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Persistent Mucus Accumulation in RAO-Affected Horses —
A Consequence of Delayed Mucous Cell Death

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Lisa Renee Bartner

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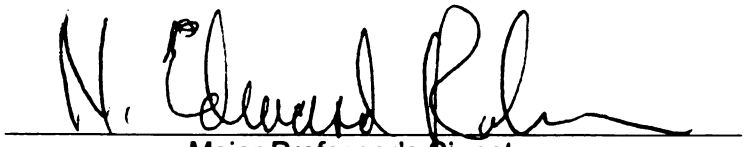

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PERSISTENT MUCUS ACCUMULATION IN RAO-AFFECTED HORSES — A CONSEQUENCE OF DELAYED MUCOUS CELL DEATH?

By

Lisa Renee Bartner

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ABSTRACT

PERSISTENT MUCUS ACCUMULATION IN RAO-AFFECTED HORSES — A CONSEQUENCE OF DELAYED MUCOUS CELL DEATH?

By

Lisa Renee Bartner

This study examined the role of delayed apoptosis of mucous cells (MC) as indicated by Bcl-2, an anti-apoptotic protein, as a contributing factor to mucus accumulation in horses affected with recurrent airway obstruction (RAO). Measurements of disease severity were collected from 6 RAO-affected and 6 control horses exposed to two different management systems (one to induce inflammation [5 days] and one to partially resolve it [7 days]) prior to euthanasia. Morphmetric analyses (Bcl-2-positivity and stored mucosubstance [Vs]) were performed on 8 bronchi. RAO-affected horses had more airway obstruction and luminal mucus than control horses under both management systems. At the time of euthanasia, RAO-affected horses had more inflammation and more Bcl-2-positive MC than control animals but no difference in MC number or Vs, and in RAO-affected animals, Vs decreased as BALF neutrophil numbers increased. Therefore, a conclusive role for Bcl-2 in prolonging MC life cannot be determined, despite Bcl-2 antibody immunoreactivity. USDA/CSREES 2002-35204-1259)

To Bubbe, Mom, and Dad for their enduring pride, love, and support.

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LIST OF ABBREVIATIONS

$\Delta\Psi_m$	Mitochondrial Inner Transmembrane Potential
ΔPpl_{max}	Maximum Change in Pleural Pressure
15-HETE	15-Hydroxyeicosatetraenoic acid
AB	Alcian Blue
AHR	Airway Hyperresponsiveness
AIF	Apoptosis Inducing Factor
ANT	Adenine Nucleotide Translocator
Apaf-1	Apoptotic Protease Activating Factor-1
ATP	Adenosine Triphosphate
Bad	Bcl-2-Antagonist of Cell Death
Bak	Bcl-2-Antagonist/Killer
BALF	Bronchoalveolar Lavage
Bax	Bcl-2-Associated X Protein
Bcl-2	B Cell Lymphoma-2
Bcl-X	Bcl-2-Related Protein
Bcl-X_L	Bcl-2-Related Protein, Long Isoform
Bcl-X_S	Bcl-2-Related Protein, Short Isoform
BH	Bcl-2 Homology Domain
Bid	BH3 Interacting Domain Death Agonist
Bik	Bcl-2-Interacting Killer
BIR	Baculovirus IAP Repeat Domains
Bok	Bcl-2-Related Ovarian Killer
cAMP	Cyclic Adenosine Monophosphate
CARD	Caspase-Activating Recruitment Domain
Caspases	Cysteine Aspartate-Specific Proteases
CF	Cystic Fibrosis
cGMP	Cyclic Guanosine 5'-Monophosphate
DAG	Diacylglycerol
DD	Death Domain
DED	Death Effector Domain
Diablo	Direct IAP Binding Protein with Low pI
DISC	Death-Inducing Signaling Complex
ECG	Electrochemical Gradient
EGFR	Epidermal Growth Factor Receptor
eNANC	Excitatory NANC
FADD	Fas-Associated Death Domain (MORT1)
FasL	Fas Ligand
GCH	Goblet Cell Hyperplasia
IAP	Inhibitors of Apoptosis Protein(s)
ICE	IL-1β-Converting Enzyme (Caspase-1)
IFN-γ	Interferon-gamma
IKK	IκB-Kinase
IL	Interleukin
iNANC	Inhibitory NANC
IP_3	Inositol Triphosphate
IP_3-R	IP_3 Receptor
LPS	Lipopolysaccharide
LTB_4	Leukotriene B_4
MARCKS	Myristoylated Alanine-Rich C Kinase Substrate
MC	Mucous Cell(s)
MCM	Mucous Cell Metaplasia
MMC	Metaplastic Mucous Cell(s)
MMP	Mitochondrial Membrane Permeabilization
MUC	Mucin Gene Abbreviation
NANC	Nonadrenergic-Noncholinergic
NF-κB	Nuclear Factor-Kappa B
OMM	Outer Mitochondrial Membrane
OVA	Ovalbumin
PAS	Periodic Acid-Schiff
PBS	Phosphate Buffered Saline
PKA	Protein Kinase A
PKC	Protein Kinase C
PKG	Protein Kinase G
PLC	Phospholipase C
PP2A	Protein Phosphatase-2A
PT	Permeability Transition
PTP	PT Pore
PTPC	Permeability Transition Pore Complex
RAO	Recurrent Airway Obstruction
RIP	Receptor Interacting Protein
ROS	Reactive Oxygen Species
sGC	Soluble Guanylyl Cyclase
Smac	Second Mitochondrial Activator of Caspases
TNF	Tumor Necrosis Family
TNFR	TNF Receptor(s)
TRADD	TNF-R1-Associated Death Domain
TRAF	TNFR-Associated Factor(s)
TRAIL	TNF Related Apoptosis-Inducing Factor
VDAC	Voltage Dependent Anion Channel
Vs	Volume Density
XIAP	X-Linked-Inhibitor-Of-Apoptosis Protein

INTRODUCTION

Recurrent airway obstruction (RAO) is an equine respiratory syndrome characterized by reversible airway obstruction that is due to bronchospasm, accumulation of mucoid secretions often containing neutrophils, and remodeling of the airway wall. Acute exacerbations of RAO are induced by exposure to organic dust usually from eating hay. When horses susceptible to this disease are in remission at pasture, there is a significantly greater amount of tracheal mucus relative to that present in control horses, as indicated by an assigned subjective mucus score. Once these animals are brought into a dusty environment, their tracheal mucus score increases significantly while the control horses' mucus scores remain unchanged.

Increased mucus in RAO horses may be due increased mucus production and secretion or decreased clearance or both. Although increased viscoelasticity and decreased clearance occur in challenged RAO horses compared to normal horses, the difference does not fully explain the existence of increased amounts of respiratory secretion (Gerber, King et al. 2000). Therefore, increased secretion of mucus must play a role in the high levels in RAO horses.

The maintenance of significantly greater quantities of mucus in RAO horses in remission leads me to ask the question: "does delayed apoptosis of mucous cells contribute to the observed mucus accumulation in RAO-affected horses?" In other words, do mucous cells have a longer life due to the abnormal prevalence of the anti-apoptotic protein, Bcl-2? I hypothesized that delayed apoptosis, as indicated by an increased presence of Bcl-2-positive mucous cells, contributes, at least in part, to an increase in mucus-secreting cells and subsequent airway mucus accumulation.

CHAPTER 1
LITERATURE REVIEW

MUCOUS APPARATUS IN AIRWAYS
STRUCTURES

The lower respiratory system can be divided into the conducting airways and the gas exchange area. The conducting airways, which serve to connect the environmental air to the alveoli, include the trachea, bronchi, and bronchioles. Gas exchange occurs in the exchange area that is comprised of the respiratory bronchioles, alveolar ducts and alveoli. The airways of the tracheobronchial tree are classified based on: (1) the morphology of the surrounding components and (2) the diversity of the cellular makeup. The distinct regional characteristics of airway morphology and cellular composition allows for separation of functions. The tracheobronchial tree conducts oxygen into alveoli and removes carbon dioxide from the blood while effectively keeping large particles, pollutants, and pathogens from entering the exchange area.

Traveling from proximal airways to distal airways, the epithelial cells change to allow for particle trapping and removal in the large central airways while prohibiting foreign molecules from entering the small, distal airways where gas exchange occurs. For example, in the trachea and bronchi, the widespread surface mucous cells (MC) provide the mucus lining of the bronchial airways that traps particles. Mucous cells are found only in larger bronchioles and decrease in numbers until they are absent in smaller bronchioles. Likewise, ciliated cells—playing the role of mucous transport—follow similar patterns as MC where they are abundant in large airways and taper off in numbers in smaller airways. Cartilage is present in large conducting airways to maintain airway patency but absent in small bronchioles. There is a positive correlation between the size of an animal and its epithelial height, and a negative

correlation between animal size and number of airway generations (Mariassy 1992). On average, the number of generations is around 23 in humans (Mariassy 1992).

The conducting airways have three layers (1) epithelium, basal lamina, and lamina propria, collectively making up the luminal mucosa, (2) the submucosa, composed of connective tissue, smooth muscle, and cartilage, and (3) the adventitia. With the exception of smooth muscle, fibroblasts, cartilage, and neural components, the airway structures are of endodermal origin (Leff and Schumacker 1993). The mucosa is the innermost, well-vascularized continuous lining (Mariassy 1992) that serves to condition the air and to protect and cleanse the lung's surface. It rests on the basement membrane composed mostly of type IV collagen and laminin, and is separated from the underlying submucosa by a smooth muscle layer, the muscularis mucosa

Luminal Mucosa

Ciliated cells

In the cartilaginous airways, trachea and bronchi, there is a pseudostratified, ciliated columnar epithelium; in non-cartilaginous bronchioles, the epithelium is ciliated columnar epithelium. In a study done in dogs by Serafini, Wanner et al (1979), ciliated cells comprised 22% of the tracheal epithelium and 25% of main bronchus, a range of 20% to 50% have been documented in trachea of other species (Wolff 1992). Ciliated cells decrease to around 5 % of total cells in smaller bronchi until the epithelium consists of mainly a single layer of cuboidal ciliated cells (Serafini, Wanner et al. 1976).

The distinctive appearance of ciliated cells is due to their apical cilia and microvilli. Each of the cilia (50 to 300) is about 6 μm in length and has a diameter of 0.3 μm; each of the micovilli (100 to 150) is 2 to 3 μm long with a 0.1 μm diameter (Alexander, Ritchie et al. 1975; Mariassy 1992). Sliding microtubules in a 9 + 2 formation with dynein arms as a motor provide

the cilia with a cyclic back-and-forth movement. Three to seven short "claws" (25 to 35 nm long) on the tips of cilia aid in movement (mechanically or chemically) of the mucus glycoproteins by catching and attaching to the underside of the gel layer during the effective stroke (Wanner, Salathe et al. 1996; Jefcoat 2002). At the base is "the necklace" region which houses the calcium-binding protein, centrin (Wanner, Salathe et al. 1996). This "mucociliary escalator" is the chief defensive mechanism of the lung.

Clara Cells

Distinct non-ciliated, secretory bronchiolar cells, also called Clara cells, serve a role of detoxification in the lung. They may also be progenitors of the ciliated cells, play a role in epithelial regeneration after injury, and contribute to the secretions of small airways, however these secretions are not thought to contain mucoid substances (Finkbeiner and Widdicombe 1992; Leff and Schumacker 1993; Wanner, Salathe et al. 1996). Horse Clara cells hold no glycogen (Mariassy 1992). In large animals, Clara cells are restricted to the non-cartilaginous bronchioles, but in small mammals, they are found in both cartilaginous and non-cartilaginous airways. These cells contain membrane-bound secretory granules, extensive smooth endoplasmic reticulum at their apex, and rough endoplasmic reticulum in the basolateral perinuclear regions (Mariassy 1992).

Serous Cells

Serous cells are mainly present in the surface epithelium of pathogen-free rodents, in animals lacking submucosal glands, and in the human fetus (Basbaum, Jany et al. 1990). In humans, they are confined, for the most part, to the submuiscosal glands after birth. Serous cells are identified by their small (600 nM) electron-dense homogenous secretory granules present on the apical portion of the cell from which they secrete neutral glycoproteins (Finkbeiner and Widdicombe 1992; Mariassy 1992; Newman, Robichaud et al. 1996; Wanner,

Salathe et al. 1996). They may also secrete lipids, lysozymes, lactoferin, and mucus proteinase inhibitors (Wanner, Salathe et al. 1996). Their cytoplasm also contains rough endoplasmic reticulum, a large vesicular nucleus and lacks smooth endoplasmic reticulum (Mariassy 1992).

Neuroendocrine Cells

Neuroendocrine cells, basal cells, brush cells, and seromucous cells occupy a relatively small component of the cellular makeup of the epithelium. Little is known about neuroendocrine cells besides their suggested function which is release of seemingly large quantities of gastrin related peptide (GRP), dopamine, and serotonin, which may play a role in reactive airway disease and pulmonary hypertension (Mariassy 1992; Plopper and Hyde 1992; Leff and Schumacker 1993).

Basal Cells

Basal cells are small (5 to 15 μm) and contact the basal lamina. Proposed functional roles for basal cells are maintenance of bronchial airways, possibly by tethering columnar epithelium to the airway wall and serving as multipotent progenitor cells (Hong, Reynolds et al. 2004).

Brush Cells

In the trachea, there are focal areas 1 mm in diameter of non-ciliated cells with either microvilli or brush-like projections on the center of the cell apex (Alexander, Ritchie et al. 1975). They may also serve as sensory receptors associated with the trigeminal nerve endings (Textbook of Veterinary Histology 5th Ed, Editors H. Dieter Dellmann and Jo Ann Eurell; Williams and Wilkins Baltimore, 1998; Authors: Donald R Adams and H. Dieter Dellmann; p 149-151). These brush cells are rare and always less than 1% of the cell population (Mariassy 1992).

Seromucous Cells

Seromucous cells are those cells that were first identified as MC but overlap with the serous cell morphology. They have an electron-lucent rim with an electron-dense core, with discrete homogenous "nucleated granules" as seen in serous cells (Mariassy 1992).

Submucosal Glands

Airway secretions come in part from submucosal glands, which play important roles in hydrating the airway surfaces, facilitating mucociliary transport, and are involved in the augmentation of the fluid matrix for the macromolecules secreted by the mucosa, including mucins, an albuminoid substance contained in mucus (Ballard and Inglis 2004). While in upper airways of some species, the greater contributor of mucus is from submucosal glands, in the horse few glands are present leaving the MC as the main supplier of mucus (Widdicombe and Pecson 2002). Developmentally, glands are derived from basal cells and appear around week 10 of gestation (Leff and Schumacker 1993). These tubuloalveolar glands empty into the airway lumen of cartilaginous airways and are usually found in tissue between the cartilage rings; they are not present in bronchioles (Finkbeiner and Widdicombe 1992; Ballard and Inglis 2004). Within the glands, there are ciliated cells, resembling surface epithelium, at the orifice of the primary duct and "nonspecified" cells in the collecting ducts (Leff and Schumacker 1993; Ballard and Inglis 2004). The serous cells at the most distal end of the tubule and MC slightly proximal carry out the exocrine function. It is suggested that, in some mammals, this arrangement enables the fluid from the serous cells to flush out the mucins secreted from the MC and that, therefore, these cells have predominant mediator functions over the MC (Ballard and Inglis 2004).

Submucosal glands in equine airways are very unevenly distributed, generally occurring about 100 μm apart in longitudinal rows of about 5, with an average frequency of 1.0/mm^2 of

mucosal surface (Widdicombe and Pecson 2002). Each gland puts out a volume of about 17 nl, this volume is only around 15% of other species (Rubin 2002).

Mucous Cells

Airway MC, also called goblet cells, participate in the homeostasis and the pathophysiology of the lung by rapidly secreting the contents of their granules and increasing in number, respectively. Their classical "goblet" shape may be merely an artifact of fixation. The granules contain both neutral and acidic mucus with sialic acid and sulfate groups within mucin glycoproteins, which determines the viscoelastic properties of the sputum (Leff and Schumacker 1993). Each granule measures about 800 nM in diameter (Wanner, Salathe et al. 1996). The granules found in these are more numerous and have a larger diameter than those in serous cells (Wanner, Salathe et al. 1996). A morphologically distinguishing feature of MC is that the cytosol is more electron-dense, relative to other epithelial cells, with electron-lucent secretory granules, indicative of mucins (Finkbeiner and Widdicombe 1992; Newman, Robichaud et al. 1996). Normal epithelium contains approximately 6,500 MC per mm^2 (30,000-50,000 goblet cells per mm^3), comprising from 5% to 20% of the epithelium in sheep, monkey, and cat tracheas, and 25% of the bronchial epithelial cells in humans and larger mammals (Rogers 2003). In central airways of humans, the ratio of ciliated columnar epithelial cells to MC is 5:1; absolute numbers of both decrease from trachea to peripheral airways (Wanner, Salathe et al. 1996). In distal airways, declining numbers of goblet cells with concurrent rise in Clara and simple cuboidal cells consequently decreases the amount of surface mucus. Goblet cells are rare in healthy bronchioles.

Airway MC are most recognizable by the presence of extensive Golgi network and secretory granules. A three-dimensional reconstruction of a tracheal goblet cell showed that each of the multiple cytoplasmic granules associates with a separate Gogi apparatus (Adler,

Hardwick et al. 1982). Mitochondria and rough endoplasmic reticulum resides between the conspicuous secretory granules in the perinuclear region, along with the Golgi complex. The large, round, dense nucleus is compressed near the basolateral membrane and composed of heterogenous chromatin. Up to 60% of the contents of a MC are membrane-bound granules; this percentage is inversely proportional to the space taken up by organelles (Mariassy 1992). However, fixation and tissue embedding do not preserve the carbohydrate component of the mucous granules leaving the cell appearing to be "empty" (Mariassy 1992).

Lamina Propria

The lamina propria is a 50 to 100 μm thick layer of connective tissue that attaches to the basement membrane. Within the lamina propria is an extensive capillary network.

Submucosa

Beneath the lamina propria is the submucosa made up of smooth muscle and connective tissue. Within the trachea and bronchi, the connective tissue contains glands, vessels, and nerves while the irregular spirals of smooth muscle partially surrounds the airway along with cartilage. In smaller airways, the thin layer of smooth muscle completely encircles the airway and the airway contains no cartilage or glands (Mariassy 1992). Mucosal and submucosal efferent innervation is provided by parasympathetic nerves to paratracheal ganglia before synapsing in airway smooth muscle, vasculature, submucosal glands, and epithelium; sympathetic neurons synapse in cervical and thoracic ganglia (Finkbeiner and Widdicombe 1992). The sympathetic and predominantly cholinergic parasympathetic fibers intermingle in the respiratory tract.

