td>Controls</td>
</tr>
<tr>
<td>Epithelium*</td>
<td>0.00039 ± 0.00019</td>
<td>0.00091 ± 0.00067</td>
<td>0.00034 ± 0.0003</td>
</tr>
<tr>
<td>WA_e</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Subepithelium**</td>
<td>0.0052 ± 0.0013</td>
<td>0.00041 ± 0.0024</td>
<td>0.00054 ± 0.00024</td>
</tr>
<tr>
<td>WA_subepi</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Smooth muscle@</td>
<td>0.0051 ± 0.0011</td>
<td>0.0011 ± 0.00039</td>
<td>0.0026 ± 0.00098</td>
</tr>
<tr>
<td>WA_sm</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Submucosa#</td>
<td>0.013 ± 0.0021</td>
<td>0.0016 ± 0.0011</td>
<td>0.0032 ± 0.0012</td>
</tr>
<tr>
<td>WA_sub</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Airway walls$</td>
<td>0.0041 ± 0.00088</td>
<td>0.00099 ± 0.00038</td>
<td>0.0011 ± 0.00034</td>
</tr>
<tr>
<td>WA_tot</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*p = 0.21

**p = 0.0001 Severe asthma group significantly different from chronic asthma and control groups.

@p = 0.005 Chronic asthma differs significantly from severe asthma group.

#P = 0.0002 Severe asthma group differs significantly from chronic asthma and control groups.

$p = 0.0003$ Severe asthma group differs significantly from chronic asthma and control groups.

(p values were determined by Kruskall-Wallis (K-W) test followed by Steel-Dwass-Critchlow-Fligner method for multiple comparisons)
Definition of abbreviations:

$W_A^e = \text{the area of the airway wall occupied by the epithelium}$

$W_A^{\text{subepi}} = \text{the subepithelial area of the airway wall}$

$W_A^{\text{sm}} = \text{the smooth muscle area of the airway wall}$

$W_A^{\text{sub}} = \text{the submucosal area of the airway wall}$

$W_A^{\text{tot}} = \text{the total area of the airway wall}$
Table 18
Expression of Fas in the inflammatory cells in the airway wall compartments of cartilaginous airways. Results presented as area fraction (Aa) mean ± SEM

<table>
<thead>
<tr>
<th>Airway wall area</th>
<th>Subject groups</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Severe asthma</td>
<td>Chronic asthma</td>
<td>Controls</td>
</tr>
<tr>
<td>Epithelium*</td>
<td>0.0028 ± 0.0024</td>
<td>0.00023 ± 0.00022</td>
<td>0.00099 ± 0.00072</td>
</tr>
<tr>
<td>Subepithelium**</td>
<td>0.0037 ± 0.00091</td>
<td>0.00037 ± 0.00031</td>
<td>0.00092 ± 0.00046</td>
</tr>
<tr>
<td>Smooth muscle@</td>
<td>0.0054 ± 0.0021</td>
<td>0.0019 ± 0.00093</td>
<td>0.0072 ± 0.0034</td>
</tr>
<tr>
<td>Submucosa#</td>
<td>0.0046 ± 0.0014</td>
<td>0.0012 ± 0.00066</td>
<td>0.0039 ± 0.0016</td>
</tr>
<tr>
<td>Airway wall$</td>
<td>0.0035 ± 0.00092</td>
<td>0.001 ± 0.00055</td>
<td>0.0029 ± 0.0011</td>
</tr>
</tbody>
</table>

*p = 0.8

**p = 0.008 Severe asthma group significantly different from chronic asthma group.

@ p = 0.9

# p = 0.7

$p = 0.5$

(p values were determined by Kruskall-Wallis (K-W) test followed by Steel-Dwass-Critchlow-Fligner method for multiple comparisons)

Definition of abbreviations:
\[ WA_e = \text{the area of the airway wall occupied by the epithelium} \]

\[ WA_{subepi} = \text{the subepithelial area of the airway wall} \]

\[ WA_{sm} = \text{the smooth muscle area of the airway wall} \]

\[ WA_{sub} = \text{the submucosal area of the airway wall} \]

\[ WA_{tot} = \text{the total area of the airway wall} \]
Table 19

Expression of Fas in the inflammatory cells in the airway wall compartments of membranous airways. Results presented as area fraction (Aa) mean ± SEM

<table>
<thead>
<tr>
<th>Airway wall area</th>
<th>Subject groups</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Severe asthma</td>
<td>Chronic asthma</td>
<td>Controls</td>
</tr>
<tr>
<td>Epithelium*</td>
<td>0.00061 ± 0.00037</td>
<td>0.0013 ± 0.00088</td>
<td>0.00041 ± 0.00039</td>
</tr>
<tr>
<td>WAe</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Subepithelium**</td>
<td>0.0082 ± 0.0016</td>
<td>0.0013 ± 0.00071</td>
<td>0.00064 ± 0.00025</td>
</tr>
<tr>
<td>WA_subepi</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Smooth muscle@</td>
<td>0.0075 ± 0.0015</td>
<td>0.0015 ± 0.00049</td>
<td>0.0041 ± 0.0015</td>
</tr>
<tr>
<td>WAsm</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Submucosa#</td>
<td>0.0074 ± 0.0013</td>
<td>0.0014 ± 0.00048</td>
<td>0.0023 ± 0.00073</td>
</tr>
<tr>
<td>WA_sub</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Airway wall$</td>
<td>0.0053 ± 0.00093</td>
<td>0.0015 ± 0.00051</td>
<td>0.0014 ± 0.00045</td>
</tr>
<tr>
<td>WA_tot</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*p = 0.1

**p = 0.0008 Severe asthma group significantly different from chronic asthma and control groups

@p = 0.002 Severe asthma group significantly different from chronic asthma and control groups