Adventia

Beneath the submucosa lies loose connective tissue, the adventia. This is part of the bronchovascular adventitia that surrounds airways and adjacent blood vessels.

AIRWAY MUCUS LINING AND THE MUCOCILIARY SYSTEM

Each day, approximately 2ml of fluid per kilogram of body weight is secreted in cats and rabbit—double in humans—for an estimated total resting secretion of 24 ml per day (Finkbeiner and Widdicombe 1992); (Wanner, Salathe et al. 1996). During chronic exposure to irritants, increased MC numbers and their increased mucin secretions can easily compromise the mucociliary system resulting in mucus trapping and obstruction of the airways, particularly if there is bronchospasm of the airway. This is especially the case in small non-cartilaginous airways where MC are present but coughing cannot adequately eliminate secretions. Therefore, removal of mucus relies on the mucociliary clearance system. Thus, in this instance, mucus ceases to have a protective function for the epithelium and instead takes on a pathophysiological role.

The main contribution of the respiratory epithelium to protection of the internal *milieu* from the external atmosphere is attributable to its ability to secrete a thin lining of fluid (average 5 μm in rats and 8 μm in humans) onto the surface of the epithelium (Hatch 1992). In 1934, Lucas and Douglas first proposed the notion that mucus contains two layers (Lucas and Douglas 1934; Wolff 1992; Wanner, Salathe et al. 1996). In some animals, including the horse, there is a mucous (gel) layer and a periciliary (sol) layer collectively forming the mucous or fluid lining. There may also be a thin layer of surfactant separating the two layers (Wanner, Salathe et al. 1996; Rubin 2002).

Having two layers of mucus makes conceptual sense: the effective stroke of the ciliary beat takes place in a mucus-free solution, the periciliary fluid, which has a depth approximately equal to the cilia length. This would permit the mechanical coupling of the cilia claws with the mucus. Simplified, it would seem that too thick of a periciliary fluid would prohibit cilia interactions with the mucus. Likewise, too thin of a sol layer would bring the mucus layer over

the cilia tips and inhibit ciliary movement through both effective and recovery strokes (Wanner, Salathe et al. 1996).

For cilia to move, mucus must be present (Eliezer, Sade et al. 1970; Wanner, Salathe et al. 1996). However, just because mucus is present, does not mean that cilia will move. Spungin and Silberberg (1984) experimentally demonstrated that tactile stimulation, like that provided by entrapment of a particle, would restart ciliary beat that was formerly in a resting or unstimulated state, if the epithelium is not deprived of mucus. The fluid moved by other cilia will influence the beat frequency of the next individual cilia. This phenomenon synchronizes the strokes of neighboring cilia, thereby minimizing the resistance of the mucus (Wolff 1992).

Clearance of mucus has been described as being "biphasic," having a fast "effective" stroke and slow "recovery" stroke in the return direction (Wanner, Salathe et al. 1996). During its recovery stroke, the cilium swings almost 180 degrees backwards closer to the cell surface, then fully extends until perpendicular to the cell surface throughout the effective stroke at a maximum velocitly of 1 mm/sec (Wanner, Salathe et al. 1996). The cilium rests shortly at the end of the cycle, before resuming the next recovery stroke. The recovery stroke is two to three times slower and does not drive the mucus; this provides propulsion in the direction of the effective stroke. Furthermore, the roots of the cilia are organizes such that the effective strokes are all in the same directions (Wolff 1992; Wanner, Salathe et al. 1996). Mucus is not likely to be uniformly moved in a "blanket" (Wolff 1992; Wanner, Salathe et al. 1996). Rather it is more likely to move in "plaques" that were produced in response to the particles first impact on the epithelium (Spungin and Silberberg 1984).

Ciliary beat frequency plays a role in mucus clearance. In the central airways, the ciliary beat frequencies ranges between 10 and 15 Hz in many mammals (Wanner, Salathe et al. 1996). Ciliary beat frequency of upper airway epithelium is modulated through at least two distinct

pathways: (1) the beta 2-adrenergic receptor produces ciliary stimulation by a pathway involving increased intracellular cAMP levels, and (2) the muscarinic receptor increases ciliary beat frequency by a mechanism involving production of prostaglandins, nitric oxide, and cGMP (Yang, Schlosser et al. 1996). Additionally, pressure (Calvet, Verra et al. 1999), PKA and PKG (Wyatt, Spurzem et al. 1998), and histamine (Schuil, van Gelder et al. 1994) have been shown to regulate ciliary beat frequency.

The highest mucus velocity is in the upper airways, and in each increasing generation, the velocity decreases. In the trachea, mucus travels at a velocity of 4 to 20 millimeters per minute (Willoughby, Ecker et al. 1991; Wanner, Salathe et al. 1996). Mucus velocity decreases in peripheral airways because there are fewer, shorter cilia, with a slower beat frequency. Additionally, MC numbers decrease with increasing generation until absent in the terminal bronchioles.

The composition of the mucus predicts its effectiveness in the entrapment and removal of particulates during invasive challenges. The airway secretions have both viscous and elastic properties to trap airborne particles and transport them up to the pharynx to be swallowed; the effectiveness of the interactions between cilia and secretions is determined by viscoelasticity of mucus. The thin fluid mucus lining in the nasal passage, trachea, and conducting airways is an aqueous solution containing high molecular weight mucous glycoproteins called mucins, and a suspension of proteins, lipids, and glycoconjugate components.

Mucus is elastic because it deforms with an applied force and that it can store energy, but because permanent deformation does not occur, mucus is not perfectly elastic (Wolff 1992). In the same respect, the viscous properties of mucus are that it flows when force is applied to it. Likewise, viscosity fails as the mucous layer gets thinner with an increased applied force (Wolff

1992; Wanner, Salathe et al. 1996). Taken together, the transport of mucus is indirectly proportional to its viscosity and directly proportional to its elasticity (Wanner, Salathe et al. 1996).

Mucus viscoelasticity is the main controlling factor of mucus transportability by cilia, rather than its biochemical characteristics. With each applied force to the mucus, viscosity decreases giving it a relaxation time up to 30 seconds long, enough time to trap and retain foreign particles (Wanner, Salathe et al. 1996). However, because the effective stroke of cilia is much faster than the recovery stroke, the cilia encounter mucus in a more solid state having a higher viscosity than if it were having a shearing force applied to it. Experiments done in 1977-1978 showed that the fastest transport rate was mucus with high elasticity and low viscosity (Shih, Litt et al. 1977). In chronic bronchitis, alternately, viscosity is the controlling factor, as elasticity does not change (Wolff 1992). Thus, decreased clearance (mucokinesis) is due to decreased mucociliary transport. In addition to rheological properties, the surface tension, thread-forming ability, and thickness of the mucus play a role in its movement (Wanner, Salathe et al. 1996; Wolff 1992).

Other factors that play a role in clearance include environmental factors, temperature and humidity, mechanical stimulation (e.g. particle deposition), pharmacological agents, infectious diseases, and chronic inflammation, which leads to MC hyperplasia and metaplasia. Particle deposition may promote gel layer formation (Art 2002). While beta-agonists, xanthine, and cholinergic agents increase ciliary activity, anesthetics cause it to decrease. Xylazine and detomidine hydrochloride given to normal horses cause tracheal clearance rates to decrease significantly, ranging from 18 to 54% (Willoughby, Ecker et al. 1991). The effects of mucolytics and expectorants have only been tested in humans (Art 2002). Bacterial diseases and purulent secretions impair mucociliary functions. Respiratory viral infections target the epithelial cells of

the lung producing desquamation, microvascular dilatation, edema, and an inflammatory cell infiltrate (Hogg 2000). Interactions between infectious agents modify the infections of a single agent, where viral infections pave the way for bacterial agents. Combined viral and bacterial infections, acting in synergy, aggravates clinical diseases by impairing the ciliary function and promoting mucus secretion (Degre 1986).

Mucus Constituents

Mucus is about 95% water and the remaining 5% is proteins, lipids, and glycoconjugate components combined. The main role of water is regulating the depth of the sol layer bathing the cilia. The important water sources are air condensation during expiration and osmotic secretion from the plasma whereas removal is through reabsorption, concentration and evaporation (Hatch 1992).

Mucins

High molecular weight glycoconjugates, or mucins, are rapidly secreted and, despite accounting for less than 2% of the wet weight, serve as a MC marker. The colocalization of the special stains alcian blue/periodic acid-Schiff (AB/PAS) indicates specific mucin components, specifically, AB stains the acidic radicals and PAS stains the neutral glycoproteins.

Mucins are 70% to 80% carbohydrates, 20% protein, and 1% to 2% sulfate with attached oligosaccharide side chains (Rubin 2002). Mucins are extremely polyanionic at a neutral pH due to sulfation of oligosaccharide and terminal sialic acid (Wanner, Salathe et al. 1996). The core of the mucin, called an apoprotein, is a single poylpetide chain (Wolff 1992) with extensive tandemly repeated amino acid sequences, largely threonine and serine. Mucins link together via disulfide bonds in an end-to-end fashion to form molecules 0.5 to 6 μm in length (Wanner, Salathe et al. 1996).

Other Proteins

Proteins in the sputum account for only 0.1% to 0.5% (Wolff 1992) of the total volume and, for the most part, are secreted directly from the plasma through tight junctions, or to a lesser extent, co-packaged with other secretions (Hatch 1992). Reabsorption and elimination of proteins is achieved through a type of endocytosis called transcytosis, which may be selective (Hatch 1992). In dogs, mucus protein concentrations are increased during pharmacological stimulation and this increased viscosity of the gel layer (Hatch 1992). The most common proteins are albumins, immunoglobulin A, lysozyme, and lactoferrin.

Carbohydrates

Carbohydrates comprise 0.2% to 3 % of the wet weight of mucus (Hatch 1992; Rogers 2003) and are the major contributors to the viscous and elastic properties (Hatch 1992; Jefcoat 2002; Rogers 2003). Specifically, the high molecular weight glycoconjugates modify the viscoelastic properties by forming a polymer matrix with strong affinity to water and positively charged ions and proteins. There are three enzymes required for biosynthesis and elimination is through ciliary action and lysozomal activity in the mucous gel. *De novo* synthesis of carbohydrates occurs in MC and submucosal glands, the latter contributes the larger percentage of airway secretion (Hatch 1992).

Lipids

Lipids represent 0.3% to 0.5% in the sol phase (Wolff 1992). They occur in saliva and synovial fluid and therefore are normal in the mucus. However, lipids collected in bronchoalveolar lavage fluid poorly serve as an indicator of mucus quantity as most lipids are from the surfactant. However, damaged epithelium releases products such as phospholipids into the airway fluid that could be an indicator of bacterial infection (Widdicombe 1995).

DNA and Actin Polymers

DNA and actin are also present in insignificant amounts normally, but become significant during inflammation (Wanner, Salathe et al. 1996) as they increase mucus viscoelasticity (Gerber, King et al. 2000). These constituents mostly arise from cellular debris such as bacteria, luminal leukocytes, and epithelial cells.

Human periciliary fluid is slightly hypoosmotic and acidic, contains les sodium and chloride, and more potassium than plasma with the salt and pH levels determining hydration of the mucus (Wanner, Salathe et al. 1996). In obstructive airway diseases, or bronchitis, and particularly during inflammatory and infectious episodes, mucus dehydration is associated with an increase in secreted molecules and with marked augmentation of DNA content. This in turn increases viscoelasticity of the surface mucus (Puchelle, de Bentzmann et al. 1995).

Purpose of Mucus

Three main functions of mucus are: (1) clearance of inhaled particulates, (2), providing a chemical screen and biological barrier and (3) protection of the lung from dehydration. The mucociliary activity is a result of the sol and gel layers.

The proteins and lipids in the mucus—acting independently or in combination—inactivate bacterial, viral, and fungal pathogens. Chemically reactive airborne pollutants undergo reactions with antioxidants in mucus. Phagocytosis by macrophages in conjunction with the mucociliary escalator and coughing are most important mechanisms for clearance of pathogens and particulates.

Prevention of dehydration relies on the maintenance of ionic composition of the mucus layer, via the active transport of ions and water in the ciliated cells, and regulation of the rate of mucin secretion in the goblet cells (Hatch 1992). Ciliated epithelial cells appear to be involved in

the secretion and absorption of ions and water into and out of the airway surface fluid (Hatch 1992).

Mucus Synthesis and Secretion

There are 19 mucin genes in humans (MUC1 through MUC19), at least eight of which are expressed in the respiratory tract. Of these, MUC2, MUC5AC (major MC mucin), and MUC5B (major submucosal gland mucin, also in goblet cells) are the main gel-forming mucins secreted in both normal and diseased airways of rats and humans (Jefcoat 2002; Gerber, Robinson et al. 2003; Rogers 2003). Equine homologues have been identified for MUC5AC and MUC2 (eqMUC5AC and eqMUC2, respectively); while eqMUC5AC expression is high, eqMUC2 has not been detected in equine lungs (Gerber, Robinson et al. 2003). No MUC5B homologue has been found in the horse, possibly due to fewer numbers of submucosal glands relative to human lung.

Mucin molecules are assembled within the extensive Golgi apparatus of the goblet cells. After the core mucin apoprotein is assembled, glycosylation and scaffold formation occur—hydroxy ions binding to threonine and serine—to allow binding of positively charged, acidic oligosaccharide side chains (Wolff 1992; Jefcoat 2002). The mucous glycoproteins influence the viscoelasticity of the mucus layer (Majima, Harada et al. 1999).

Once synthesized, the mucins are tightly packaged into granules. Mucin molecules are polyanionic so that upon secretion, there is rapid and massive expansion. However, in order to first be tightly packaged into granules, this biochemical restriction must be overcome. These repulsive forces are overcome by interlacing a positively charged ion amongst the negative mucin polymer matrix (Nguyen, Chin et al. 1998; Rogers 2003). This arrangement gives the mucin "ion-exchange properties" (Nguyen, Chin et al. 1998) to allow for rapid exocytosis of the granule

contents into the airway lumen; exocytosis of one mucin granule takes less than 100 milliseconds (Rogers 2003) and it will expand up to 600-fold in the airway lumen (Rogers 1994).

Since mucus is required for ciliary beat, it is possible that ciliated cells can specifically or nonspecifically signal mucus secretion. While the former is unlikely (Wanner, Salathe et al. 1996), airway epithelial cells *are* capable of producing many substances shown to modulate mucus secretion in a nonspecific manner. Extensive studies have investigated these substances, including, nitric oxide (Runer and Lindberg 1999; Jain, Rubinstein et al. 1993), prostaglandins (Gayner and McCaffrey 1998), and leukotriene C_4 (Schuil, van Gelder et al. 1994).

Newman, Robichaud et al. (1996) categorized the stage or characteristics of exocytosis into three types that have been used in subsequent studies (Rogers 2003). Simple exocytosis is "constitutive secretion" in which a single granule fuses with the plasma membrane and is secreted. Compound exocytosis is due to fusion of membranes of multiple granules intracellularly and with the plasma membrane soon after. Apocrine-like secretion is the loss of the central apical granules *and* some components of the cytoplasm.

The secretory process relies on synergistic events of concurrent activation of both protein kinase C (PKC) and cyclic GMP-dependent protein kinase G (PKG) and translocation of intracellular granule to the internal surface of the apical membrane (Jefcoat 2002; Rogers 2003). The key regulator mediating this dynamic process is myristoylated alanine-rich C kinase substrate (MARCKS) (Li, Martin et al. 2001). Binding of a mucin secretagogue to its receptor initiates the sequence of events illustrated in Figure 1 (Li, Martin et al. 2001).

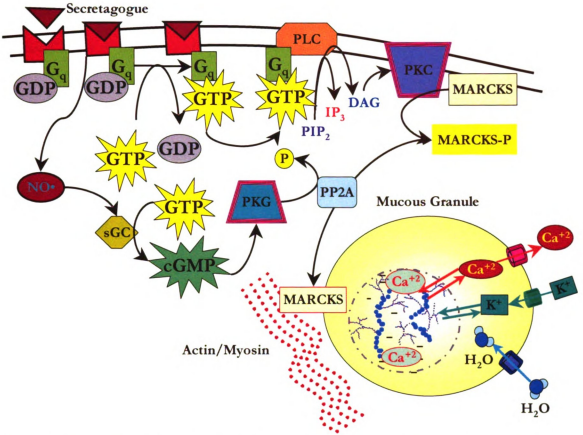

Figure 1. Mucous Granule Movement and Exocytosis. Binding of a mucin secretagogue to its receptor ultimately activates both PKC and PKG; dual activation is required for a robust secretory response. The ligand-receptor complex activates phospholipase C (PLC), which results in the production of inositol triphophste (IP_3) and diacylglycerol (DAG), an activator of PKC. Increases in IP_3 stimulates calcium-release from the intracellular stores (i.e. mucin polymer matrix and intracellular free-calcium). Activated PKC phosphorylates membrane-bound myristoylated alanine-rich C kinase substrate (MARCKS), leading to its subsequent translocation into the cytoplasm. The membrane association of MARCKS is phosphorylation dependent; in a phosphorylated state MARCKS is cytoplasmic while in a dephosphorylated state MARCKS is membrane-bound. At the same time, activation of PKG, via the nitric oxide-cyclic GMP pathway, activates cytoplasmic protein phosphatase-2A (PP2A), which dephosphorylates MARCKS. After dephosphorylation by PP2A, MARCKS recovers its membrane-binding abilities and becomes stabilized on membranes of cytoplasmic mucin granules. Simultaneously, MARCKS interacts with actin and myosin, acting as a tether, linking the granules to the intracellular contractile apparatus mediating granule movement to the apical membrane and consequently exocytotic release. (NOTE: Images in this thesis are presented in color).

After docking of the granule to periphery of the cell, it must go through a priming process before opening and releasing its mucins onto the airway epithelium (Figure 2) (Rogers 2003). After the initiation of exocytosis, there is an efflux of calcium from the mucin matrix via a Ca^{+2}/K^{+} exchanger on granule membrane (Rogers 2003). As mentioned above, intragranular calcium, sequestered in the mucin polymer matrix serves as one calcium

extracellular sodium. There may also be a free cystolic calcium pool (Nguyen, Chin et al. 1998). Nguyen, Chin et al. 1998 devised a model to illustrate the complex mechanisms involved in the storage and release of intracellular calcium from these two probable stores (Nguyen, Chin et al. 1998). The steps are described in Figure 2. The calcium efflux creates an "electrostatic repulsion", due to the high number of anions, that rapidly expands the mucin polymer matrix (Rogers 2003). Further enhancement for the expulsion of mucins is the simultaneous uptake of water and "hydration" of the mucus. This process is likened to a Jack-in-the-box requiring no more than 100 milliseconds to complete, and mucus expands up to 600-fold due to hydration (Wanner, Salathe et al. 1996; Rogers 2003).

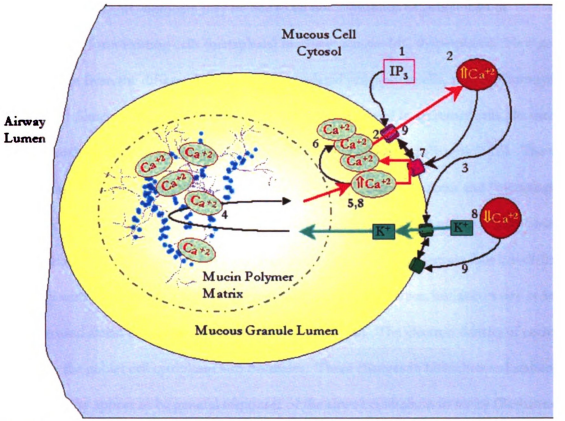

Figure 2. Mucin Granule–Cytosol Calcium Exchange Dynamics and Exocytosis. (1) IP_3 binds to IP_3-R channel and (2) there is release of resting, free calcium from the granule lumen into cytoplasm and calcium in cytosol increases. (3) Localized icreases in cytosol [calcium] trigger opening of a calcium-activated potassium channel and potassium influx that maintains electroneutrality and (4) mobilizes lumenal matrix-bound calcium via Ca^{+2}/K^{+} ion exchange (also increasing the calcium diffusion gradient for release). (5) Calcium increases in the granule lumen (6) promote calcium efflux, which increases cytosolic [calcium] and decreases luminal [calcium]. (7) Large increases in calcium concentration in cytosol cause inactivation of IP_3-R while (8) the calcium-activated potassium channel remains open, luminal [calcium] rises and cytosolic [calcium] decreases (via diffusion or buffering). (9) The calcium-activated potassium channel is inactivated and the IP_3-R is reactivated for new cycle (Nguyen, Chin et al. 1998). (NOTE: Images in this thesis are presented in color).

Mucous Cell Metaplasia/Goblet Cell Hyperplasia

After either acute or chronic exposure to an environmental challenge, there is airway remodeling, characterized by thickening of the mucosa, increased bronchial vascularity, and hypertrophy of the smooth muscle (Rogers 2002). The plasticity of the epithelium allows for an increase in the number of epithelial cells and/or replacement of large patches of mucociliary epithelium by metaplastic MC given its ability to proliferate, differentiate, and change the proportions of specific cell (Basbaum and Jany 1990).

Increases in MC numbers may be due to de novo synthesis of goblet cells or transformation form existing cells (metaplasia) resulting in more MC (hyperplasia). New goblet cells may arise from the differentiation of non-granulated progenitor cells, and less from goblet cell division. Since no increase in mitotic rates has been observed in secretory cells, the increase in mucous cell numbers is attributed to hyperplasia and/or metaplasia (Rogers 2003). The term "hyperplasia" is usually reserved for large airways where MC normally occur and "metaplasia" is specific to the origin of MC in small airways less than 2 mm in diameter where MC are absent in healthy lungs. This is not to say that metaplasia cannot occur in large airways, but it is difficult to identify with confidence. Mucous cells increase not only in number, but also in size as seen from increased alcian blue-staining (Basbaum and Jany 1990). The electron density of secretory granules in the goblet cell cytoplasm also decreases. These changes in histochemical staining and electron density appear to be general responses of the airway epithelium to injury (Basbaum and Jany 1990).

The progenitor cells—serous, basal, and Clara cells—have the capacity to divide followed by differentiation into "mature" ciliated or secretory cells. Some investigators hypothesize that Clara and serous cells differentiate into goblet cells. This is because non-ciliated, electron lucent mucus granule-containing cells display many similarities as Clara cells (Hayashi, Ishii et al. 2004). While others claim that basal cells are progenitors for MC after injury, which in turn are progenitors for ciliated cells. When MC are absent, as in the peripheral airways, Clara cells serve as progenitor cells for ciliated cells (Wanner, Salathe et al. 1996). However, it is unclear if these 'new' cells maintain a Clara cell disposition or if they become genuine MC.

Airway Repair and Regeneration

Under normal conditions, mucosal cell turnover is very slow resulting less than 1% of all epithelial cells turning over in a 24 hour period (Wanner, Salathe et al. 1996). Within 12 hours after minor injury, this process speeds up considerably so that in approximately 2 weeks a highly differentiated epithelium is restored (Wanner, Salathe et al. 1996).

When 90 percent of epithelium is removed from chicken trachea, mucociliary function is restored in 14 days and complete epithelial restoration in 30 days (Battista, Denine et al. 1972). Half of the mucociliary activity can be restored by regeneration of only 10 percent of ciliated cells, suggesting a functional reserve for the mucociliary apparatus (Wanner, Salathe et al. 1996).

Tesfaigzi (2003) categorized the airway epithelial recovery process into five steps: (1) epithelial cells migrate to the damaged area within minutes; (2) epithelial cells begin the processes of proliferation and differentiation to replace injured epithelial cells and to establish squamous or MC metaplasia, respectively; (3) apoptosis of some cells occurs in order to allow restoration of the epithelium by the newly proliferated epithelial cells; (4) once reduction of cell numbers occurs, to that of an unexposed epithelium, normal proportions of cell types are restored; (5) epithelial cells can develop a memory for chronic exposure. This last point is important because it permits a quick response to minimize further injury (Tesfaigzi 2003).

There appears to be a Th1 cytokine profile involved in the resolution of mucous cell metaplasia (MCM) with IFN-γ crucial for induction of MC apoptosis (O-Quan Shi, Fischer et al. 2002). IFN-γ induces Fas-mediated apoptosis in airway epithelial cells (Wen, Madani et al. 1997), probably by increasing the expression of Bax, a pro-apoptotic member of the Bcl-2 family of proteins (Tesfaigzi, Fischer et al. 2002). It is unclear if 1) only newly formed cells express Bax, or 2) if pre-existing cells can also undergo apoptosis during the resolution of MCM (Tesfaigzi, Fischer et al. 2002).

PHYSIOLOGICAL CELL DEATH: PROGRAMMED SUICIDE OR NECROSIS

Life is defined by death. Death at a cellular level plays many roles having widespread biological significance in defense, differentiation and development, proliferation/homeostasis, and aging. Cell death occurs through two courses of action, one physiological (apoptosis) and the other accidental (necrosis), each having distinguishing features. The relative rates of these processes determines whether the cell undergoes apoptosis or necrosis as the primary means of removal (Kroemer, Dallaporta et al. 1998). According to Kroemer et al (1998), both methods go through a two-step process involving the mitochondria (Figure 3).

The morphological characteristics of apoptosis are caused by activation of cysteine proteases, termed caspases, which leads to chromatin condensation, nuclear fragmentation (pyknosis), cytoplasmic blebbing, cell shrinkage, and breakup into small membrane-bound vesicles called apoptotic bodies (Reed 2000). Release of these apoptotic bodies gave this form of programmed cell death its name; "apoptosis" is from the Greek, meaning "to fall away from" (Reed 2000). The apoptotic bodies express phosphotidylserine making them recognizable for phagocytosis. Because they are phagocytized and there is no release of cellular contents, there is no inflammation.

There is evidence for an alternative nonapoptotic programmed cell death pathway ('paraptosis) that does not fulfill morphological criteria of apoptosis; however, since it requires gene expression, it is not necrosis either (Sperandio, de Belle et al. 2000).

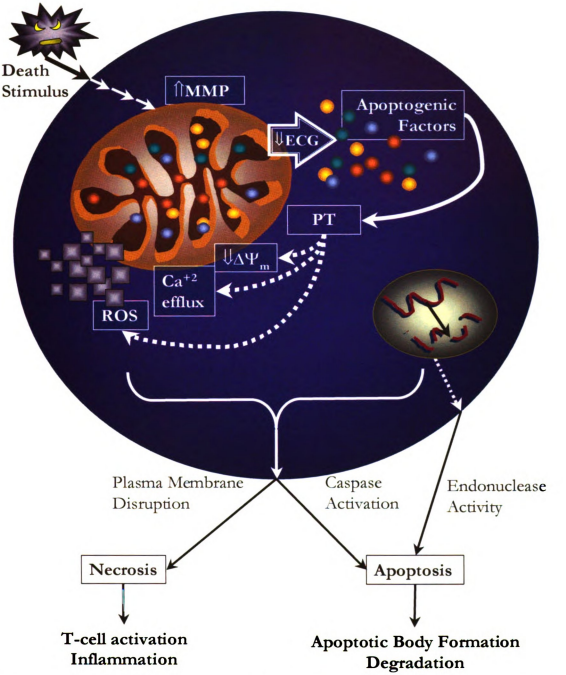

Figure 3. The Mitochondrial Regulator: "Bioenergetic Catastrophe" Or Subtle Disappearance? Stage 1(solid arrows): a multitude of stimuli set off a signal transduction pathway to the mitochondrial membrane, increasing its permeability. This mitochondrial membrane permeabilization (MMP) releases sequestered apoptogenic factors (including endonucleases) into the cytosol and abolishes the electrochemical gradient (ECG) in the membrane, leading to mitochondrial dysfunction and the second stage (dotted arrows). Organellar breakdown leads to loss of plasma membrane integrity (necrosis) and/or activation of proteases and endonucleases (apoptosis). These consequences, indicated by dotted arrows, include: (a) collapse of mitochondrial inner transmembrane potential ($\Delta\Psi_m$), (b) calcium efflux, (c) increased reactive oxygen species (ROS), indicative of uncoupling of electron transport chain. Additionally, caspase-activating proteins are released from the intermitochondrial space. (NOTE: Images in this thesis are presented in color).

NECROSIS

Death by necrosis is un-programmed and occurs in acute, non-physiological injury (Hetts 1998). Cells passively (i.e. energy is not required) take up extracellular fluid, swell, and undergo lysis releasing their cytoplasmic and nuclear contents (Hetts 1998). This release results in an inflammatory response involving maturation of dendritic cells into antigen presenting cells to stimulate $CD4^+$ T helper cells and $CD8^+$ cytotoxic T lympthocytes (Schultz and Harrington 2003). Although important in acute injury and certain inflammatory responses, this is not the normal mechanism for cell death (Hetts 1998).

APOPTOSIS

Apoptosis is a highly organized process that has been evolutionarily conserved from worms to mammals. In contrast to necrotic cell death, apoptosis is a non-inflammatory, energy-dependent form of genetically programmed cell death (Hetts 1998). It occurs under normal, physiological circumstances where cells commit suicide 'for the good of the organism,' although apoptosis also can play a primary or secondary role in pathological states.

Defects in programmed cell death can lead to disease, either with cell accumulations, as in cancer or restenosis, or with an excess loss of cells, seen in a stroke, heart failure, AIDS, or neurodegeneration (Reed 2000). Specific stimuli induce apoptosis, necrosis occurs instead because there is failure by the cells to maintain homeostasis following damage (Schultz and Harrington 2003).

The apoptotic process is divided into four phases. In the first phase, the cell receives a stimulus and "decides" whether or not to commit. This stimulus provokes an apoptotic response which ultimately leads to activation of the effectors: cysteine-dependent aspartate-

specific proteases called capsases (Vaux and Strasser 1996). The signal can be external such as ligation of surface receptors, lack of survival signals, contradictory cell cycle signals, and developmental death signals; the signal may also be internal from drugs, toxins, or radiation that may cause DNA damage due to a defect in repair mechanisms, or cytotoxic drug treatments (Vaux and Strasser 1996).

After stimulus detection, signal transduction pathways reach death effector machinery to begin the second phase. Many components of the apoptotic machinery are ready and waiting for activation and, for this reason, tight regulation and multiple checkpoints ensure appropriate execution. At this point, in the "execution phase", activation of proteases and positive and negative regulators occurs (Vaux and Strasser 1996). Apoptosis is carried out by caspases; these proteases are ready in the cytosol as precursors called pro-caspases. A death signal leads to proteolytic cleavage of pro-caspases to release the active caspase, which then cleaves the next pro-caspase to release its active caspase in a caspase cascade. Completion of this pathway depends on the ratios of positive and negative regulators.

Finally, in the postmortem phase, morphological characteristics appear (chromatin condensation, DNA degradation) and apoptotic bodies are formed. Changes in the makeup of cell surface proteins and lipids occur, specifically, externalization of phosphatidylserine, to flag the apoptotic bodies for phagocystosis is the earliest detectable morphological event (Liu, Brouha et al. 2004). After phagocytosis, lysosomes degrade the packaged bodies.

Caenorhabditis elegans-A Genetic Model of Cell Death

It was not until the late 1980s to early 1990s that cell death was hypothesized to be a normal fate during development. This idea developed through study of *C. elegans* and finding that not only is apoptosis a normal process, but it is a very precise one at that. During this

nematode's development, specific genes are activated to kill exactly 131 cells and leave 959 cells (Hetts 1998). These and subsequent studies have provided the basics for apoptosis in higher multicellular animals, as the homology is widespread, in the mechanisms and the molecules. Genes discovered in *C. elegans* are extremely conserved from worm to humans (Figure 4). The four sequential steps of apoptosis—commitment, signal transduction, execution, and phagocytosis and degradation—have been described using *C. elegans.*

The protein products of the ced-3 gene implement the execution of apoptosis. These are cysteine aspartyl proteases (caspases) that are activated after cleavage behind a conserved aspartate and go on to cleave their substrates, including polyadenosine diphosphate ribose polymerase, components of nuclear membrane, and endonucleases (responsible for cleaving the apoptotic cell's DNA) (Reed 2000). Mutations of *ced-3* gene block all developmentally programmed cell deaths (Yuan, Shaham et al. 1993); the animals age normally with normal life-spans and no apoptosis (Vaux and Strasser 1996). This would suggest that different processes than those for programmed cell death regulate aging. Perhaps during evolution of multicellular organisms, aging evolved *after* the two lineages (germ and somatic) formed (Vaux and Strasser 1996). Likewise, defense and development mechanisms may have evolved earlier in single-cell organisms.

There are two *ced* (cell death abnormal) genes involved in carrying out apoptosis and one inhibiting it. Upstream to CED-3 is the *ced-4* gene. *Ced-4* receives the death commitment signal, binds to and cleaves pro-CED-3 and releases active CED-3 (Hetts 1998). The antagonist for apoptosis, CED-9, is a multifunctional protein. *Ced-9* can inhibit both *ced-3* and *ced-4*; CED-*9* is localized to the mitochondrial membranes and by binding to CED-4, it prevents the activation of pro-CED-3 by anchoring CED-4 away (Hetts 1998).

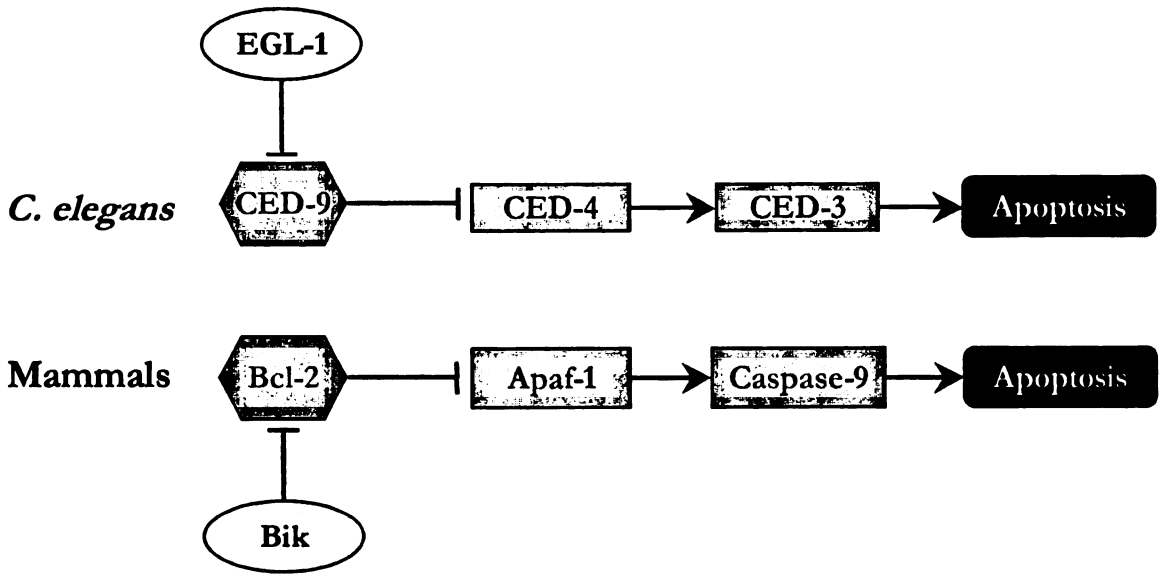

Figure 4. Apoptosis pathways in *C. elegans* and Mammals. Homologies occur between the nematode, *C. elegans*, and mammals in proteins and in mechanism. Ced-4/Apaf-1 binding is required to activate Ced-3/Caspase-9 for apoptosis to occur. Ced-9/Bcl-2 interacts with Ced-4/Apaf-1 and sequesters it away from Ced-3/Caspase-9, thus inhibiting apoptosis. Egl-1/Bik binds to and inhibits Ced-4/Apaf-1. (modified from Adams and Cory 1998).

Cell Death in Higher Mammals

Studies of cell death in higher mammals showed that commitment to death occurs through some ubiquitous pathways but many times, the pathways are specific for cell type, stimulus, or even time (Hetts 1998). It is far more beneficial for an organism to have many apoptotic pathways than a single one; if only one pathway was present, all the cells would die from a given signal. Fortunately, with the array of triggers that are present in the environment, only a specific subset of the cells undergo programmed cell death while other are spared (Hetts 1998). These mechanisms for survival (or death) allow for independent regulation of pathways that converge at a common endpoint, namely the caspase cascade and morphological characteristics of apoptosis (Vaux and Strasser 1996).

Several differences between the worm and mammals are present. In the worm, Ced-4 binds directly to Ced-3, but in mammals, Apaf-1 (Apoptotic Protease Activating Factor-1) cannot directly bind to caspase-9. It requires a co-factor to mediate Apaf-1-caspase-9 interactions, identified as cytochrome c. The second variation concerns Bcl-2 and its

involvement as a death suppressor. Although in the worm model Ced-9 binds to Ced-4, Bcl-2 has not been shown to bind directly to Apaf-1 (Moriishi, Huang et al. 1999). Instead, its main function may be through indirect constraints via prevention of mitochondrial release of cytochrome c (Kluck, Bossy-Wetzel et al. 1997; Yang, Liu et al. 1997). This is still up for debate as Bcl-2 is seen to retain its pro-survival activity even with cytochrome c in the cytosol (Brustugun, Fladmark et al. 1998).