#p = 0.0002 Severe asthma group significantly different from chronic asthma and control groups

$\ p = 0.001$ Severe asthma group significantly different from chronic asthma and control groups

(p values were determined by Kruskall-Wallis (K-W) test followed by Steel-Dwass-Critchlow-Fligner method for multiple comparisons)

Definition of abbreviations:
$WA_e = \text{the area of the airway wall occupied by the epithelium}$

$WA_{\text{subepi}} = \text{the sub-epithelial area of the airway wall}$

$WA_{\text{sm}} = \text{the smooth muscle area of the airway wall}$

$WA_{\text{sub}} = \text{the submucosal area of the airway wall}$

$WA_{\text{tot}} = \text{the total area of the airway wall}$
Table 20

Expression of FasL in the epithelium of cartilaginous and membranous airways combined. Data are presented as area fraction (Aa) mean ± SEM.

<table>
<thead>
<tr>
<th>Subject groups</th>
<th>Aa (mean ± SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Severe asthma</td>
<td>0.19 ± 0.026 *</td>
</tr>
<tr>
<td>Chronic asthma</td>
<td>0.14 ± 0.031*</td>
</tr>
<tr>
<td>Controls</td>
<td>0.051 ± 0.015</td>
</tr>
</tbody>
</table>

*p = 0.0004 Severe asthma and chronic asthma significantly different from controls

(p value for significant difference between the subject groups determined by single factor ANOVA followed by Least Significant Difference (LSD) method for multiple comparisons)
Table 21

Expression of FasL in the epithelium of cartilaginous and membranous airways when analyzed separately. Data are presented as area fraction (Aa) mean ± SEM.

Area fraction (Aa) of FasL positive cells in the airway epithelium (mean ± SEM)

<table>
<thead>
<tr>
<th>Subject groups</th>
<th>Aa mean ± SEM</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>C</td>
<td>M</td>
<td></td>
</tr>
<tr>
<td>Severe asthma</td>
<td>0.24 ± 0.032*</td>
<td>0.18 ± 0.017**</td>
<td></td>
</tr>
<tr>
<td>Chronic asthma</td>
<td>0.058 ± 0.011</td>
<td>0.15 ± 0.024**</td>
<td></td>
</tr>
<tr>
<td>Controls</td>
<td>0.041 ± 0.014</td>
<td>0.054 ± 0.0076</td>
<td></td>
</tr>
</tbody>
</table>

* p = 0.0004 Severe asthma significantly different from chronic asthma and control groups

** p = 0.0008 Severe and chronic asthma significantly differs from controls

C = cartilaginous, M = membranous

(p value for significant difference between the subject groups determined by single factor ANOVA followed by Least Significant Difference (LSD) method for multiple comparisons)
Table 22
Expression of FasL in the inflammatory cells in the airway wall compartments of cartilaginous and membranous airways combined. Results presented as area fraction (Aa) mean ± SEM

Area fraction (Aa) of FasL positive inflammatory cells in the airway wall compartments

(mean ± SEM)

<table>
<thead>
<tr>
<th>Airway wall area</th>
<th>Subject groups</th>
<th>Severe asthma</th>
<th>Chronic asthma</th>
<th>Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Severe asthma</td>
<td>Chron ic asthma</td>
<td>Controls</td>
</tr>
<tr>
<td><strong>Epithelium</strong></td>
<td></td>
<td>0.0013 ± 0.00051</td>
<td>0.052 ± 0.0021</td>
<td>0.0011 ± 0.00053</td>
</tr>
<tr>
<td>WA</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Subepithelium</strong></td>
<td></td>
<td>0.0039 ± 0.0011</td>
<td>0.0072 ± 0.0031</td>
<td>0.0052 ± 0.0013</td>
</tr>
<tr>
<td>WA_{subepi}</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Smooth muscle</strong></td>
<td></td>
<td>0.0085 ± 0.0023</td>
<td>0.016 ± 0.0036</td>
<td>0.0089 ± 0.0019</td>
</tr>
<tr>
<td>WA_{sm}</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Submucosa</strong></td>
<td></td>
<td>0.012 ± 0.0033</td>
<td>0.024 ± 0.012</td>
<td>0.014 ± 0.0034</td>
</tr>
<tr>
<td>WA_{sub}</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Airway wall</strong></td>
<td></td>
<td>0.0054 ± 0.0014</td>
<td>0.011 ± 0.0021</td>
<td>0.0052 ± 0.00091</td>
</tr>
<tr>
<td>WA_{tot}</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*p = 0.7  
**p = 0.9  
@p = 0.4  
#p = 0.5  
$p = 0.2  

There was no statistically significant difference in the FasL expressing inflammatory cells in the airway wall compartments of cartilaginous and membranous airways of the study groups.
(p values determined by Kruskall-Wallis test followed by Steel-Dwass-Critchlow-Fligner method for multiple comparisons)

Definition of abbreviations:

\( W_{ae} \) = the area of the airway wall occupied by the epithelium

\( W_{\text{subepi}} \) = the sub-epithelial area of the airway wall

\( W_{\text{sm}} \) = the smooth muscle area of the airway wall

\( W_{\text{sub}} \) = the submucosal area of the airway wall

\( W_{\text{tot}} \) = the total area of the airway wall
Table 23

Expression of FasL in the inflammatory cells in the airway wall compartments of cartilaginous airways. Results presented as area fraction (Aa) mean ± SEM