Even with such great similarities, many pathways exist for commitment to death and many components of "the executioner" exist (Hetts 1998). Although all cells have the capacity to undergo cellular suicide, not all are susceptible (Chao and Korsmeyer 1998). A cell's apoptotic threshold depends on the ratio of positive and negative regulators.

There are two principal pathways for the initiation of apoptosis. One is the 'intrinsic' pathway controlled by the mitochondria and the Bcl-2 family of proteins to regulate activation of caspase-9. The second pathway is the 'extrinsic' pathway mediated by death receptors belonging to the tumor necrosis factor (TNF) family and CD95 (also known as Fas or APO-1) and the resultant activation of caspase-8. Although initiated by separate signals, one at the plasma membrane (extrinsic) and the other at an organelle (intrinsic) level, crosstalk occurs and the two pathways converge for a common outcome: activation of downstream proteases termed caspases.

Caspases

Caspases are cysteine aspartyl-specific proteases that are responsible for the morphological characteristics associated with apoptosis; they are the conserved downstream machinery that carries out the execution phase of programmed cell death. Caspase-1 or ICE (IL-1β-Converting Enzyme), was the first homologue of Ced-3 to be identified in mammals

(Miura, Zhu et al. 1993). Regardless of pathway of activation or trigger, the same endpoint is reached at a caspase cascade. Caspases are present in most, if not all, nucleated cells (Hetts 1998).

Caspases are synthesized in precursor form of zymogens, called pro-caspases, containing a large (p10) N-terminal prodomain and a small (p20) catalytic subunit, sometimes separated by a linker peptide (Reed 2000). While both domains are common among caspases, the N-terminal prodomain differs greatly (Sprick and Walczak 2004).

There at least 14 caspases in mammals and these form two fundamentally different subgroups based on amino acid similarities of the prodomain (Reed 2000). The first group is the upstream, or 'initiator', caspases, characterized by a long N-terminal prodomain, which provide protein interaction modules to allow for recruitment of and interaction with pro-caspases at an activating protein complex. Caspases included in this group are caspases-1, -2, -4, -5, -8, -9, -10, -11, and -12 (caspases-11 and -12 are in mice only, caspases-4 and -5 in humans only). The N-terminal prodomain of caspases-8 and -10 has a death effector domain (DED) and the other caspases listed have N-terminal prodomains with a caspase-activating recruitment domain (CARD) to enable activation of the second group, the downstream 'effector' caspases (Sprick and Walczak 2004). Caspases-1, -4, -5, -11, and -12 are involved in processes other than apoptosis and will not be discussed in further detail. Caspase-2 is activated during cytotoxic stress and is required for the permeabilization of mitochondria and the release of apoptosis-inducing molecules (Lassus, Opitz-Araya et al. 2002). Pro-caspases-8 and -10 are recruited and bind to the adapter protein FADD/MORT1 via their DED regions to complete the formation of the scaffold, called a DISC (Death Inducing Signaling Complex), to which additional pro-caspases can bind to and autocatalytically cleave and activate caspases (Boldin, Goncharov et al. 1996; Muzio, Chinnaiyan et al. 1996). Therefore, caspase-8 is regarded as the 'apical caspase' in

this pathway. Caspase-9 is the apical caspase in the intrinsic pathway and its recruitment to a complex analogous to the DISC forms the apoptosome. Unlike the DISC-activated caspase-8, caspse-9 likely requires the apoptosome to carry out its functions.

The second group of effector caspases, also called executioner caspases, is responsible for the majority of the cellular destruction. These caspases, caspases-3, -6, and -7 have a short N-terminal prodomain having an unknown function (Reed 2000). These caspases depend on the upstream, initiator caspases for proteolytic processing and activation.

Downstream pro-caspases require post-translational activation by an activated initiator caspase. A model for initiator caspase activation has been proposed for each pathway. In the extrinsic pathway, the mechanism for activation has been proposed in the 'induced proximity model' where the DISC serves as a scaffold to which pro-caspases are recruited, cleaved, and the active caspases then cleave other inactive zymogens to release active enzymes in a proteolytic cascade, that has been likened to the complement cascade or blood clotting (Hetts 1998). This '*trans*-processing' model assumes a very important fact: zymogens are not completely inactive; they have weak yet measurable proteolytic activity that is at around 1 percent of fully active enzymes for procaspase-8 (Denault and Salvesen 2002).

A somewhat similar activation pathway to the induced proximity model is described for the intrinsic pathway. After the release of cytochrome c, a caspase-activating protein, from the mitochondria, it binds to ATP or dATP, with Apaf-1 and pro-caspase-9 to form the so-called apoptosome. Procaspase-9 is a monomer and its dimerization leads to its release of caspase-9 in much the same manner as caspase-8 in the extrinsic pathway (Denault and Salvesen 2002). Caspase-9 goes on to activate the effector pro-caspases-3, -6, and -7 to amplify the death signal.

Activation of executioner caspases occurs in a two-step mechanism. First, proteolysis separates the large and small subunits; this is normally carried out by an active initiator caspase (i.e. DISC-activated caspase-8 or apoptosome-activated caspase-9) (Sprick and Walczak 2004). Even after this initial cleavage step, the enzyme is still inhibited by its own prodomain. In the second, autocatalytic step, the prodomain is removed and the active effector caspase is ready.

The Mitochondria-Associated (Intrinsic) Pathway and Bcl-2

A role for mitochondria in apoptosis was first illustrated in *Xenopus* oocyte extracts where apoptosis required a membrane fraction that was enriched with mitochondria (Newmeyer, Farschon et al. 1994). Induction of apoptosis by the mitochondria-dependent pathway can be in response to many stimuli including growth-factor withdrawal, irradiation, *myc* overexpression, hypoxia, UV radiation, chemotherapeutic agents, oxidants, and calcium overload. A lack of exogenous signals causes activation of an endogenous default death program, the intrinsic pathway. Proteins associated with the outer mitochondrial membrane (OMM) regulate cytochrome c leakage and its integrity. Release of cytochrome c induces formation of a complex analogous to the DISC of the TNF pathway, the apoptosome, and activation of the most upstream protease, caspase-9 (Li, Nijhawan et al. 1997).

In a similar mechanism to the close proximity model involving the DISC complex, the apoptosome aides in the cleavage of pro-caspase-9. Activated caspase-9 cleaves and activates caspase-3, the first caspase common to both the intrinsic and extrinsic pathways. However, caspase-9 is not released into the cytosol after cleavage (Reed 2000). While Apaf-1 requires a cofactor (i.e. cytochrome c) to bind to pro-caspase-9 (Hengartner 1998), it was shown that pro-caspase-9 is not required for the active complex (Acehan, Jiang et al. 2002). Furthermore, the apoptosome may serve to amplify caspase activity instead of to initiate it because Bcl-2 is shown

to regulate caspase activation independently of the apoptosome (Marsden, O'Connor et al. 2002).

The BCL-2 Family

Bcl-2, isolated from a B-cell lymphoma, was first described in 1984 as an oncogene, on the human chromosome t (14:18), that prolongs cell survival (Pegoraro, Palumbo et al. 1984; Vaux, Cory et al. 1988). Most of these members are associated with the membranes of mitochondria, endoplasmic reticulum, and nucleus, anchored by a stretch of hydrophobic residues near the carboxyl terminal (Schendel, Xie et al. 1997; Reed 2000).

Bcl-2 gene expression may result in an inhibition of cell division cycle progression from the G1 to S phases resulting in a 30%-60% increase in the length of G1 phase (Mazel, Burtrum et al. 1996), although conflicting evidence was seen in rats (Tesfaigzi, Harris et al. 2004). With confidence, it can be said that Bcl-2 proteins either positively or negatively control cell death commitment, independent of caspases, and they regulate the release of cytochrome c (Reed 2000), although the functional mechanisms are unclear. Several theories have been proposed: (1) Bcl-2 binds directly to Apaf-1, sequestering it away from caspases and/or (2) Bcl-2 prevents cytochrome c, as well as other apoptogenic proteins, from escaping the confines of the mitochondria, or (3) both events occur (Hetts 1998).

In the first theory, borrowed from *C. elegans*, Bcl-2 directly controls caspase activation. However, Moriishi, Huang et al (1999) demonstrated that Apaf-1 does not bind to Bcl-2 proteins. The second scenario gives Bcl-2 family members a more central role in which they guard mitochondrial activation. Many groups have found Bcl-2 to protect mitochondrial integrity and control cytochrome c release (Kluck, Bossy-Wetzel et al. 1997; Shimizu, Narita et al. 1999). Likewise, as mentioned above, others argue against this involvement for Bcl-2 (Marsden, O'Connor et al. 2002). There is also some discrepancy whether Bcl-2 and the close

homolog Bcl-x_L may also be able to inhibit Fas-mediated apoptosis. While some groups claim Fas-induced pathways are separate from Bcl-2 regulated pathways (Huang, Hahne et al. 1999; Robinson, Jeffcoat et al. 2002) others disagree (Armstrong, Aja et al. 1996).

Bcl-2 acts both upstream and downstream from cytochrome c; cytochrome c activates caspases, and activated caspases, in turn, stimulate cytochrome c release. In one experiment, Bcl-2 failed to block Bax-induced cytochrome c release, regardless of co-localizing with Bax, and the cells survived with cytochrome c in their cytoplasm showing Bcl-2 working downstream of cytochrome c (Rosse, Olivier et al. 1998). Another group found that Bcl-2 blocked apoptosis induced by injected cytochrome c (Zhivotovsky, Orrenius et al. 1998). Alternatively, Bcl-2 and Bcl-x_L can prevent cytochrome c release from the mitochondria (Kluck, Bossy-Wetzel et al. 1997; Finucane, Bossy-Wetzel et al. 1999); they set a threshold in the amount of cytochrome c required to activate caspases (Cosulich, Savory et al. 1999). The ability of Bcl-2 to act upstream and downstream of cytochrome c allows it to suppress caspase activation at several distinct steps (Cosulich, Savory et al. 1999).

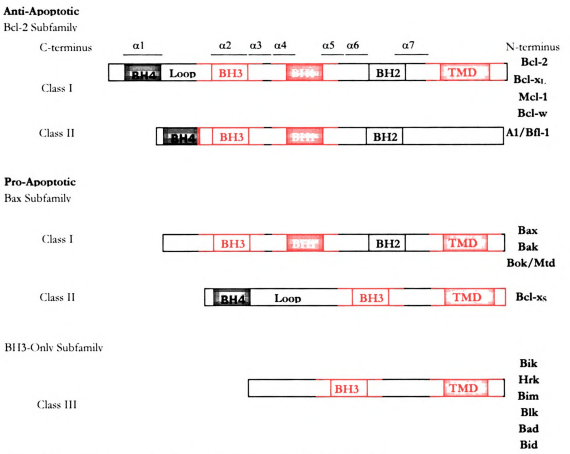

Figure 5. The Bcl-2 Family. (modified from Adams and Cory 1998; Reed 2000).

The Bcl-2 protein family is a large group of more than 20 members categorized by the presence of at least one of four (BH1-BH4) conserved motifs called Bcl-2 homology (BH) domains (Figure 5). There are two functional classes of proteins, those that promote survival and those that promote cell death, and three structural subgroups, the anti-apoptotic Bcl-2-like proteins (the Bcl-2 subfamily), the pro-apoptotic Bcl-2-like proteins (the Bax subfamily) and the pro-apoptotic BH3-only proteins (Table 1). After sub-dividing them in this manner, two facts become evident: (1) the anti-apoptotic proteins contain at least three BH domains—BH1, BH2, and BH3—and (2) the pro-apoptotic protein must have, at minimum, the BH3 domain (Figure 5) (Kelekar and Thompson 1998).

Anti-Apoptotic	Pro-Apoptotic	
Bcl-2 Subfamily	**Bax Subfamily**	**BH3-Only Proteins**
Bcl-2	Bax	Bad
Bcl-x_L	Bak	Bid
Bcl-w	Bok/Mtd	Bik
Mcl-1	Diva	BimL
Bfl-1/A1	Bcl-x_S	Blk
NR-13		Hrk
BHRF1		Bmf
LMW5-HL		Noxa
ORF16		Spike
KS-Bcl-2		Puma
E1B-19K		BNIP3
CED-9		BimL
		EGL-1

Table 1. Categorization of BCL-2 Family Members. Members can be categorized into two groups based on function and three groups based on structure.

Further division of these groups, based on their homology with Bcl-2, places them into two anti-apoptotic and three pro-apoptotic classes (Figure 5). The pro-survival proteins making up class I (Bcl-2, Bcl-x_L, and Mcl-1) contain a loop region and are α-helical pore-like proteins (Reed 2000). Other members of the Bcl-2 subfamily lack the 'loop' domain, putting them into class II. Although, they contain all four BH domains, their functions are different. For instance, the class I Mcl-1 behaves like Bcl-2 in a cytoprotective manner, while the function of the class II protein, A1, remains unknown (Vaux and Strasser 1996).

Of the pro-death proteins, class I contains members of closest relation to Bcl-2 (Bax, Bak, and Mtd/Bok), having BH1, BH2, and BH3 domains and transmembrane domains but lacking the loop regions. These proteins may interact with and antagonize the anti-apoptotic members (via the BH3 domain) or act independently of them. Both classes II and III pro-death proteins must interact with survival proteins to elicit their apoptotic promoting function. Proteins in Class III are members containing only the central short (9 to 16 residue) BH3

domain, and thus called 'BH3-only proteins', act as sentinels over some organelles and processes (Adams and Cory 1998; Sprick and Walczak 2004). Although normally inactive, stimulation causes BH3-only proteins to become activators for pro-apoptotic members, therefore, in order for the BH3-only proteins to act on the mitochondria, Bax and Bak must be present (Sprick and Walczak 2004). However, they are unable to dimerize with pro-apoptotic proteins. BH3-only proteins contain amphipathic α-helix that fits into hydrophobic "crevices" of anti-apoptotic proteins like Bcl-2 and Bcl-x_L (Reed 2000).

Alternative splicing of the Bcl-x results in two distinct bcl-x mRNAs, protein products from which are the longer, anti-apoptotic Bcl-x_L and the shorter, pro-apoptotic Bcl-x_S proteins (Boise, Gonzalez-Garcia et al. 1993). Bcl-x_S, can be placed in its own class II having only BH3 and BH4 and the transmembrane domain, however it has been placed in the Bcl-2 subfamily because it is shares the BH4 domain (Tsujimoto 1998). Bcl-x_S has also been categorized as part of the Bax subfamily (Tsujimoto 1998).

Regulation of Cell Death Machinery

Regulatory mechanisms among Bcl-2 members vary. A recurring theme in apoptosis is the important role protein-protein interactions play and as expected, Bcl-2 proteins are no exception. These interactions are involved in dimerization, sequestration through proteolytic cleavage via caspases, and phoshorylation by kinases.

Pro- and anti-apoptotic Bcl-2 family members can homodimerize and/or heterodimerize and one may titrate the other's function. In this manner, the ratio of inhibitors to activators can establish a cell's susceptibility to death triggers. For instance, heterodimerization of Bax and Bcl-2 allow one to regulate the other's function. Although heterodimerization is not required for survival function, it is for apoptotic function.

Through X-ray crystallography and NMR methods, the structures of Bcl-2 proteins have provided insight into the architectural basis for dimerization (Chittenden, Flemington et al. 1995; Muchmore, Sattler et al. 1996). The three-dimensional structure of Bcl-x_L showed that there are two central hydrophobic α-helices, α5 and α6, surrounded by five amphipathic helices, α1, α2, α3, α4 and α7, arranged so that the BH1, BH2, and BH3 domains are closely juxtaposed, forming a hydrophobic pocket on the surface. This pocket seems to serve a receptor-like function by interacting with the hydrophobic face of the BH3-containing α2 helix of the Bcl-2 protein it needs to bind (Reed 2000). In a monomeric state, the hydrophobic side chains of the amphipathic α2 helix are pointed inward, making these residues unavailable for protein interaction (Sattler, Liang et al. 1997). Thus, structural changes, such as rotation of the α2 helix, may be required before BH3 can take part in dimerization. Such a rotation is a strong possibility based on the prediction that the α2 helix is flanked by highly flexible loops on either end.

There seems to be some degree of selectivity and hierarchy of dimerization. Using yeast-two hybrid systems, Bax homodimers were shown to induce cell death, heterodimerization of Bcl-2 and Bax inhibit death by sequestration of Bax function, and Bcl-x_S binding to Bcl-2 does not allow Bcl-2 to neutralize Bax and cell death occurs (Sato, Hanada et al. 1994). Bcl-2/Bcl-2, Bcl-2/Bax, Bax/Bax, and Bax/Bcl-x_L interactions have been proven in mammalian cells (Sedlak, Oltvai et al. 1995).

Mutagenic studies have identified the BH1, BH2, and BH3 regions to be critical in dimerization and induction of apoptosis. Mutations in BH1 and BH2 that disrupt the repressive functions of Bcl-2 or Bcl-x_L also disrupt their abilities to dimerize with Bax or Bak (Yin, Oltvai et al. 1994; Chittenden, Flemington et al. 1995). Thus, BH1 and BH2 domains are essential for dimerization with pro-apoptotic proteins and the survival function of Bcl-2 and Bcl-x_L.

Although Bax and Bak contain BH1 and BH2, the BH3 domain appears to contribute to their apoptotic function and interactions with pro-survival proteins (Kelekar and Thompson 1998). Bcl-x_S lacks both BH1 and BH2 regions and does not interact with pro-apoptotic members.

Cytosolic members may lack the C-terminal transmembrane domain, as in the BH3-only proteins (Bid, Bim, and Bad), or have a regulatory C-terminal domain (Bax). While Bax is free in the cytosol, its C-terminal transmembrane domain is masked (Nechushtan, Smith et al. 1999) and upon receipt of apoptotic trigger, possibly pH (Khaled, Kim et al. 1999), it undergoes a conformational change, exposing the C- and N-terminals for insertion into the OMM and regulation of cell death machinery.

Proteolytic cleavage by caspases is required for activation of some BH3-only proteins. This is evident in caspase-8-induced translocation of truncated Bid to the mitochondrial membrane. Removal of the N-terminal 52 amino acids exposes a hydrophobic core and the BH3 dimerizing domain, both of which are required for insertion into the OMM (Schendel, Azimov et al. 1999) and mitochondrial leakage. Thus, tBid links the death receptor pathway to the mitochondrial pathway. Other proteins, such as Bim and Bad, are sequestered in the cytoplasm. Bim associates with the dynein light-chain of microtubules; once freed, Bim dimerizes with anti-apoptotic Bcl-2 members via their BH3 domains (Reed 2000).

The phosphorylation state of some members determines their colocalization. In a phosphorylated state, Bad is inactive and is bound to the cytosolic protein 14-3-3, which interacts with signaling enzymes (Nagata 1997). Dephosphorylation of Bad activates it and enables dimerization with Bcl-2 or Bcl-x_L to promote apoptosis. Kinases actively regulating Bcl-2 members include Akt, PKA, Raf1, Rsk1, Pak1 (Reed 2000).

Transcriptional regulation for members varies. Bcl-2 and Bax are under Stat3 and p53 control (Miyashita, Krajewski et al. 1994) while, Bcl-x_L expression partly depends on p53, Stat5,

and NF-κB activation (Merchant, Loney et al. 1996; Tsukahara, Kannagi et al. 1999; Bureau, Vanderplasschen et al. 2002).

OMM Maintenance, Pore Forming Ability, and Mitochondrial Dysfunction

Members of the Bcl-2 family of proteins maintain mitochondrial homeostasis and OMM integrity either directly (e.g. ion-channel forming activity) or indirectly—via OMM proteins like those of the Voltage Dependent Anion Channel (VDAC)—to regulate leakage of cytochrome c and other apoptogenic factors from the mitochondria (Donovan and Cotter 2004). Loss of this results in a permeability transition (PT), loss of membrane potential and swelling, allowing the rapid release of cytochrome c.

Crystal structures of Bcl-x_L and Bcl-2 demonstrate similarities to pore forming bacterial toxins, such as colicins A1 and E1 and diphtheria toxin (Muchmore, Sattler et al. 1996), in the BH1- and BH2-flanked helices α5 and α6, which are speculated to participate in pore formation (Kelekar and Thompson 1998). This indicates survival proteins, as well as Bax and tBid may form ionic pores that allow electrochemical homeostasis in organelles, primarily the mitochondria (Hetts 1998) (Antonsson, Conti et al. 1997; Luo, Budihardjo et al. 1998). Homologous and non-homologous protein-protein interactions are likely to regulate pore formation. For example, interaction with the co-chaperone, survival protein BAG-1 enhances Bcl-2 pore formation, while interactions with Bcl-X_S and Bad interferes with it (Schendel, Xie et al. 1997). In any case, it is unclear if these channels directly control cytochrome c release.

Bax must oligomerize in order to form pores (Antonsson, Montessuit et al. 2000), doing so in a membrane potential- and pH-dependent manner (Antonsson, Conti et al. 1997). Not only do pores formed by Bax abolish Bcl-2 channel formation, they can directly induce cytochrome c release, instead of indirectly by inhibiting regulators of caspases (Jurgensmeier, Xie et al. 1998), and allow the transport of ions or proteins across the membrane, opposite to Bcl-2

(Schendel, Xie et al. 1997). At a neutral pH, Bax channels are mildly cation selective having a permeability ratio of sodium to chloride of 2:1 (Antonsson, Conti et al. 1997) but overall, Bax pores display a much broader range of pH than Bcl-2 channels (Schlesinger, Gross et al. 1997).

The ability of Bcl-2 family members to interact with PT pore (PTP) proteins (e.g. VDAC) on the OMM allows the formation of much larger pores than would otherwise have been allowed with Bcl-2 alone (Reed 1997; Hengartner 2000). Bid, the BH3-only protein that links the death receptors to the membrane pathway, can trigger cytochrome c release in purified mitochondria after cleavage by caspase-8 by closing the VDAC, resulting in decreased metabolite exchange between mitochondria and the cytosol resulting in mitochondrial dysfunction, without swelling and PT (Luo, Budihardjo et al. 1998; Rostovtseva, Antonsson et al. 2004). Although Bax has been shown to induce cytochrome c release, Bid is far more potent in releasing 100% of mitochondrial cytochrome c at a 500-fold lower concentration than Bax, which causes only 20% of the total cytochrome c to be released (Luo, Budihardjo et al. 1998). Bid may also oligomerize with a pro-apoptotic member (Bax) to trigger cytochrome c release and/or inactivate an anti-apoptotic member (Luo, Budihardjo et al. 1998).

Completion of programmed cell death, regardless of stimulus and pathway, relies on disruption of the OMM and the *rapid* release of various caspase-activating factors into the cytosol. Most apoptosis-inducing conditions involve induction of mitochondrial PT, a critical early event (Zamzami, Susin et al. 1996) due to the opening of a large conductance channel termed the PT pore (PTP), and the disruption of the mitochondrial inner transmembrane potential ($\Delta\Psi_m$). During PT, there is a sudden increase in the $\Delta\Psi_m$ and this allows molecules up to 1.5 kD to enter the intermembrane space. Maintenance of $\Delta\Psi_m$ relies on impermeability of the inner mitochondrial membrane; thus, PT leads to disruption of $\Delta\Psi_m$. Collapse of $\Delta\Psi_m$ is

accompanied by immediate shutdown of biological homeostasis in the mitochondria (i.e. uncoupling of oxidative phosphorylation and the generation of ROS) (Attardi and Schatz 1988; Zamzami, Marchetti et al. 1996). The increased ROS oxidize lipids, proteins, and nucleic acids to cause further disruption of $\Delta\Psi_m$ (Marchetti, Decaudin et al. 1997). These changes occur before apoptotic nuclear events (Zamzami, Marchetti et al. 1996) and in enucleated cells (Jacobson, Burne et al. 1994; Schulze-Osthoff, Walczak et al. 1994), indicating this mitochondrial dysfunction is independent of the nucleus.

After discussing disruption of the inner mitochondrial membrane, the next question to ask is how these larger molecules breach the OMM. The most prominent scenario involves the opening of the PTP (Green and Reed 1998). The PTP, also called the megachannel, normally appears to regulate matrix calcium, pH, $\Delta\Psi_m$, and volume, functioning as a calcium-, voltage-, pH-, and redox-gated channels with several levels of conductance (Jacotot, Costantini et al. 1999). At high levels of conductance, the mitochondrion undergoes irreversible $\Delta\Psi_m$ dissipation and matrix swelling. Opening of this nonselective channel causes volume dysregulation in the mitochondria caused by equilibrium of ions between the matrix and inner membrane space, dissipation of H^+ gradient across the membranes, and uncoupling of the electron transport chain and eventually, expansion of the matrix due to it hyperosmolarity (Green and Reed 1998). Because the inner membrane is convoluted and folded into cristae, its large surface area can handle the expansion. Unfortunately, the same is not true for the OMM and it will rupture as a result, releasing caspase-activating proteins into the cytosol. Bcl-2 family members regulate PTP opening and closing. Bcl-2 can prevent PT while Bax induces PT and apoptosis (Zamzami, Susin et al. 1996). Opening of the PTP seems to be required for Bax-, p53-, TNF-, and glucocorticoid-induced death (Jurgensmeier, Xie et al. 1998).

The effects of cytochrome c likely depend on the cell type (Green and Reed 1998). Dying cells that have a pre-existing excess of cytochrome c are able to maintain electron transport, oxygen consumption, and ATP production with docked cytochrome c, even after the released cytochrome c activates caspases. These cells experience apoptotic demise. On the other hand, in cells with an excess of endogenous caspase inhibitors, despite cytochrome c release there is failure to induce caspase-independent apoptosis. The mitochondria experience loss of electron transport, PT, Ψ_m collapse and inner membrane swelling, and mitochondrial rupture tending towards necrosis.

The Death Receptor (Extrinsic) Pathway

Once extracellular binding of a membrane-bound or soluble ligand to death receptors occurs, the signals are transmitted via a death domain on the cytoplamsic tail that activates the proteases homologous to CED-3, capsases (14 have been described to be involved in the cell death pathway) (Schultz and Harrington 2003). Caspase-8 is considered the apical caspase for the extrinsic pathway.

In the death receptor pathway, apoptotic signals are transmitted via the TNF family, with at least 16 members, including TNFR1, TNFR2, Fas, NGFR, CD40, CD27, CD30, DR3, TNF-related apoptosis-inducing ligand (TRAIL), and DR6 death receptors (Schultz and Harrington 2003); (Nagata 1997). Most is known about the Fas (CD95 or Apo 1) receptor and TNF receptor-1 (TNFR-1) (p55 or CD120a); these receptors share cytoplasmic region similarities, along with TNFR2 (Nagata 1997).

As we have seen with caspase activation and Bcl-2 proteins, protein to protein interactions play a prominent role in conducting a death signal from its origin to its destination in most, if not all, apoptotic pathways. The intermolecular domains required for these interactions are described in Appendix 1.

The Fas receptor is central in signaling apoptosis and activating the transcription factor nuclear factor kappa B (NF-κB). This transcription factor protects cells from apoptosis by inducing the expression for pro-survival factors, such as the inhibitor of apoptosis protein (IAP) family (Schultz and Harrington 2003). The Fas receptor ligand, FasL, is inactive in its membrane-bound form (Nagata 1997). After proteolysis by metalloprotienases it is released from activated $CD4^+$ and $CD8^+$ T cells in its functional, soluble form (Nagata 1997). Binding of FasL to Fas induces trimerization of the Fas receptor (Nagata 1997). Activated Fas recruits FADD/MORT1 via interactions between death domains (DD), which activate caspase-8 (FLICE/MACH) through its DED. Similarly to FasL, TNF is normally in an inactive, membrane-associate form; upon proteolysis by metalloproteinases, it is converted into its functional, soluble form and binds to TNFR1 or TNFR2 (Nagata 1997). TNFR trimerize and the DD of TRADD, an adapter protein, interacts with the receptor's DD. This complex attracts additional proteins, FADD/MORT1 and RIP.

FADD/MORT1-induced apoptosis can be counteracted by RIP-induced transcription of NF-κB. TNFR1-mediated IKK (IκB kinase) activation requires both RIP and TRAF2 proteins, possibly because RIP stabilizes the interaction between TRAF2 and IKK and mediates the activation of IKK (Devin, Cook et al. 2000). Transcription of NF-κB can also occur through the TNFR2 receptor in some cells, but it is more likely to use the major signaling receptor, TNFR1 (Nagata 1997). Thus, since both receptors can trigger the two major functions of TNF—activation of NF-κB and induction of apoptosis—some overlap does exist (Hsu, Huang et al. 1996). NF-κB has been shown to inhibit TNF-mediated apoptosis by inducing transcription of Bcl-x_L, A1, cIAP, etc. (Wang, Cusack et al. 1999). Alternatively, overexpression

of RIP protein was shown to induce both NF-κB and apoptosis (Hsu, Huang et al. 1996). This is suggested to be possible because, in low RIP conditions, TRAF2 binds and induced NF-κB transcription and survival due to uncoupling of the death pathway. Once death triggers are received, an increase in RIP associated with TRADD bridges TNFR to FADD/MORT1 and the apoptotic machinery (Pimentel-Muinos and Seed 1999). Thus, the dual functions of TRADD may cause TNFR to "nullify" their own apoptosis-inducing activity.

RECURRENT AIRWAY OBSTRUCTION IN HORSES

Recurrent airway obstruction (RAO) is a chronic, recurring inflammatory lung disease, most commonly found in horses stabled during the winter in environmental conditions prone to having dust and allergens present in the air (Gerber 1973; Cook 1976). Neutrophilic infiltration into the airways, mucus accumulation within the airways, bronchospasm, and airway hyperreactivity (AHR) are hallmark features of this disease (Obel and Schmiterlöw 1948; Schatzmann, Buergi et al. 1973; Nicholl 1978; Robinson, Derksen et al. 1996). Described below are: the etiology, clinical signs, lesions, pathophysiology and pathogenesis, treatments, and indices of severity for RAO in the equine lung.

ETIOLOGY

Many factors present in the horse's environment elicit an airway response and disease exacerbation. These pro-inflammatory agents include thermophilic molds and actinomycetes *(Aspergillus fumigatus, Thermoactinomyces vulgaris,* and *Faenia rectivirgula)* mold spores, endotoxins, proteinases, forage mites, and particles of feed grains, feces, dander, and pollen that become aerosolized around the horse's breathing zone while it is feeding (McPherson, Lawson et al. 1979a; Derksen, Robinson et al. 1988; McGorum, Dixon et al. 1993a). When RAO-susceptible

horses are exposed to such agents, they develop inflammation, AHR, and obstruction, whereas horses not affected with RAO do not have the same severity of inflammation and do not develop obstruction or AHR. Changing the management of RAO-susceptible horses to either keeping them on pasture or feeding them pellets or grass silage and bedding them on shavings greatly reduces the dust exposure (Woods, Robinson et al. 1993; Vandenput, Votion et al. 1998) and severity of disease exacerbation (Thomson and McPherson 1984). When RAO-affected animals are allowed to be at pasture for 1 or 2 weeks, their BALF leukocyte count is no different from that of control horses and airway obstruction is minimal (Derksen, Scott et al. 1985). The time taken for horses to become asymptomatic correlates with age, duration of illness, and severity of disease as measured by the non-elastic work of breathing (Thomson and McPherson 1984). There may be genetic predisposition for horses to develop RAO, although the inheritance pattern in unclear (Marti, Gerber et al. 1991). Breed, gender, body weight, and season are not factors in disease susceptibility (McPherson, Lawson et al. 1979).

Exposure to the dusty conditions described above causes acute inflammation, with neutrophil accumulation in the airway lumina. Many believe repeated exposure and inflammation causes MC proliferation and that this aids in protecting the lung through increased mucus secretion, presumably to trap and transport inhaled particles. Normally useful, this becomes a problem in combination with the edematous airways resulting in thickened walls, decreased airway diameter, and mucus plugs.

One consequence of RAO to horses is poor gas exchange, indicated by low PaO_2 (hypoxemia) (Robinson, Derksen et al. 1996). Interestingly, this is not accompanied by hypoventilation, or increased $PaCO_2$, despite the horse having to work harder to breathe air through obstructed airways (bronchospasm) to maintain tidal volume. The drop in PaO_2, due to ventilation/perfusion mismatching, causes an increase in respiratory rate. The most common

and effective treatments for horses affected with RAO are environmental management to reduce dust exposure, bronchodilators (Murphy, McPherson et al. 1980; Robinson, Derksen et al. 1993), and corticosteroids (LaPointe, Lavoie et al. 1993).

Clinical Signs

RAO-affected horses display all or some of the following clinical signs during acute exacerbations of disease: 1) difficult breathing characterized by expiratory abdominal effort (heaving), 2) increased breathing sounds with wheezing, and 3) increased bronchial secretions (Gillespie and Tyler 1969). In more severe instances, these 'abnormal findings' worsen and horses develop chronic cough, double expiratory effort, and hypertrophy of the external abdominal oblique muscle ("heaves line") (Breeze 1979). Copious amounts of mucus are visible in the trachea within 24 hours of exposure and are maintained in RAO-affected horses, even at pasture (Gerber, Straub et al. 2001).

Lesions

Originally, RAO was thought to be an emphysema-like disease because the lungs do not collapse when the chest is opened (Lowell 1964; Thurlbeck and Lowell 1964; Tyler, Gillespie et al. 1971). However, unlike emphysema where failure of collapse is due to the loss of elastic tissue in the alveolar septa, failure of collapse in RAO is due to the trapping of gas behind mucus plugs and the consequential hyperinflation of alveoli (Robinson, Derksen et al. 1996).

The primary lesion of RAO is inflammation in small diameter (<2 mm) airways with widespread epithelial hyperplasia and metaplasia (Breeze 1979). Inflammation, largely consisting of luminal neutrophils, is a prominent feature of RAO. Peribronchial infiltration of lymphocytes, plasma cells, mast cells and few eosinophils have been documented in addition to

the intraluminal neutrophils (Breeze 1979; Fairbairn, Page et al. 1993; Robinson, Derksen et al. 1996). Mast cells may be found in the intercellular clefts (Kaup, Drommer et al. 1990a).

Mucous cell metaplasia (MCM) has been described in bronchioles of affected horses, where MC are normally absent, and the changes increase with disease severity (Kaup, Drommer et al. 1990b). This becomes more problematic farther out the tracheobronchial tree as the diameter of airways decreases, contributing to the stasis of mucus. Thus, the small diameter together with increased mucus viscoelasticity (Gerber, Lindberg et al. 2004) and the resultant decreased tracheal transport rates (Coombs and Webbon 1987; Turgut and Sasse 1989) expedite the formation of plugs composed of mucus and neutrophils (Breeze 1979) in more peripheral airways. Descriptive pathology studies report some fibrosis in small diameter airways, necrosis of type I cells, their replacement with type II cells, and acinar overinflation in alveoli (Kaup, Drommer et al. 1990b). In larger airways, focal loss of ciliated cells followed by replacement with undifferentiated cells leading to development of a hyperplastic epithelium is described (Kaup, Drommer et al. 1990a). This was also seen in smaller airways (Winder, Gruenig et al. 1989). While many of these changes correlate with severity of disease, some horses show ciliary malformations that appear to be independent of disease severity (Kaup, Drommer et al. 1990a).

In larger airways, remodeling occurs (i.e. hyperplasia and/or hypertrophy of mucosa, submucosa, and smooth muscle) that coincides with neutrophilic infiltration (Robinson, Derksen et al. 1996) so that only a small amount of smooth muscle contraction is required to cause in a big change in airway diameter. This exaggerated narrowing of the airways or, nonspecific AHR, contributes to airway obstruction; airway remodeling is most likely due to the inflammatory response (Figure 6).