**Area fraction (Aa) of FasL positive inflammatory cells in the airway wall compartments (mean ± SEM)**

<table>
<thead>
<tr>
<th>Airway wall area</th>
<th>Subject groups</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Severe asthma</td>
<td>Chronic asthma</td>
<td>Controls</td>
<td></td>
</tr>
<tr>
<td>Epithelium*</td>
<td>0.0025 ± 0.0016</td>
<td>0.0037 ± 0.0024</td>
<td>0.00035 ± 0.00021</td>
<td></td>
</tr>
<tr>
<td>WAE</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Subepithelium**</td>
<td>0.0019 ± 0.00049</td>
<td>0.0049 ± 0.0023</td>
<td>0.0024 ± 0.00089</td>
<td></td>
</tr>
<tr>
<td>WAsubepi</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Smooth muscle@</td>
<td>0.0056 ± 0.0016</td>
<td>0.016 ± 0.0055</td>
<td>0.0061 ± 0.0027</td>
<td></td>
</tr>
<tr>
<td>WAsm</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Submucosa#</td>
<td>0.0039 ± 0.0011</td>
<td>0.011 ± 0.0041</td>
<td>0.0041 ± 0.0015</td>
<td></td>
</tr>
<tr>
<td>WAsub</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Airway walls$</td>
<td>0.0032 ± 0.00069</td>
<td>0.0084 ± 0.0032</td>
<td>0.0029 ± 0.0011</td>
<td></td>
</tr>
<tr>
<td>WAtot</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*p = 0.4

**p = 0.2

@p = 0.04 Chronic asthma significantly different from severe asthma and control groups

#p = 0.1

$p = 0.1

(p values determined by Kruskall-Wallis test followed by Steel-Dwass-Critchlow-Fligner method for multiple comparisons)

Definition of abbreviations:

WAE = the area of the airway wall occupied by the epithelium
WA_{subepi} = the subepithelial area of the airway wall
WA_{sm} = the smooth muscle area of the airway wall
WA_{sub} = the submucosal area of the airway wall
WA_{tot} = the total area of the airway wall
Table 24

Expression of FasL in the inflammatory cells in the airway wall compartments of membranous airways. Results presented as area fraction (Aa) mean ± SEM

**Area fraction (Aa) of FasL positive inflammatory cells in the airway wall compartments (mean ± SEM)**

<table>
<thead>
<tr>
<th>Airway wall area</th>
<th>Subject groups</th>
<th>Severe asthma</th>
<th>Chronic asthma</th>
<th>Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Epithelium</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>( WA_e )</td>
<td>0.0012 ± 0.00055</td>
<td>0.0058 ± 0.0017</td>
<td>0.0013 ± 0.00055</td>
<td></td>
</tr>
<tr>
<td><strong>Subepithelium</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>( WA_{subepi} )</td>
<td>0.0053 ± 0.00097</td>
<td>0.0077 ± 0.0025</td>
<td>0.0045 ± 0.00098</td>
<td></td>
</tr>
<tr>
<td><strong>Smooth muscle</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>( WA_{sm} )</td>
<td>0.014 ± 0.0022</td>
<td>0.018 ± 0.0028</td>
<td>0.013 ± 0.0021</td>
<td></td>
</tr>
<tr>
<td><strong>Submucosa</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>( WA_{sub} )</td>
<td>0.01 ± 0.0016</td>
<td>0.014 ± 0.0022</td>
<td>0.0089 ± 0.0014</td>
<td></td>
</tr>
<tr>
<td><strong>Airway wall</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>( WA_{tot} )</td>
<td>0.0072 ± 0.0011</td>
<td>0.011 ± 0.0017</td>
<td>0.0055 ± 0.00081</td>
<td></td>
</tr>
</tbody>
</table>

*p = 0.5

**p = 0.8

@p = 0.8

#p = 0.9

$p = 0.5

There was no statistically significant difference in FasL expressing inflammatory cells in the airway wall compartments of membranous airways of the subject groups.
(p values determined by Kruskall-Wallis test followed by Steel-Dwass-Critchlow-Fligner method for multiple comparisons).

Definition of abbreviations:

\( WA_e = \) the area of the airway wall occupied by the epithelium

\( WA_{subepi} = \) the subepithelial area of the airway wall

\( WA_{sm} = \) the smooth muscle area of the airway wall

\( WA_{sub} = \) the sub-mucosal area of the airway wall

\( WA_{tot} = \) the total area of the airway wall
APPENDIX II

Graph 1

Effect of postmortem time intervals and temperature conditions on TUNEL staining of airway epithelium (RT = room temperature)

TUNEL positive cells/100 cells (mean ± SEM)

Time points in hours

0hrs  6hrs  12hrs  18hrs  24hrs  36hrs

48hrs  72 hrs
Graph 2

Effect of postmortem time interval and temperature conditions on TUNEL staining of airway smooth muscle (RT = room temperature)
Cumulative frequency distribution for membranous airways in the subject groups

The frequency distribution of airway size (Pbm) was similar in the subject groups (ANOVA)
Cumulative frequency distribution for cartilaginous airways in the subject groups

The frequency distribution of airway size (Pbm) was different in the subject groups
p = 0.04 (ANOVA)
Graph 5

Relationship between the square root of epithelial area and the basement membrane perimeter of cartilaginous airways

- △ Severe asthma
- ★ Chronic asthma
- ● Controls
Graph 6

Relationship between the square root of sub-epithelial area and the basement membrane perimeter of cartilaginous airways
Graph 7

Relationship between the square root of smooth muscle area and the basement membrane perimeter of cartilaginous airways

- △ Severe asthma
- ● Chronic asthma
- ⋯ ○ Controls
Graph 8
Relationship between the square root of total airway wall area and the basement membrane perimeter of cartilaginous airways.

- △ Severe asthma
- ● Chronic asthma
- ○ Controls
Relationship between the square root of epithelial area and the basement membrane perimeter of membranous airways

- △ Severe asthma
- ◆ Chronic asthma
- ⋅⋅○ Controls
Graph 10
Relationship between the square root of sub-epithelial area and the basement membrane perimeter of membranous airways.