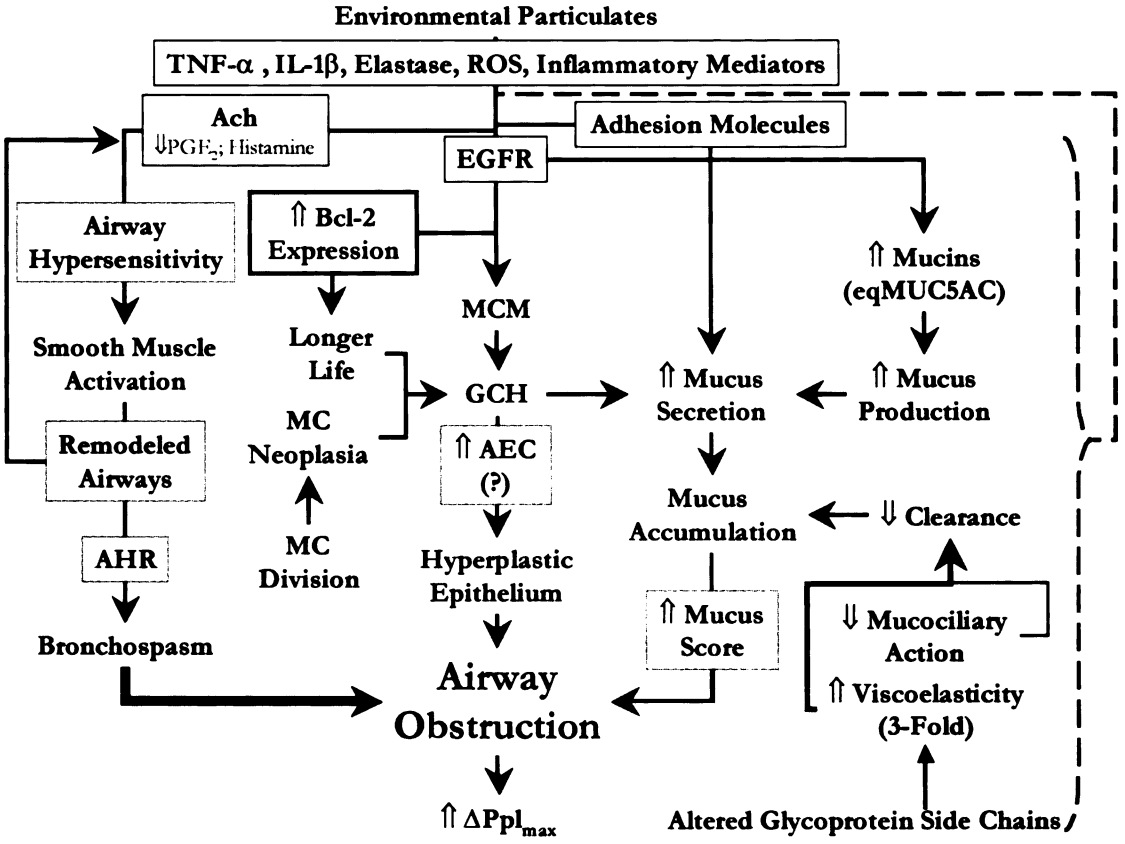

Figure 6. A flow diagram of possible mechanisms leading to the progression and development of disease in RAO-susceptible horses. Accumulation of neutrophils and their products is an early feature of the disease, happening in three to five hours after a challenge. Activated neutrophils generate IL-1β and TNF-α, which activate NF-κB, which in turn induces their transcription initiating a positive feedback loop (not shown). TNF-α also increases EGFR expression causing MCM, mucus secretion, and mucin synthesis. Neutrophil products likely increase mucus viscoelasticity and mucin (MUC5AC) expression (via TNF-α-induced EGFR expression and ROS-induced transactivation), which subsequently decrease the clearance of and increases the production of mucus, respectively. Concurrently, neutrophil elastase, a well-known MC secretagogue, induces mucus secretion by interacting with MC (via adhesion molecule ICAM-1). Together, possibly with decreased clearance (due to decreased mucociliary transport and increased viscoeleasticity), accumulated mucus in the airway, and if it occurs, increased numbers of MC (due to longer survival time, proliferation of existing MC, and/or differentiation of progenitor cells into MC), contributes to the visible increased mucus score. Increased luminal mucus and airway remodeling potentiate airway obstruction due to bronchospasm. In addition to neutrophil products, 'bronchspastic' neurotransmitters (ACh), inflammatory mediators, and nonspecific irritants (dust) stimulate hypersensitive airway smooth muscle to contract. Because of remodeling and increased thickness of the airway walls, this exaggerated contraction (AHR) greatly reduces luminal diameter (bronchospasm) around mucus plugs to cause airway obstruction that is measurable as the maximal change in pleural pressure (ΔPpl_{max}) during tidal volume at rest. Arrow thickness denotes known, relative contributions. Dotted line represents possible pathways. Solid boxes are mediating factors; dotted boxes are consequential effects. Components highlighted in yellow are the focus of this thesis. (NOTE: Images in this thesis are presented in color).

PATHOPHYSIOLOGY AND PATHOGENESIS

Inflammation

While RAO-susceptible horses are in remission, macrophages and lymphocytes are the main white blood cells found in the bronchoalveolar lavage fluid (BALF) of horse lungs (Derksen, Scott et al. 1985; McGorum, Dixon et al. 1993). During an acute exacerbation, neutrophils infiltrate the lung between three and five hours after the onset of exposure (Fairbairn, Page et al. 1993) and are detectable in BALF at five hours (McGorum, Dixon et al. 1993). In general, percentage of neutrophils increases with severity of disease; however, neutrophils can be present without dyspnea (Fairbairn, Page et al. 1993). Likewise, because neutrophilic accumulation is not always observed in the above allotted time frames, neutrophils may not be required for airway obstruction early on and may have more prominent roles later in disease pathogenesis (Fairbairn, Page et al. 1993).

Paramount in the pathogenesis of RAO is neutrophil infiltration into the airway lumen. Specific mechanisms for neutrophil chemotaxis involve macrophages and lymphocytes that produce and secrete cytokines. IL-8, a strong neutrophil chemoattractant, correlates tightly with the percentage of neutrophils in tracheal aspirates of asthmatics (Ordonez, Shaughnessy et al. 2000) and has been found in the BALF of RAO-affected horses (Giguere, Viel et al. 2002). However, since neutrophilic influx can occur in the presence of low IL-8, other mediators must be involved in neutrophil chemotaxis. During disease exacerbation of RAO-affected horses, IL-1β and TNF-α are increased in the BALF (Giguere, Viel et al. 2002). The latter proinflammatory cytokines increase ICAM-1 expression in endothelial and epithelial cells (Tosi, Stark et al. 1992), which, in concert with elevated levels of both mRNA and protein of IL-8, may contribute to the observed neutrophilia in diseased horses. Activated neutrophils of RAO-affected horses generate IL-1β and TNF-α, which activate NF-κB, which in turn induces their

transcription initiating a positive feedback loop (Bureau, Delhalle et al. 2000). The persistent NF-κB expression in RAO-affected horses (Bureau, Bonizzi et al. 2000), which may partially explain higher cytokine gene expression (Giguere, Viel et al. 2002) may explain the neutrophilic nature of RAO inflammation. Another proinflammatory mediator, leukotriene B_4 (LTB_4), that is related to neutrophil-mediated tissue damage, is increased by tumor necrosis factor and interleukins as well as complement fragments and endotoxins (Crooks and Stockley 1998). Production of LTB_4 may be increased in RAO-affected horses and also may contribute to neutrophil infiltration into the lung (Lindberg, Robinson et al. 2004). LTB_4 stimulates the activation and production of NF-κB and IL-8 cytokine (Aoki, Qiu et al. 1998) and may prolong the life of neutrophils (Lee, Lindo et al. 1999). Hence, LTB4 promotes transcription of inflammatory genes and cytokines.

There is a debate as to whether RAO has a polarized cytokine profile and if it is Th1, Th2, or some combination. In support for a predominantly Th2 response, Komai et al (2003) have found this cytokine profile to be responsible for airway remodeling and increased mucus secretion, via MUC5AC induction, in mice (Komai, Tanaka et al. 2003). Increased expression of cytokines implicated in the Th2 response, such as IL-4, IL-5, and IL-13, suggest RAO in horses may be an allergic condition like asthma (Lavoie, Maghni et al. 2001; Bowles, Beadle et al. 2002; Cordeau, Joubert et al. 2004). Additionally in animal models, IgE generation and its maintenances in BALF and serum (Halliwell, McGorum et al. 1993; Komai, Tanaka et al. 2003) relies on IL-4 and IL-13.

Alternatively, IFN-γ, IL-8, IL-12 (Ainsworth, Grunig et al. 2003) and several forms of IgGs have been found in serum and BALF samples (Ainsworth, Appleton et al. 2002) of RAO horses refuting a polarized Th2 cytokine profile and suggestive of a Th1 type response. Finally,

it may be that RAO-affected horses have a Th0 response, characteristic of both Th1 and Th2 cytokines (Ledru, Lecoeur et al. 1998).

Neutrophils can have a large impact on lung function and can indirectly induce airway obstruction by releasing ROS, proteases, and inflammatory mediators, which cause increased mucus secretion as well as other possible outcomes (Figure 6). The products of neutrophils are described as causing 'adverse changes' in mucus rheology, inducing mucus hypersecretion, increasing mucin gene expression, and causing glycoprotein alterations to mucin molecules.

MEASUREMENTS OF DISEASE SEVERITY

Maximal Changes in Pleural Pressure (ΔPpl_{max})

Placement of an esophageal balloon distal to the heart is commonly used to measure of maximal changes in pleural pressure (ΔPpl_{max}) during tidal breathing (Derksen and Robinson 1980; Robinson, Derksen et al. 1999). The determinants for ΔPpl_{max} are: pulmonary resistance (R_L), tidal volume (V_T), dynamic compliance (C_{dyn}), and air flow rate (V).

$$\Delta Ppl_{max} = V_T/C_{dyn} + Flow*R_L$$

In RAO-affected horses, increased ΔPpl_{max} is indicative of airway obstruction (Figure 6). The airway obstruction increases R_L and decreases C_{dyn}. Respiratory rate increases but tidal volume remains constant, thus horses must increase minute ventilation to move air through obstructed airways at increased flow rates resulting in larger differences in pleural pressure. The increased R_L, decreased C_{dyn}, and increased flow rate all contribute to the increase in ΔPpl_{max}. Horses having a ΔPpl_{max} greater than 15 cm H_2O are considered to have RAO (Robinson 2001). Normal horses have ΔPpl_{max} of 5 to 10 cm H_2O.

Clinical Score

A subjective clinical score with a significant relationship to changes in lung function, has been validated (Robinson 2001). Upon clinical examination, two scores ranging from 1 to 4 are assigned indicating the degree of visible abdominal effort (abdominal score) and flaring of the nostrils (nasal score). These two scores are totaled (total clinical score) and serve as a good measure of disease severity when the total score is greater than 5 as compared to measurements of pulmonary resistance and dynamic elastance (Costa, Seahorn et al. 2000; Robinson, Olszewski et al. 2000). However, scores lower than 5, implying minimal impairment of lung function, fail to reflect low-grade airway obstruction detected with lung function tests (Robinson, Olszewski et al. 2000).

Mucus Score

Severe mucus accumulation is one of the most notable findings in RAO-affected horses (Costa, Seahorn et al. 2000). Recently, a subjective scoring system—via tracheal endoscopy—was shown to be good measure of mucus volumes and it was reproducible between blinded observers (Gerber, Straub et al. 2004). This is a good indicator for the presence of disease, but not necessarily the severity of the disease.

Bronchoalveolar Lavage Fluid (BALF)

Collection of BALF is a useful investigative tool for the quantification of inflammatory cell infiltration into the airways and analysis of the airway lining fluid. In both control and RAO-affected horses, a single BALF sample is representative of the entire lung (McGorum, Dixon et al. 1993). During collection, passage of an endoscope into the lung allows sampling of peripheral airways, through which, typically of 1-3 aliquots of 100 ml of sterile physiological saline are instilled and aspirated. From the pooled samples, total and differential white blood cell counts may be determined to assess total leukocyte density.

During disease exacerbation in RAO-affected horses, neutrophils increase to significantly higher levels than those of controls, with an insignificant increase in eosinophils and mast cells (Derksen, Scott et al. 1985; Winder, Grunig et al. 1991; Robinson 2001; Gerber, Lindberg et al. 2004). The number of neutrophils present in BALF aspirated from the lungs is a good indicator to the degree of airway inflammation present. RAO-affected horses generally have greater than 20% neutrophils in BALF (Robinson 2001). In control horses, the primary leukocytes present are alveolar macrophages (60%) and lymphocytes (35%), while neutrophils, eosinophils, and mast cells are negligible (Robinson 2001).

Colleted BALF provides a more useful diagnostic aid over transtracheal washes in evaluating RAO, even if the amount of BALF recovered is very small (Pickles, Pirie et al. 2002). In control horses, there is less variability in leukocyte populations in BALF compared to transtracheal washes while in RAO-affected horses, the clinical usefulness of cytological evaluations of transtracheal aspirates may be limited (Derksen, Brown et al. 1989). Though increased leukocytes, found by cytology performed on BALF, allows early detection of inflammatory respiratory disease, is not specific for RAO (Couetil, Rosenthal et al. 2001).

MUCUS ACCUMULATION AND MUCOUS CELL METAPLASIA IN RAO

Mucus accumulation contributes to airway obstruction (Figure 6) and leads to changes in breathing strategy, abnormal sounds upon auscultation, and the overall appearance of clinical signs. Persistent mucus accumulation is a consistent finding in RAO-affected horses before, during, and after exacerbation of disease (Gerber, Lindberg et al. 2004). This may be a consequence of changes in the rates of production, secretion and clearance.

Increased production and secretion may result from one or all of the following: the presence of secretagogues (neutrophils and their products), induction of mucin gene expression

(eqMUC5AC), or increased MC numbers. Increased mucin gene expression has been repeatedly shown in many animal models, including horses (Fischer and Voynow 2000; Takeyama, Dabbagh et al. 2000; Gerber, Robinson et al. 2003). Neutrophils play a central role in MC degranulation and mucin secretion. Neutrophil elastase and other factors (e.g. IL-8, IL-13, ROS, EGF, TNF-α) are important inducers of mucin production and secretion (Agusti, Takeyama et al. 1998; Takeyama, Agusti et al. 1998; Nadel, Takeyama et al. 1999).

During exacerbations of RAO, there is indeed a decreased rate of mucociliary clearance in horses with RAO compared to control horses (Turgut and Sasse 1989) accompanied with a three-fold increase in mucus viscoelasticity, apparently a result of alterations to the glycoprotein side chains of mucins, within 24 hours of exposure (Gerber, King et al. 2000; Jefcoat, Hotchkiss et al. 2001). The latter causes decreased clearability and thus stasis and accumulation of mucus. Increased mucus viscoelasticity also results from actin and DNA released from degranulated neutrophils, which aggregate, increase the viscoelasticity, and affect both ciliary and cough clearability of mucus (Gerber, King et al. 2000; Pietra, Guglielmini et al. 2000). Decreased mucociliary action is also likely to play a role in decreased clearance. While in remission, however, there is no difference in mucus rheology (i.e. viscoelasticity) between RAO-affected and control horses but there was up-regulation of mucin genes that may increase mucin secretion and overcome mucociliary clearance mechanisms to contribute to the persistent changes in mucus transport (Gerber, Lindberg et al. 2004). Thus mucus accumulation in remission is likely due to hypersecretion i.e. mucin upregulation, and perhaps to increased numbers of MC.

Goblet cell hyperplasia (GCH) is a common finding in many respiratory diseases. Qualitative findings of GCH and MCM in bronchi and bronchioles, respectively, have been reported numerous times where existing and newly formed cells can differentiate into mucus-

producing cells (Winder and von Fellenberg 1988; Kaup, Drommer et al. 1990b; Nyman, Lindberg et al. 1991; Lakritz, Wisner et al. 1995; Costa, Seahorn et al. 2000; Shi, Fischer et al. 2002). This may be due to proliferation (i.e. MCM and neoplasia), to prolonged cell life [increased anti-apoptotic factors (Bcl-2)], or to a combination. Activation of epidermal growth factor receptor (EGFR) can also lead to MCM and eventually GCH (Lee, Takeyama et al. 2000). Initiation of neutrophil-induced oxidative stress by cytokines, such as IL-13, causes phosphorylation of the EGFR (Figure 6) (Takeyama, Dabbagh et al. 2000; Shim, Dabbagh et al. 2001). Increased expression of EGFR by TNF-α (Nadel 2001) and activation of its tyrosine kinase in airways increases the synthesis of mucin MUC5AC mRNA and protein (Takeyama, Dabbagh et al. 1999; Takeyama, Jung et al. 2001). Morphometric techniques can be applied to measure the amount of store intraepithelial mucins (Vs) in the airways in order to assess mucus production (Harkema and Hotchkiss 1992). A role for Bcl-2 in MCM is suggested by the observation that Bcl-2 expression occurs in ~20–30% of metaplastic MC (MMC) in epithelium of ozone-challenged rats (Tesfaigzi, Hotchkiss et al. 1998; Tesfaigzi, Fischer et al. 2000) and that MCM does not resolve as long as Bcl-2 is expressed (Foster, Gott et al. 2003).

Apoptosis of MC

Determining mechanisms for maintenance of MCM is an ongoing topic of investigation in both spontaneous and induced airway disease. One thought that has gained interest, as a causative mechanism is prolonged life of MC. Challenges with ozone, endotoxin, and allergens in rats, increase Bcl-2 expression in epithelial cells in the proximal septum of the rat nose and different regions of the lungs (Tesfaigzi, Fischer et al. 2000; Tesfaigzi 2002; Foster, Gott et al. 2003; Tesfaigzi, Harris et al. 2004). These observations suggest a role for Bcl-2 in MCM, possibly by inhibiting progression of the cell division cycle (Mazel, Burtrum et al. 1996). However, more recently published was the observation that approximately one-half of the MMC

are derived from proliferating cells and the other half differentiate from pre-existing epithelial cells (Tesfaigzi, Harris et al. 2004). Moreover, since Bcl-2 is expressed in MMC derived from pre-existing and proliferating cells, Bcl-2 may not have a cell cycle regulatory function, at least not one that was seen in rat epithelial cells (Tesfaigzi, Harris et al. 2004). The expected role for Bcl-2 is maintenance of MCM.

SUMMARY

This literature review describes the mucous apparatus in airways, physiological cell death and the pathology and diagnosis of RAO in horses. Increased and persistent mucus accumulation is a common finding of RAO. The contribution of increased numbers of MC has not been investigated. This thesis addresses GCH as a possible consequence of delayed apoptosis. I hypothesized that delayed apoptosis, as indicated by an increased presence of Bcl-2-positive MC, contributes, at least in part, to an increase in the number of mucus-secreting cells and airway mucus accumulation.