- ▲ Severe asthma
- ♦ Chronic asthma
- ... Controls

Square Root of sub-epithelial area

Perimeter of basement membrane (Pbm) (mm)
Graph 11

Relationship between the square root of smooth muscle area and the basement membrane perimeter of membranous airways

- △ Severe asthma
- ◇ Chronic asthma
- ⋆ ⋀ Controls
Graph 12

Relationship between the square root of total airway wall and the basement membrane perimeter of membranous airways

- △ Severe asthma
- ● Chronic asthma
- •••• Controls

Square root of total airway wall vs. Perimeter of basement membrane (Pbm) (mm)
Graph 13

Measured ($Abm$) and idealized luminal area ($Abm^*$) of the membranous airways vs. perimeter of the basement membrane in subjects who had severe asthma.
Graph 14

Measured (Abm) and idealized luminal area (Abm*) of the membranous airways vs. perimeter of the basement membrane in subjects who had chronic asthma
Graph 15

Measured (Abm) and idealized luminal area (Abm*) of the membranous airways vs. perimeter of the basement membrane in the control subjects.
Graph 16

TUNEL staining in the epithelium of cartilaginous and membranous airways

*p = 0.0004 severe asthma significantly different from chronic asthma and controls (ANOVA)
Graph 17

TUNEL staining in the epithelium of cartilaginous (C) and membranous (M) airways when analyzed separately

*p = 0.01 severe asthma significantly different from chronic asthma and controls (ANOVA)

**p = 0.004 severe asthma significantly different from chronic asthma (ANOVA)
Expression of Fas in the epithelium of cartilaginous and membranous airways.

* p = 0.04 severe asthma significantly different from chronic asthma and controls (ANOVA)
Fas expressing epithelial cells in cartilaginous (C) and membranous (M) airways when analyzed separately.

\[ p = 0.2 \text{ no difference between the subject groups in cartilaginous airways (ANOVA)} \]

\[ p = 0.2 \text{ no difference between the subject groups in membranous airways (ANOVA)} \]
Fas expressing inflammatory cells in the wall compartments of cartilaginous and membranous airways in the subject groups.

(abbreviations: please see materials and methods)

*p = 0.0001
#p = 0.005
$\text{p} = 0.0002
‡\text{p} = 0.0003

(Kruskall Wallis test)
Graph 21

Fas expressing inflammatory cells in the wall compartments of cartilaginous airways
(abbreviation: please see materials and methods)

SA = Severe asthma
CA = Chronic asthma
C = Controls
Graph 22

Fas expressing inflammatory cells in the wall compartments of membranous airways (abbreviation: please see materials and methods)

SA = Severe asthma
CA = Chronic asthma
C = Controls
Graph 23

Expression of FasL in the epithelium of cartilaginous and membranous airways

*p = 0.0004 severe asthma and chronic asthma significantly different from controls (ANOVA)
Graph 24

FasL expressing epithelial cells in cartilaginous (C) and membranous (M) airways when analyzed separately.

* \( p = 0.0004 \) severe asthma significantly different from chronic asthma and controls (ANOVA)

** \( p = 0.0008 \) severe asthma and chronic asthma significantly different from controls (ANOVA)
Graph 25

FasL expressing inflammatory cells in the wall compartments of cartilaginous and membranous airways in the subject groups.
(abbreviations: please see materials and methods)

no difference between the subject groups (Kruskall Wallis test)
Graph 26

FasL expressing inflammatory cells in the wall compartments of cartilaginous airways (abbreviation: please see materials and methods)

SA = Severe asthma
CA = Chronic asthma
C = Controls
Graph 27

FasL expressing inflammatory cells in the wall compartments of membranous airways
(abbreviation: please see materials and methods)

SA = Severe asthma
CA = Chronic asthma
C = Controls

p = 0.5
p = 0.8
p = 0.9
Graph 28

Mean of the morphometric measurements (mm) from the two observers for each airway

% difference = diff. b/w two measurements / mean of the measurements x 100

mean of the morphometric measurements (mm) for each airway from the two observers
Graph 29

Mean of the number of positive points counted by the two observers for each airway

% difference = diff. b/w two measurements / mean of the points counted X 100

mean of the number of positive points counted by two observers for each airway

- TUNEL
- ▲ FasL (epith)
- □ FasL (inflamm.cells)
- ● Fas (epi)
- X Fas (inflamm.cells)
Graph 30

Mean of the two morphometric measurements (mm) by one observer for each airway

% difference = diff. b/w two measurements / mean of the measurements x 100

mean of the two morphometric measurements (mm) from one observer for each airway
Figure 2. Molecular pathways of Fas-FasL mediated apoptosis

FasL → Fas → FADD → DISC → Caspase 8 → Caspase 3 → Caspase 6

Caspase 1 → Caspase 10 → Caspase 7 → Caspase 9 → Procaspase 9 → Apaf-1 → dATP → Cyto c → Mitochondria

Perforin → GraB → Plasma membrane

Pro-apoptotic:
Bax
Bad
Bak
Bcl-X_s
Bcl-X_L
Bcl-2
A1
Mcl-1

Pro-survival:

Nucleus → PARP → apoptosis
Figure 4

Catalyzed Signal Amplification (CSA) System...... how it works

1

Primary Ab, 15 min.

2

Biotinylated 2° Ab, 15 min.

3

Streptavidin-Biotin/HRP 15 min.
Figure 4. cont.

CSA System........................................amplification step

4

Biotinyl-tyramide, 15 min.

5

Streptavidin/HRP, 15 min.

6

DAB, 5 min.
Figure Legends

Figure 1.