CHAPTER 2
PERSISTENT MUCUS ACCUMULATION—A CONSEQUENCE OF DELAYED MUCOUS CELL DEATH IN RAO-AFFECTED HORSES

ABSTRACT

This study examined the role of delayed apoptosis of mucous cells (MC) as indicated by Bcl-2, an anti-apoptotic protein, as a contributing factor to mucus accumulation in horses affected with recurrent airway obstruction (RAO). Measurements of disease severity were collected from 6 RAO-affected and 6 control horses exposed to two different management systems (one to induce inflammation [5 days] and one to partially resolve it [7 days]) prior to euthanasia. Morphmetric analyses (Bcl-2-positivity and stored mucosubstance [Vs]) were performed on 8 bronchi. RAO-affected horses had more airway obstruction and luminal mucus than control horses under both management systems. At the time of euthanasia, RAO-affected horses had more inflammation and more Bcl-2-positive MC than control animals but no difference in MC number or Vs, and in RAO-affected animals, Vs decreased as BALF neutrophil numbers increased. Therefore, a conclusive role for Bcl-2 in prolonging MC life cannot be determined, despite Bcl-2 antibody immunoreactivity. USDA/CSREES 2002-35204-1259)

MATERIAL AND METHODS

ANIMALS

Six control and six RAO-affected adult horses (eight males and four females; ages 10 to 29, weights 399 to 538 kg) were used in this study. The RAO-affected animals were from a herd of such horses maintained by the Pulmonary Laboratory at Michigan State University and met the criteria defined at the International Workshop on Equine Chronic Airway Disease (Robinson 2001). That is, in the presence of organic dust in a stable they had airway obstruction that was decreased by administration of atropine (0.02 mg/Kg IV) (Figure 7).

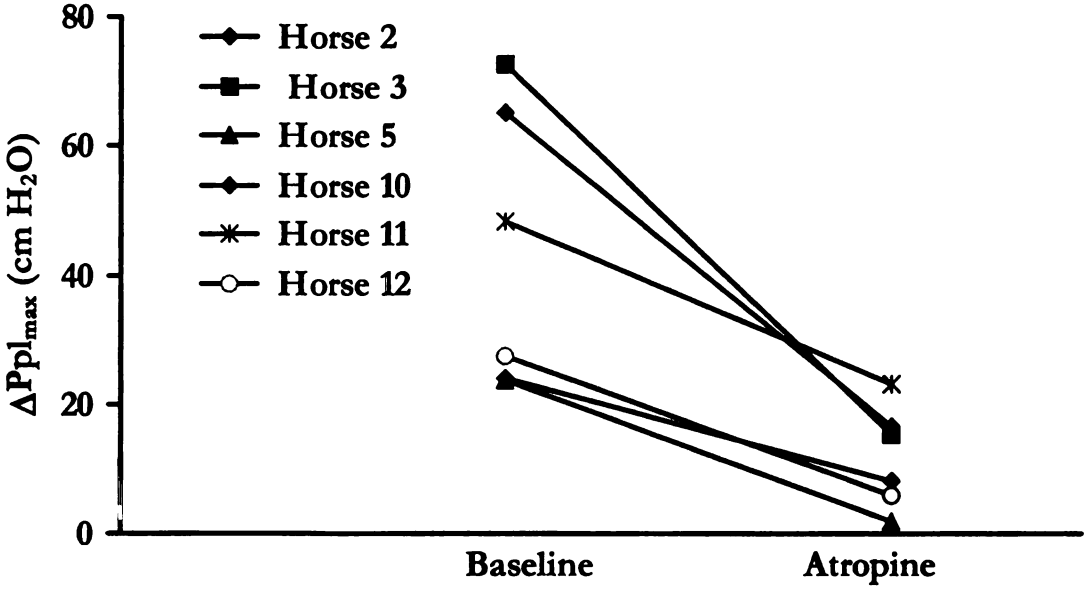

Figure 7. Effect of atropine (0.02 mg/kg IV) on the maximal change in pleural pressure (ΔPpl_{max}) during tidal breathing in six stabled horses with a history of RAO. Atropine decreased ΔPpl_{max} in all horses.

Horses in the control group had no known history of chronic airway disease and developed no clinical signs of obstructive airway disease when stabled.

EXPERIMENTAL DESIGN

In this blinded study (Figure 8), which was approved by the Animal Use and Care Committee of MSU, each horse and sampling time-point was assigned a random code. Horses were studied in pairs of one RAO and one control. After being at pasture for a minimum of two weeks (average of 8.5 weeks), one horse from the pair was stabled, fed poor quality hay and bedded on straw for 5 days. These stabling conditions induce airway inflammation in the RAO-susceptible animals. At this point, the horse's diet and bedding were changed to pellets and shavings, respectively, in order to reduce the severity of airway obstruction and inflammation in the RAO-affected horses (Thomson and McPherson 1984; Vandenput, Votion et al. 1998). After 7 days on pellets and shavings (day 12), the horse was euthanized. On days 5 and 12, the severity of airway obstruction was quantified by measurement of the maximal change in pleural pressure during tidal breathing (ΔPpl_{max}), bronchoalveolar lavage was performed, and a tracheal mucus accumulation score was assigned. This protocol was repeated the following week with the other horse from the pair. The sequence of the pairs—RAO-affected and control—was randomized.

Horses were euthanized with pentobarbital (86 mg/kg IV). The thorax was opened; the lungs were dissected away from other structures, extracted, and grossly examined. From each of eight regions (Figure 9), samples of peripheral parenchyma and an adjacent cartilaginous bronchus (2-6 mm diameter) were collected. The sequence in which the regions were sampled was randomized a priori.

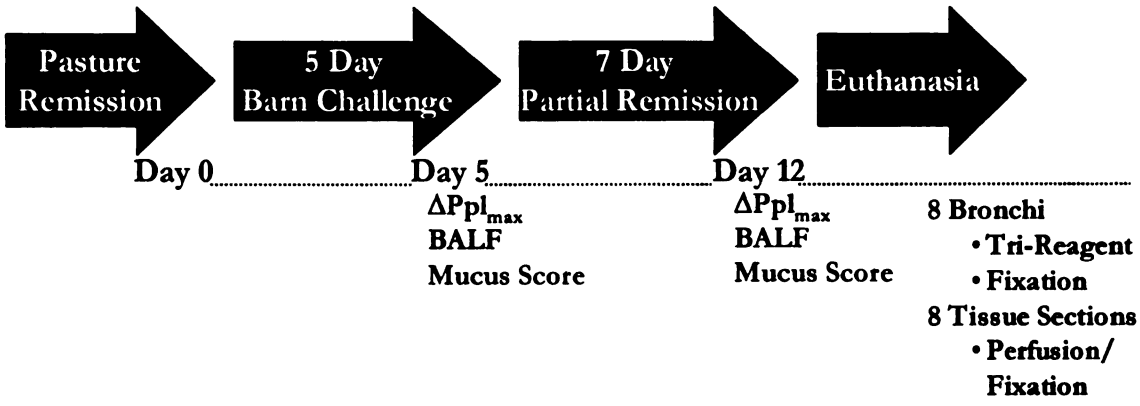

Figure 8. Experimental Design. One horse of each pair was brought into the stable, fed research hay and bedded on straw for 5 days. At the end of the 5-day environmental challenge, samples for ΔPpl_{max}, BALF, and mucus score were collected. The management was changed to pellets and shavings for 7 days to induce a partial remission of disease. At the end of the twelfth day, samples for ΔPpl_{max}, BALF, and mucus score were collected once more and the horse was euthanized. Post-mortem samples included 8 bronchi and 8 tissue sections.

MEASUREMENT OF ΔPPL_{MAX}

Horses were intubated with an esophageal balloon (Trojan condom, Carter-Wallace Inc, NY) sealed over the end of a 240-cm long polyethylene catheter (3 mm internal diameter, 4.4 mm external diameter) (PE240, Becton, Dickinson & Co, Franklin Lakes, NJ) with lateral holes drilled in the portion covered by the condom. The catheter was inserted so that the balloon was placed caudal to the heart base but cranial to the diaphragm. This location provides a good estimate of the pressure within the pleural cavity at that location (Derksen and Robinson 1980). Pressure changes within the balloon were detected by means of a pressure transducer (Model DP/45-35, Validyne, Northridge, Ca) and were recorded on a portable physiograph (Dash model II, Astro-Med, West Warwick, RI). The equipment was calibrated daily against a water manometer. The fluctuations in esophageal pressure were recorded for 20 consecutive breaths. The differences between the peak inspiratory and peak expiratory pressures were calculated to derive the ΔPpl_{max}.

TRACHEAL MUCUS SCORE

Horses were intravenously sedated with xylazine hydrochloride (1.1 mg/kg) and butorphanol tartrate (0.02 mg/kg) approximately five minutes before the bronchoscopy was performed with a 3-m endoscope (Olympus GIF 300, Olympus America Inc., Melville Maryland, USA). The bronchoscope was passed via the nares into the trachea where the amount of accumulated mucus was scored using a subjective grading system (Figure 11C) that is reproducible between observers (Gerber, Straub et al. 2004).

BRONCHOALVEOLAR LAVAGE

The endoscope was passed further into the trachea and into a bronchus. Once wedged, three-100 ml aliquots of sterile Dulbecco's phosphate buffered saline (PBS) were infused into the lungs and aspirated after each aliquot infusion and the samples were pooled. Leukocyte density per microliter BALF was assessed in a 10-μl aliquot by use of a hemacytometer. The percentage of each type of white blood cell was determined from counting 200 cells on a cytocentrifuged preparation stained with Wright-Giemsa. The percentage of each cell type and the total cell counts were used to calculate the total number of each cell type present per μl BALF.

POST-MORTEM PROCESSING

Parenchyma from each region of lung (Figure 9) was securely clamped using two curved bowel-clamps, tips touching to form two sides of a triangle, the third side being the lung margin. One large cartilaginous bronchus (2 to 6 mm diameter) also was dissected (1 cm length) from each region adjacent to the clamped region. A small cross-section (3 mm in length, 500 mg) of this bronchus was submerged into 5 ml of tri-reagent and stored at –80°C

for future use. The remainder of the bronchus was fixed by immersion in 1% paraformaldehyde/0.1% glutaraldehyde solution (pH 7.4) for 45 minutes, and was then transferred to 30% ethanol and washed three times in a fresh ethanol bath every five minutes. The fixed bronchus was stored in the final wash of 30% ethanol at room temperature.

The parenchymal sample was separated from the remaining lung tissue by incising along the outside of the clamps, which were then removed. The lung 'pouch' was carefully perfused with 1% paraformaldehyde/0.1% glutaraldehyde solution (pH 7.4); through a 19-gauge needle, inserted into the parenchymal pouch and connected via tubing to a system that maintained a constant perfusion pressure of 30 cm H_2O. After 15 minutes, the perfusion apparatus was disconnected from the lung tissue, and the lung sample remained an additional 30 minutes in a bath of the fixative solution before undergoing three ethanol washes and being stored in the last wash. Blocks of tissue and bronchi were embedded in paraffin and 5μm sections were cut. Two slides were prepared; one stained with Alcian-Blue (AB)-Positive Acid Shiff (PAS) and the other immunohistochemically stained for Bcl2 and counterstained with AB.

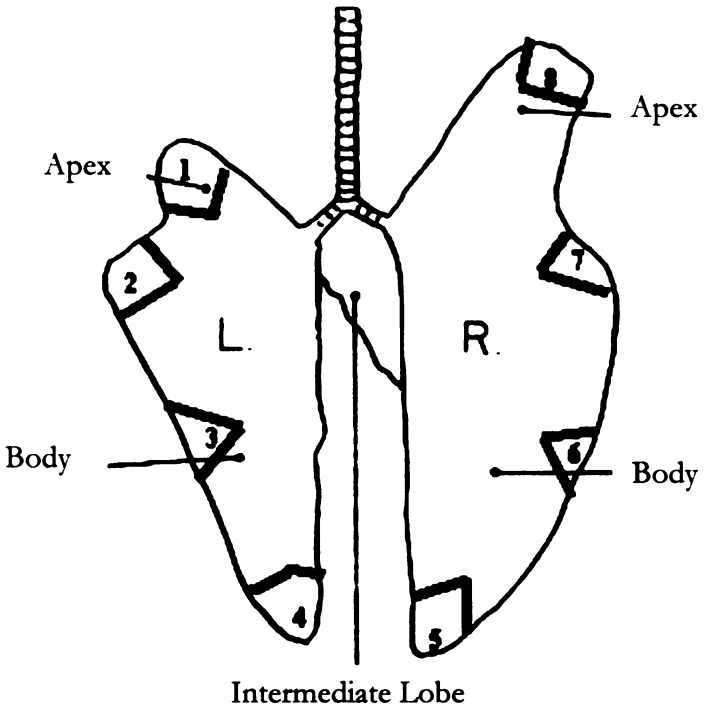

Figure 9. Regions of horse lung from which sections were taken. Sequence of sampling was randomized for each horse. Black lines specify location of clamps for the isolation of parenchymal sections.

IMMUNOHISTOCHEMISTRY

For immunohistochemical examination, sections of lung tissue were deparaffinized and rehydrated by routine methods. Antigen retrieval was accomplished by incubation of slides in antigen retrieval solution (Dako, Carpinteria, CA) in a steamer at 98°C (Black&Decker) for 20 min followed by gradual cooling of slides within the retrieval solution for 20 min at room temperature. Endogenous peroxidase was blocked for 5 min at room temperature with 3% hydrogen peroxide. Endogenous avidin and biotin activity was blocked by incubating the slides in an avidin and bition blocking reagents (Dako, Carpinteria, CA) for 10 min each at room temperature. Non-specific immunoglobulin binding was blocked by incubation of slides for 10 min at room temperature with a protein-blocking agent (Dako, Carpinteria, CA) prior to application of the primary antibody. Sections were stained in a Dako autostainer apparatus. The slides were incubated with the primary

antibody against Bcl-2 (PharMingen) at a dilution of 1:200 for 30 minutes at room temperature. A streptavidin-immunoperoxidase staining procedure (Dako, Carpinteria, CA) was used for immunolabeling (Webster, Kiupel et al. 2004). The immunoreaction was visible with Nova Red (Vector Laboratories). Sections were counterstained with alcian blue at a pH of 2.5 and Gill's 3 haematoxylin. Positive immunohistochemical controls included hyperplastic lymph nodes and lymph nodes with malignant lymphoma from archived equine tissues. For negative controls, the primary antibodies were replaced with TBS buffer (Figure 10).

Sections serial to those airways quantified for MC and Bcl-2 were stained with Alcian Blue (pH 2.5)/periodic acid-Schiff (AB/PAS) reagent for stored acidic and neutral mucin components as described previously (Harkema and Hotchkiss 1993).

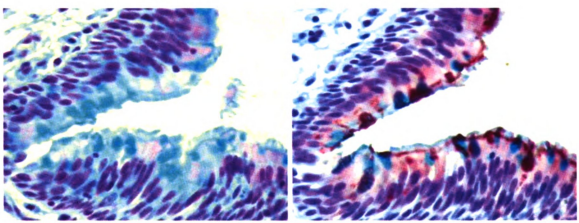

Figure 10. Negative control (left) and positive control (right) for Bcl-2 in a RAO-affected horse. (NOTE: Images in this thesis are presented in color).

MORPHOMETRIC QUANTIFICATION OF MC AND BCL-2 EXPRESSION

Morphometric analysis of airway mucosa and Bcl-2-positivity was performed using the Scion Image program (Scion Corporation, Frederick, MD). The regions of mucosa used for counting mucus-containing cells met the following conditions: 1) the basal lamina was intact, 2) the section of airway wall was transverse rather than oblique so that the apical

membrane of MC was aligned with the adjacent surface epithelium. The length of basal lamina underlying the surface epithelium was calculated from a contoured line on the digitized image of the airway epithelium and the number of Bcl-2-positive and negative MC per millimeter of basal lamina were counted. Total MC numbers were also quantified from AB/PAS stained tissue to confirm numbers.

Quantification of Intraepithelial Stored Mucosubstance

Using Scion image analysis software, pixel density and threshold of intensity were adjusted to determine the volume density (Vs) and the areas of acidic and neutral (AB/PAS-stained) stored mucosubstance along a measured perimeter as described previously (Harkema, Plopper et al. 1987; Harkema, Plopper et al. 1987). Data were expressed as the mean volume density (Vs; nl/mm^2 of basal lamina.) of AB/PAS-positive mucosubstance ± SEM.

Statistical Analysis

Data were examined and if not normally distributed, log10 transforms were made. A two way repeated measures ANOVA was used to determine the effects of 1) time and disease on ΔPpl_{max}, mucus score, total and differential cell counts, and 2) the effects of disease and lung region on the mucous cell counts and their Bcl-2 expression. When significant main effects were detected ($p<0.05$) multiple comparisons were made using the Student-Newman-Keuls method. Because of the small number of animals, significance of the interaction term was set at $p<0.1$. The Vs in the two groups of horses was compared by the Student's t-test. Statistical analyses were performed by means of a computer software program (SigmaStat, SPSS Science, Chicago, IL).

RESULTS

INDICES OF DISEASE SEVERITY

Maximum Changes in Pleural Pressure (ΔPpl_{max})

On day 5, RAO-affected horses had significantly greater ΔPpl_{max} compared with control horses (Figure 11A). On Day 12, the mean ΔPpl_{max} had decreased but continued to be significantly greater than in controls. The ΔPpl_{max} of control horses was unchanged between days 5 and 12.

Although RAO-affected horses had significantly greater ΔPpl_{max} than controls (Figure 11A), they greatly ranged in values among horses (Figure 11B). Two horses had ΔPpl_{max} greater than 40 cm H_2O at day 5, two had intermediate values, and two had low values. The decrease in ΔPpl_{max} that occurred between day 5 and day 12 was positively correlated with its magnitude on day 5.

Tracheal Mucus Score

The RAO-affected horses had significantly higher mucus scores (>2.5) than controls on both days 5 and 12 (Figure 11D). There was no effect of time in either group. Unlike ΔPpl_{max}, in which the trend in RAO-affected horses was to decrease between days 5 and 12, the change in mucus score was highly variable (Figure 11E).

Cytology of Bronchoalveolar Lavage Fluid (BALF)

The only statistically significant change in leukocyte numbers was in neutrophils (Table 2, Figure 11F). On day 5, control and RAO-affected horses had 11.8 ± 5.1 and 216.3 ± 204.7 (mean ± SEM) neutrophils/μl BALF, respectively. This difference was not significant because one RAO-affected animal had a low neutrophil count (9 cells/μl) (Figure

11G). On day 12, the neutrophil count for RAO-affected animals was statistically unchanged from Day 5 (102.7 ± 48.4). In control horses, the count on Day 12 was 1.0 ± 0.6, significantly less than controls on Day 5 and RAO-affected animals on Day 12. The mean count of macrophages, lymphocytes, mast cells and eosinophils was 22.5 ± 6.0, 51.1 ± 16.8, 2.5 ± 1.3, and 0.03 ± 0.03, respectively.

Table 2. BALF total and percent neutrophils, macrophages, lymphocytes, eosinophils, mast cells and total cell count on days 5 and 12 in RAO-affected and control horses.