**Fas-FasL interactions in initiating apoptosis.** Coexpression of Fas and FasL in the airway epithelium and the surrounding inflammatory cells suggests a possible role for an autocrine, paracrine or juxtacrine loop causing self or adjacent cell death. The metalloproteinase cleaves the FasL molecule into soluble FasL which is functionally active and may work locally via cell-cell interaction.

Figure 2.

**Molecular pathways involved in Fas-FasL mediated apoptosis.** Binding of Fas ligand to Fas receptor initiates the recruitment of death inducing signaling complex (DISC) causing the dimerization and activation of caspase 8. Granzyme B (GraB) cleaves caspase 10 which then activates caspase 3 and 7. Pro-apoptotic stimuli release cytochrome c from the mitochondria. Cytochrome c in conjunction with adenosine triphosphate (ATP) promotes Apaf-1 mediated caspase 9 activation which then cleaves caspase 3. Caspase 3 cleaves caspase 6 and is also responsible for cleaving various proteins like nuclear lamins thereby disabling important cellular structural and repair processes.

Figure 3.

**TUNEL assay** In this assay terminal deoxynucleotidyl transferase (TdT) binds to exposed 3'-OH ends of DNA fragments generated in response to apoptotic signals and catalyzes the addition of biotin-labeled and unlabeled deoxynucleotides. Biotinylated nucleotides are detected using ABComplex (avidin-biotin-alkaline phosphatase complex). Substrate-chromogen solution consisting of Naphthol-ASBI phosphate and New fuschin reacts with the labeled sample to generate an insoluble (deep red-pink) colored precipitate at the site of DNA fragmentation.
Catalyzes Signal Amplification (CSA) The specimens were first incubated with 3% hydrogen peroxide and then with a protein block. This was followed by incubation with mouse primary antibody or negative control reagent followed by sequential incubations with biotinylated secondary antibody, streptavidin-biotin-peroxidase complex, biotinyl tyramide (amplification reagent), streptavidin peroxidase and DAB which resulted in a brown colored precipitate at the antigen site.
Criteria for airway selection

Include

Integrity of the basement membrane

Exclude

Presence and distribution of the smooth muscle

Aspect ratio < 3.3
Figure 6. Morphometric measurements of airway wall components and compartments
Manual tracing of the airway wall components using Image Pro-Plus software, to determine the morphometric dimensions.
Arrows represent the perimeters that were traced.
Pi = luminal perimeter
Pbm = perimeter of basement membrane
Pmi = perimeter inner smooth muscle border
Pmo = perimeter outer smooth muscle border

Figure 7. Point counting using a grid mask and manual tags
Point counting using a grid mask and manual tags (Image Pro-Plus software).
Arrows represent different classes of manual tags to count several objects of interest simultaneously in the field of view.

Figure 9. Complete occlusion of the airway lumen by mucous plug
Tissue section from a severe asthma subject showing complete occlusion of the airway lumen by mucus plug. Arrows point to the cellular debris in the mucus mesh.
Figure 8

Effects of postmortem delay time interval and temperature conditions on TUNEL staining of lung tissue (72 hrs)

TUNEL staining of lung tissue following 72 hrs. of postmortem delay at room temperature (B), 37°C (C) and 4°C (D). Intense dark brown TUNEL staining of the nuclei is seen (arrows). DAB (di-amino benzadine) substrate chromogen was used for the TUNEL assay in this experiment. Several airway epithelial cells that were completely detached from the basement membrane were identified (no fill arrows). Negative control (A) was generated by the omission of TdT (Terminal deoxy nucleotidyl transferase) enzyme. All bars = 200 μm.
Figure 10. Extent of basement membrane denudation of the airway sections from the study subjects

Airway sections from subjects who had severe asthma (A), subjects who had chronic asthma (B) and the control subjects (C) Arrows show significant airway epithelial damage and denudation in A versus less extensive epithelial damage in B and almost none in C. There are only some basal epithelial cells in some areas in (B) and (C). Bars = 200 μm
Figure 11. TUNEL assay (positive and negative control)
The positive and negative controls for TUNEL assay. Positive control (A) was generated by treating the tissue sections with DNAse and the negative control (B) was generated by omission of the TdT (Terminal deoxynucleotidyl transferase) enzyme. Remainder of the steps were similar for the assay.
Bar = 200 μm

Figure 12. TUNEL staining in study subjects
Detection of apoptosis in the airway sections obtained from subjects who had severe asthma (A), subjects who had chronic asthma (B) and the control subjects (C). Numerous apoptotic nuclei were detected in A, while there were fewer in B and much less in C. All bars = 200 μm
Figure 13. Fas immunohistochemical staining for control tissues

Tissue sections from normal human liver, prostate gland and uterus were stained for Fas by immunohistochemical methods using mouse monoclonal IgG antibody and Catalyzed Signal Amplification (CSA) system of detection. Thick arrows show positive intense brown staining for Fas (panel A, C and E). Samples stained by isotype matched IgG do not show brown staining (panel B, D and F). There is some brown background staining (thin arrows) in negative control samples. Bar = 200 μm.
Figure 14. FasL immunohistochemical staining of control tissues

Immunohistochemical staining of tissue sections obtained from normal human testis, prostate gland and uterus using polyclonal IgG FasL antibody and avidin-biotin alkaline phosphatase (ABC) conjugate. Arrows show deep pink positive staining for FasL (A, C and F). Negative controls treated by isotype matched non-specific IgG does not show any staining (B, D and E). All bars = 200 μm.
Figure 15 and 16. Expression of Fas and FasL in the airway epithelium of study subjects.
Airway sections from subjects who had severe asthma (A), subjects who had chronic asthma (B) and the control subjects (C). Significant epithelial damage is seen in A (thick arrows), substantial numbers of epithelial (thin arrows) and inflammatory cells (thinner arrows) expressing Fas and FasL are seen in these tissue sections (A & B). Staining for Fas and FasL in the epithelium and in the inflammatory cells in the submucosa is seen in A, B and C. All bars = 200 μm
APPENDIX IV

References


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APPENDIX V

Detailed Research Methodology

V.1 TUNEL assay

TdT-FragEL™ DNA Fragmentation Detection Kit cat # QIA33 Oncogene™ Research Products Boston, MA.

The manufacturer’s protocol was modified and optimized according to the study samples.

Materials provided by the manufacturer

- **Proteinase K**: 2 mg/ml Proteinase K in 10 mM Tris pH 8.0.
- **5X TdT Equilibration Buffer**: 1 M sodium cacodylate, 0.15 M Tris, 1.5 mg/ml BSA, 3.75 mM CoCl$_2$, pH 6.6.
- **TdT Labeling Reaction Mix**: Mixture of labeled and unlabeled deoxynucleotides at a ratio optimized for DNA fragment end labeling with TdT.
- **TdT enzyme**: Terminal Deoxynucleotidyl Transferase
- **Stop Buffer**: 0.5 M EDTA, pH 8.0.
- **Blocking Buffer**: 4% BSA in PBS
- **Control Slides**: two slides of HL60 promyelocytic leukemia cells and HL60 cells incubated with 0.5 µg/ml actinomycin D for 19 hours to induce apoptosis.

Materials required but not provided

- **Xylene, Hemo-de®**: should be used in the fume hood
- **Isopropanol**: 100%, 90%, 70%
- **Wash buffer**: 1X TBS(Tris-buffered saline 20 mM Tris pH 7.6, 140 mM NaCl).
- **10 mM Tris**: pH 8.0
- **1 mM MgSO$_4$ in 1X TBS**: for use in generating positive control
- **DNaseI**: for use in generating positive control
• **ABComplex/AP**: (for detection) code # K 0376 (DAKO® Diagnostics Canada Inc., Mississauga, Ontario)

• **Chromogen substrate solution**: Naphthol AS-BI phosphate, dimethylformamide, 1X TBS pH 8.7, 1M levamisole, sodium nitrite, 5% New Fuschin.

• **$dH_2O$**

• **Wheaton dishes or coplin jars**: glass or plastic with slide holders

• **Wash bottle or beaker**: for rinsing

• **Humidified chamber**

• **37°C incubator**

• **Glass coverslips**

• **Permanent mounting medium**: such as Permount® or Entellan

• **Microscope**

• **Precision pipetters**: 2-20 µl, 20-200 µl and 200-1000 µl with disposable tips

• **Microcentrifuge tubes**

• **Parafilm®**

• **Absorbent wipes**

• **Ice**

**Methodology**

*Tissue fixation, deparaffinization and rehydration*

• Single tissue sections were placed on silanized glass slides

• Sections were dried overnight at 37°C

• Tissue sections were baked at 65°C for an hour prior to staining

• The slides were immersed in Hemo-de for 10 minutes.

• This step was repeated using fresh Hemo-de for a second 10 minute incubation.
• The first two steps of deparaffinization procedure were carried out in the fume hood. The rest of the procedure was carried out at the bench.

• The slides were immersed in 100% isopropanol for 5 minutes. This step was repeated using fresh 100% isopropanol for another 5 minutes.

• The slides were then sequentially immersed in 90% and 70% isopropanol for 5 minutes each time.

• All the above steps were carried out at room temperature.

• Slides were left in dH₂O for 5 minutes

• Slides were rinsed briefly with 1X TBS and carefully dried around the specimen.

• It is extremely important to keep the tissue specimens hydrated and moist at all times during or between the staining steps

• The specimens were encircled using a hydrophobic slide marker to help contain small reaction volumes around the specimen.

• The slides were placed in the humidified chamber and covered with a lid to provide a humidified environment.

• All the following steps of the assay were carried out with the slides placed in the humidified chamber.

**Permeabilization of specimen**

• Proteinase K (stock 2 mg/ml) was diluted 1:50 in 10 mM Tris pH 8 (mix 2 µl of 2 mg/ml Proteinase K plus 98 µl 10 mM Tris per specimen).

• The entire specimen was covered with 100 µl of 40 µg/ml Proteinase K and incubated at 37°C for an hour.

• The slides were rinsed with 1X TBS 3 times.
• Excess liquid was gently tapped off and the glass slides were carefully dried around the specimen.

**Generation of positive control**

• The entire specimen was covered with 100 μl of 0.1 mg/ml of DNase1 (stock 0.8 mg/ml) in 1X TBS/1 mM MgSO₄ (1:8 dilution; 12.5 μl DNase1 + 87.5 μl 1X TBS/1 mM MgSO₄ per specimen).

• The specimens were incubated at room temperature for 30 minutes.

• Slides were rinsed with 1X TBS, gently tapping off excess liquid gently and carefully drying the glass slide around the specimen.

**Equilibration and labeling reaction**

• 5X TdT Equilibration buffer was diluted 1:5 with dH₂O (20 μl 5X buffer was mixed with 80 μl dH₂O per specimen)

• The entire specimen was covered with 100 μl of 1X TdT Equilibration buffer.

• Specimens were incubated at room temperature for 20 minutes while preparing the labeling reaction mixture.

• The working TdT labeling mixture was prepared as follows:

  The contents of TdT labeling reaction mix were lightly vortexed

  The TdT enzyme was pulse-spinned prior to opening

  For each sample to be labeled, 57.0 μl of TdT labeling reaction mix was added to 3.0 μl of TdT enzyme in a clean microfuge tube on ice.

• The TdT equilibration buffer was carefully blotted from the specimen taking care not to touch the specimen.

• Sixty μl of TdT labeling reaction mix was applied onto each specimen (except the negative control).
• Sixty μl of TdT labeling buffer only was applied onto the negative control slide.
• The specimen was covered with a piece of Parafilm® to evenly distribute the reaction mixture and prevent loss due to evaporation during incubation.
• One end of the Parafilm® was folded up to aid in its application and removal.
• The tissue slides were incubated at 37°C for 1 hour and 30 minutes in the humidified chamber.

**Termination of labeling reaction**

• The stop buffer was pre-warmed to room temperature.
• The Parafilm® coverslip was removed and the slides were rinsed with 1X TBS.
• The entire specimen was covered with 100 μl of stop buffer and incubated for 5 minutes at room temperature.
• Slides were rinsed with 1X TBS tapping off excess liquid and carefully drying the glass slide around the specimen.

**Detection**

• One hundred μl of Blocking buffer was applied and the specimens were incubated at room temperature for 10 minutes.
• ABComplex was prepared 1:100 dilution in 1X TBS half an hour prior to its application.
• The Blocking buffer was carefully blotted from the specimen and 100 μl per specimen of ABComplex was immediately applied.
• The specimens were incubated at room temperature for 30 minutes.

**Preparation of chromogen substrate** (this was prepared fresh in a fume hood and was used immediately)

<table>
<thead>
<tr>
<th>Chemical</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Naphthol-ASBI phosphate</td>
<td>20 mg</td>
</tr>
<tr>
<td>Dimethylformamide</td>
<td>0.3 ml</td>
</tr>
</tbody>
</table>
1X TBS pH 8.7 (0.05 M Tris, 0.15 M NaCl) 50 ml
1M Levamisole 50 μl
Sodium nitrite 200 mg
5% New Fuschin 0.1 ml
dH₂O 5 ml

- Sodium nitrite 200 mg was dissolved in 5 ml dH₂O in a small beaker.
- In a conical flask, 0.1 ml New Fuschin was added to 0.25 ml of sodium nitrite solution.
- The above solution was agitated for 30-60 seconds.
- Fifty ml 1X TBS pH 8.7 and 50 μl levamisole was added to the above solution in the conical flask.
- Twenty five mg of naphthol-ASBI phosphate was dissolved in 0.3 ml of dimethylformamide in an Eppendorf tube and was dissolved by gentle vortexing.
- Naphthol-ASBI phosphate solution was added to the above solution in the conical flask.

Application of chromogen substrate
- The slides were washed in 1X TBS twice for 5 minutes each time and the excess fluid was tapped off gently.
- The substrate solution was filtered directly onto the slides to cover the specimen completely.
- The slides were incubated at room temperature for 20 minutes.

Mounting
- The slides were rinsed with dH₂O thoroughly and air dried.
- A glass coverslip was mounted over the specimen using the permanent mounting medium (Entellan)
- Slides were air dried for the mounting medium to solidify and examined under the microscope.
V.2 Immunohistochemical staining for FasL

Materials required

- **Water bath**: temperature to be stabilized between 95-98°C.
- **Sodium citrate**: 10 mM pH 6.0 (Sigma)
- **dH₂O**
- **Wash buffer**: 1X TBS, (50 mM Tris and 140 mM NaCl pH 7.6)
- **Blockers**: 1) Universal Blocker (cat. # CD 310082 DAKO® Diagnostics Canada Inc., Mississauga, Ontario)
  2) DAKO® Biotin Blocking System (code # X 0590 DAKO® Diagnostics Canada Inc., Mississauga, Ontario)
- **Primary antibody**: goat polyclonal IgG FasL 20 µg/ml (N-20 sc-834-G Santa Cruz Biotechnology Inc., Santa Cruz, CA) 1:70 dilution
- **Secondary antibody**: biotinylated rabbit anti-goat IgG (code # E 0466 DAKO® Diagnostics Canada, Mississauga, Ontario) 1:250 dilution
- **Negative control**: rabbit immunoglobulin fraction (code # X 0903 DAKO® Diagnostics Canada, Mississauga, Ontario) 1: 40,000 dilution
- **Diluting buffer**: 1X TBS + 1% BSA
- **ABComplex/AP**: 1:100 dilution (described previously)
- **Chromogen substrate solution**: (same as described for TUNEL assay)
- **Permanent mounting medium (Entellan) and glass coverslips**
- **Microscope**
- **Wheaton dishes or Coplin jars**: glass or plastic with slide holders
- **Wash bottle or beaker**: for rinsing
- **Humidified chamber**
• **Precision pipetters:** 2-20 μl, 20-200 μl and 200-1000 μl with disposable tips

**Methodology**

*Tissue fixation, deparaffinization and rehydration*

(same as described for TUNEL assay)

*Heat induced epitope retrieval*

• A water bath with temperature stabilized between 95-98°C was set up.
• A covered Wheaton dish containing 10 mM sodium citrate pH 6.0 was placed in the water bath for 10 minutes to pre-heat.
• A slide holder containing tissue slides was immersed in the covered Wheaton dish containing sodium citrate and was incubated in the water bath for 30 minutes.
• Slides were cooled down for 20 minutes at room temperature still immersed in sodium citrate.
• Slides were rinsed with dH₂O and then with TBS.
• Immunohistochemical staining of the tissue sections was carried in the humidified chamber at room temperature.

*Blocking*

• Universal Blocker was applied to cover the entire specimen and the slides were incubated for 20 minutes.
• Slides were rinsed in TBS
• Biotin Block – Tissue sections were incubated for 15 minutes with avidin solution. Avidin solution was then rinsed off with TBS.
• Slides were incubated for 15 minutes with biotin solution. Biotin solution was washed off with TBS.

*Antibody application*
• Primary antibody solution or negative control solution was applied and the slides were incubated for one hour.

• Slides were rinsed gently with TBS 3 times for 5 minutes each time.

• Secondary antibody solution was applied to cover the entire specimen, slides were incubated for 30 minutes.

• Slides were rinsed 3 times for 5 minutes each time with TBS.

Detection

• ABComplex/AP diluted 1:100 was applied to the slides and incubated for 30 minutes.

• Following this step, slides were rinsed with TBS 3 times for 5 minutes each time.

Application of chromogen substrate

(same as described for TUNEL assay)

Mounting

(same as described for TUNEL assay)

V.3 Immunohistochemical staining for Fas

DAKO® Catalyzed Signal Amplification (CSA) System, Peroxidase for mouse primary antibodies (code # K 1500 DAKO® Diagnostics Canada Inc., Mississauga, Ontario)

The manufacturer’s protocol was optimized according to study tissues.

Materials included in this system

• Hydrogen peroxide: 3% hydrogen peroxide in water.

• Protein block: serum-free protein in Phosphate Buffered Saline (PBS) with 0.015 M sodium azide.

• Link antibody: biotinylated rabbit anti-mouse immunoglobulins in Tris-HCl buffer containing carrier protein and 0.015 M sodium azide
- **Streptavidin-biotin Complex:** (prepare 30 minutes prior to use) 40:1 dilution
  
  Reagent A – Streptavidin in PBS buffer containing an anti-microbial agent

  Reagent B – Biotin conjugated to horseradish peroxidase in PBS buffer containing an anti-microbial agent

  Diluent – PBS buffer containing carrier protein and an anti-microbial agent

- **Amplification reagent:** Biotinyl tyramide and hydrogen peroxide in PBS containing carrier protein and an anti-microbial agent.

- **Streptavidin peroxidase:** Streptavidin conjugated to horseradish peroxidase in PBS containing carrier protein and an anti-microbial agent.

- **Substrate tablets:** DAB chromogen tablet containing 10 mg 3, 3 diaminobenzidine tetrachloride (DAB) packaged with desiccant.

- **Substrate:**

  Tris buffer concentrate: Tris HCl buffer concentrate pH = 7.6

  Hydrogen peroxide: 0.8% hydrogen peroxide in water

*Materials required but not provided*

- Permanent mounting medium (Entellan) and glass coverslips

- Microscope

- Wheaton dishes or coplin jars: glass or plastic with slide holders

- Wash bottle or beaker: for rinsing

- Humidified chamber

- Precision pipettes: 2-20 µl, 20-200 µl and 200-1000 µl with disposable tips

- Water bath: temperature to be maintained between 95-98°C.

- dH₂O

- Wash buffer: 1X TBST, (50 mM Tris and 140 mM NaCl pH 7.6 + 0.1% Tween 20)
- **Blockers:** 1) Universal blocker (same as protein blocker) 2) Biotin blocking system (described previously)

- **Primary antibody:** mouse monoclonal IgG κ isotype Fas 823 μg/ml (code # M 3554 DAKO® Diagnostics Canada Inc., Mississauga, Ontario) 1:4000

- **Target retrieval solution:** 10X concentrate (code # S 1699 DAKO® Diagnostics Canada Inc., Mississauga, Ontario).

- **Antibody diluting buffer:** (cat. # CD 200082 DAKO® Diagnostics Canada Inc., Mississauga, Ontario).

**Methodology**

*Tissue fixation, deparaffinization and rehydration*

- (same as described for TUNEL assay)

*Heat induced epitope retrieval*

- A water bath was heated to 95-98°C.

- A Wheaton dish was filled with 1:10 diluted target retrieval solution pH 6.0 and was placed in the water bath to preheat for 10 minutes.

- Sections were immersed in preheated target retrieval solution in water bath and incubated for 30 minutes.

- The container with slides was removed from water bath and allowed to cool at room temperature for 20 minutes.

- Sections were rinsed with dH₂O and TBS.

- Immunohistochemical staining was performed on sections in the humidified chamber at room temperature.

**Step 1**

- Hydrogen peroxide was applied to sections and incubated for 5 minutes.
• Sections were rinsed with dH₂O and then with TBST.

**Step 2**

• Biotin blocking system – sections were sequentially incubated with avidin solution for 10 minutes, washed with TBST, incubated with biotin solution for 10 minutes, washed with TBST.

**Step 3**

• Protein block was applied and sections were incubated for 5 minutes.
• Excess liquid was tapped off gently, sections were not rinsed.

**Step 4**

• Primary antibody or negative control solution was applied to cover the specimens and incubated for 45 minutes.
• Sections were washed with TBST twice for 5 minutes each time

**Step 5**

• Specimens were covered with link antibody and incubated for 20 minutes followed by 2 washes with TBST, each wash was for 5 minutes.

**Step 6**

• Prepared streptavidin-biotin complex was applied to specimens and incubated for 15 minutes, sections were washed twice with TBST.

**Step 7**

• Following each wash, excess liquid was tapped off and slides were wiped carefully around the specimen to keep reagents within the marked area.
• Specimens were covered with amplification reagent and incubated for 15 minutes.
• Slides were washed as before

**Step 8**

• Specimens were incubated with streptavidin-peroxidase for 15 minutes.
Following incubation, slides were washed with TBST as before and then rinsed twice with dH₂O.

**Step 9** (prepared and applied in the fume hood)

- Prepared chromogen substrate was applied to cover specimen and incubated for 5 minutes.
- Sections were rinsed gently with dH₂O, substrate chromogen waste was collected in a hazardous materials container for proper disposal.
- Tissue slides were air dried

**Step 10**

- Specimens were mounted and coverslipped with a permanent mounting medium
- Mounted slides were air dried allowing the mounting medium to solidify
- Sections were examined under the microscope.