		BALF Cytologies	
	Day	**RAO-Affected Horses (n=6)**	**Control Horses (n=6)**
% Neutrophils	5	44.9± 12.3[b]	17.7± 8.6
	12	40.3± 14.5[b]	1.6± 1.0[a]
Total Neutrophils (Cells/µl)	5	216.3± 204.7	11.8± 5.1
	12	102.7± 48.4[b]	1.0± 0.6[a]
% Macrophages	5	14.8± 4.3[b]	29.8± 4.4
	12	11.4± 2.7[b]	37.5± 7.3
Total Macrophages (Cells/µl)	5	23.0± 8.0	21.7± 3.9
	12	19.3± 6.1	25.8± 5.8
% Lymphocytes	5	34.1± 8.3	50.7± 5.6
	12	46.6± 13.3	53.3± 6.7
Total Lymphocytes (Cells/µl)	5	46.3± 13.5	37.1± 6.1
	12	75.9± 32.4	45.2± 15.1
% Eosinophils	5	0.2± 0.2	0.0± 0.0
	12	0.1± 0.1	0.2± 0.2
Total Eosinophils (Cells/µl)	5	0.0± 0.0	0.0± 0.0
	12	0.0± 0.0	0.1± 0.1
% Mast Cells	5	2.3± 1.0	1.8± 0.7
	12	1.7± 0.1	7.4± 4.5
Total Mast Cells (Cells/µl)	5	2.0± 1.0	1.5± 0.7
	12	1.4± 0.9	4.9± 2.6
TCC (Cells/µl)	5	292.9± 211.2	72.1± 7.1
	12	199.2± 52.1	77.1± 18.3

Values are means ± SEM
[a] Significantly different from day 5 within group
[b] Significantly different from control at same point

Goblet Cell Numbers

AB-PAS staining was present in MC of control and RAO horses (Figure 12A). There was no significant disease or regional effect on MC numbers (Figure 12B and 6C) in either AB/PAS or Bcl-2/AB stained tissue.

STORED MUCOSUBSTANCE IN AIRWAY MUCOUS CELLS

There was no significant difference between groups in Vs (Figure 12D). In the RAO-affected horses, Vs was negatively correlated ($r = -0.886$, $p=0.033$) with the log10 of the number of neutrophils in BALF (Figure 12E).

BCL-2 STAINING AIRWAY MUCOUS CELLS

In comparison to control horses, RAO-affected horses had noticeably more Bcl-2-positive MC in their epithelium (Figure 13A). Bcl-2 staining, seen as Nova Red counter-stain, was so abundant it masked the AB. However, morphological characteristics and serial sections stained with AB-PAS (Figure 12A) ensured these were MC.

In every region, the bronchi of RAO-affected horses contained significantly more Bcl-2-positive MC per millimeter of basal lamina ($p=0.011$) than controls (Figure 13B). Because there was no difference in total MC between the two groups, the Bcl-2-positive MC constituted a significantly ($p= 0.009$) greater percentage of MC in RAO-affected animals. Examination of large second-generation bronchi and bronchioles demonstrated comparable levels of Bcl-2 expression in each horse (Figure 14A-F).

Figure 13C suggests that horses with more than 10 neutrophils per microliter of BALF (day 12) expressed Bcl-2 in their MC while those with fewer neutrophils (including one RAO-affected animal) had very little observable Bcl-2 staining. There was no correlation between Bcl-2 positivity and Vs (data not shown).

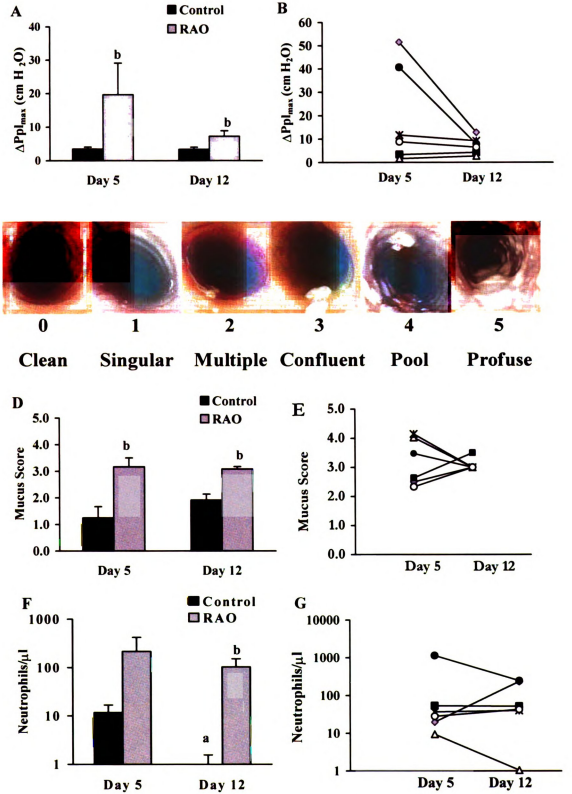

Figure 11. (A) Maximum changes in pleural pressure in RAO-affected and control horses on days 5 and 12. Values are means ± SEM of RAO (n=6) and control (n=6) horses after a 5-day hay/straw challenge (day 5) and

after 7 days on pellets/shavings (day 12). There was a significant difference between diseased and control horses on both days, but no effect of time in either group. (B) Individual values of ΔPpl_{max} in the six RAO-affected horses at days 5 and 12. There was considerable variability in ΔPpl_{max} at day 5; horses with the greatest values decreased most by day 12. (C) Subjective tracheal mucus score. The scoring system is as follows: 0=no visible mucus, 1=single or a few mucus globules; 2=many mucus globules that are beginning to coalesce, 3=many globules and a continuous mucous stream; 4=thick continuous stream and many coalescent mucus globules around the wall of the trachea; and 5=mucus stream fills about one quarter of the tracheal lumen. (D) Tracheal mucus score in RAO-affected and control horses on days 5 and 12. Values are means ± SEM of RAO (n=6) and control (n=6) horses after a 5-day hay/straw challenge (day 5) and after 7 days on pellets/shavings (day 12). There was a significant difference between diseased and control horses on both days, but no effect of time in either group. (E) Individual values of mucus score in the six RAO-affected horses at days 5 and 12. There was no consistent change in mucus score between the two days. (F) Total neutrophils per micoliter BALF of control and RAO-affected horses on days 5 and 12. Values are means ± SEM of RAO (n=6) and control (n=6) horses after a 5-day hay/straw challenge (day 5) and after 7 days on pellets/shavings (day 12). On day 12, the total neutrophils of control horses was significantly less than the day 5 value and significantly different from the RAO-affected horses. RAO-affected horses did not change from day 5 to day 12. (G) Individual values of neutrophils per micoliter BALF in the six RAO-affected horses at days 5 and 12. There were no consistent changes in neutrophils between the two days. (NOTE: Images in this thesis are presented in color).

[a] Significantly different from day 5 within group; [b] Significantly different from control at same point

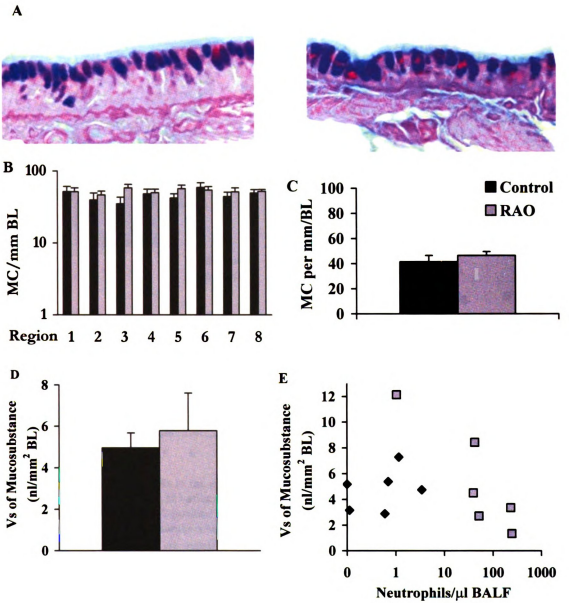

Figure 12. (A) Alcian Blue/Positive Acid-Schiff (AB-PAS) stained epithelium of a representative control (left) and RAO-affected horse (right). (B) Number of mucous cells (MC) per millimeter of basal lamina (BL). and (C) mean numbers of MC. No significant regional or disease effects were detected. Values are means ± SEM of RAO (n=6) and control (n=6) of MC in each region of lung. (D) Stored intraepithelial mucosubstance (Vs) in large cartilaginous airways of control and RAO-affected horses. There were no significant differences between groups. (E) Relationship between the total neutrophils per microliter BALF and Vs in control and RAO-affected horses. In RAO-affected horses, there was a negative correlation between the numbers of neutrophils and the Vs. Black diamonds (♦) represent control horses; grey squares (■) represent RAO-affected horses. Values are means ± SEM. (NOTE: Images in this thesis are presented in color).

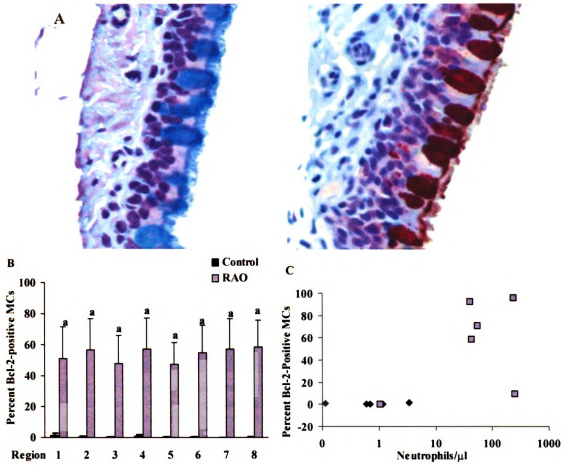

Figure 13. (A) Bcl-2 staining cells in the epithelium of a representative control (left) and RAO-affected horse (right). Bcl-2 staining in the RAO-affected horse was unmistakably more abundant than that seen in the control horse. (B) Percent Bcl-2-positive mucous cells (MC) present in each region of lung. RAO-affected horses had significantly greater numbers and percentages of Bcl-2-positive MC compared to controls for each region of lung sampled. Values are means ± SEM of RAO (n=6) and control (n=6) horses in each region of lung. (C) Percent Bcl-2-positive mucous cells (MC) in relation to total neutrophils per microliter BALF in RAO-affected and control horses. Horses having more than 10 neutrophils per microliter of BALF tended to have Bcl-2 staining in more than half of their MC while those horses with less inflammation had less expression. The one RAO-affected horse with similar numbers of neutrophils as controls had a similar percentage of Bcl-2-positive MC as the controls. Black diamonds (♦) represent control horses; grey squares (■) represent RAO-affected horses. (NOTE: Images in this thesis are presented in color).

[a] Significantly different from control in the same region.

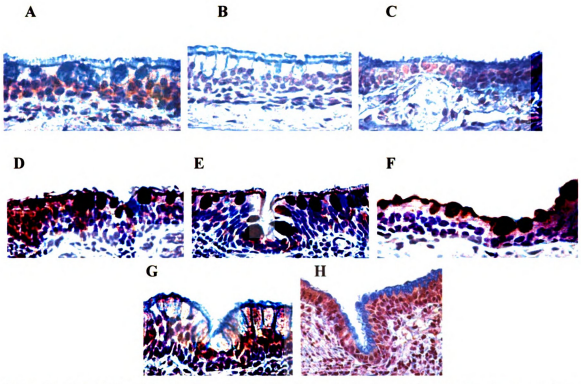

Figure 14. Bcl-2/AB staining in three airway generations. (A-C) A second-generation airway, a 4-6 mm diameter conducting airway, and a non-conducting airway of a control horse, (D-F) a second-generation airway, a 4-6 mm diameter conducting airway, and a non-conducting airway of a RAO-affected horse, (G, H) a 4-6 mm diameter conducting and a non-conducting airway from "horse 5." (NOTE: Images in this thesis are presented in color).

DISCUSSION

Although the expression of Bcl-2 by MC has been well established in rodents challenged with LPS, ozone, and allergens, its expression in spontaneously occurring airway disease has only been examined in cystic fibrosis (CF) (Harris, Fischer et al. 2005). In the latter, Bcl-2 was expressed in MC of CF patients but not of controls. The present study examined the differences in expression of MC Bcl-2 between control horses and those with RAO, an environmentally induced inflammatory obstructive airway disease. Bcl-2 was expressed in more than half of bronchial MC in RAO-affected animals but expression was trivial in the MC of controls that had undergone the same challenge.

The design of the experiment incorporated an organic dust challenge (hay feeding) known to induce inflammation and obstruction in RAO-susceptible animals followed by reduction of dust exposure (pellet feeding). Because there is little stored mucin in some RAO-affected horses during periods of acute inflammation, the week in the low-dust environment allowed time for both synthesis and storage of mucins and for GCH. As expected, the 5-day dust challenge resulted in airway obstruction and accumulation of mucoid secretions in RAO-affected animals—their ΔPpl_{max} and tracheal mucus scores were significantly greater than in controls. Although there were these functional differences between the two groups of animals, there was no significant difference in the severity of inflammation on Day 5. Others also have reported that control animals develop neutrophilic airway inflammation without changes in lung function in response to a similar dust challenge (Tremblay, Ferland et al. 1993; Gerber, Straub et al. 2004). After one week in the lower-dust environment, inflammation had significantly waned in controls but not in RAO-affected animals so that at the time of euthanasia, control animals had significantly fewer neutrophils in BALF than did the RAO-affected horses.

At the time of euthanasia, there were significant differences in Bcl-2 expression between the two groups of horses. In RAO-affected animals, Bcl-2 was expressed, on average, in over 40 percent of MC whereas less than 1 percent of cells expressed Bcl-2 in controls. Because I was concerned that disease may not be uniform throughout the lung, I examined eight different regions and found the Bcl-2 expression to be similar among bronchi. Although Bcl-2 expression was virtually absent in all control animals, there was some variation between individual RAO-affected horses. The animal that had very little inflammation on both Days 5 and 12 had Bcl-2 levels like a control animal in both large and small airways (Figure 14F and 6G). Figure 13C shows that Bcl-2 was only expressed in animals in which the neutrophil count exceeded 10 cells/μl and in RAO-affected animals, expression tended to increase with severity of neutrophilic inflammation.

A similar association between the magnitude of Bcl-2 expression in MC and numbers of neutrophils in airways also has been reported in rats challenged with two doses of LPS (Foster, Gott et al. 2003). However, neutrophils are not essential for Bcl-2 expression because, in the latter model, neutrophil depletion did not reduce Bcl-2 expression. The immune mechanisms of RAO are not well understood but TNF-α, IL-1, IL-4, IL-5, IL-8, IL-13 and IFN- γ are increased during the development and/or maintenance of the disease (Joubert, Silversides et al. 2001; Cordeau, Joubert et al. 2004). Furthermore there is evidence that oxidant stress is increased during acute exacerbations of RAO (Kirschvink, Smith et al. 2002). Many of these factors have been associated with changing levels of Bcl-2. In particular, TNF-α induces cell death by reducing the Bcl-2/Bax ratio (Luo, Xia et al. 2005) but this effect can be inhibited by IL-1 and NF-κB-induced up-regulation of Bcl-2 expression via degradation of the IκB inhibitor (Van Antwerp, Martin et al. 1996; Bours,

Bonizzi et al. 2000). The latter pathway is of interest in the case of the horse because NF-κB activity in airway brushings is positively correlated with the magnitude of airway obstruction and is maintained by persistent inflammation as indicated by TNF-α expression (Bours, Bonizzi et al. 2000). Another possibility is that increased Bcl-2 expression is induced by activation of the 15-lipoxygenase (Tang, Chen et al. 1996) pathway because the lungs of horses with RAO produce more 15-HETE than do controls (Gray, Derksen et al. 1992).

Although Bcl-2 was expressed in a greater percentage of MC in RAO-affected horses with neutrophilic inflammation, this was not associated with an increase in either the number of MC or the amount of stored mucins (Vs). One possible explanation for this observation is that the horses were euthanatized before GCH had occurred. This is unlikely because, in rats, MCM develops within 3 to 5 days of an allergen, LPS or ozone challenge (Tesfaigzi, Fischer et al. 2000). If the time course of events is similar in horses, the up to 12-day period of inflammation should have been sufficient to cause GCH. The negative correlation of Vs with neutrophil numbers ($r = -0.886$, $p=0.033$) in RAO horses supports neutrophil products being potent secretagogues. For this reason, I may not have been able to identify empty MC and could therefore have underestimated the MC numbers in horses with heaves. Assuming that there was adequate time for GCH and that all MC were identified, what is the role for Bcl-2? My initial hypothesis that Bcl-2 prolonged MC life and thereby increased MC numbers is clearly not supported by the data. An alternative role for Bcl-2 would therefore be prevention of premature MC death due to the oxidant stress and other factors produced during the inflammatory response. Over expression of Bcl-2 has been shown to induce accumulation of the active form caspase-9 in the mitochondria, rendering the cells resistant to the redox stress (Katoh, Tomimori et al. 2004). As neutrophils of RAO-affected horses produce reactive oxygen species (ROS), Bcl-2 may protect their MC from dying, while MC

of control horses undergo apoptosis, despite the generation of fewer ROS (Art, Kirschvink et al. 1999).

With regard to the MC numbers, they were similar to those reported in other species. The absence of GCH agrees with an earlier description of inconsistent morphological changes in the epithelium of larger airways of horses with COPD, a term that includes horses with RAO (Kaup, Drommer et al. 1990).

The presence of Bcl-2 in three different airway generations indicates that RAO is a condition that affects the entire tracheobronchial tree and is not limited to the peripheral airways although the inflammation may be most obvious in that region. Further support for the generalized nature of RAO is provided by the observations of up-regulation of eqMUC5AC (Gerber, Robinson et al. 2002) at all airway levels and the absence of iNANC function in large airways (Robinson, Derksen et al. 1996).

This study addressed the role of increased MC numbers, as a result of delayed death, as a potential cause of accumulated amounts of mucoid secretions in the airways of RAO-affected animals. My data indicate that MC numbers are not increased but that stored mucins are released in response to neutrophilic inflammation in RAO-affected horses. The increased expression of eqMUC5AC is necessary to replenish the secreted mucins (Gerber, Robinson et al. 2003). Finally the decreased mucus clearability further contributes to accumulation of secretions (Coombs and Webbon 1987).

APPENDIX

Activation of caspases require cleavage after an aspartate residue at an active cysteine in the middle of the conserved motif, QACRG present in all proteases, (Vaux and Strasser 1996). This cleavage results in the large (~20 kd) and small (~10 kd) subunits and their subsequent assembly into heterotetramers (Vaux and Strasser 1996). Each heterotetramer consists of two large and two small subunits with two active sites per molecule (Reed 2000). Substrates observed in humans are both nuclear and cytoplasmic proteins for DNA repair and replication, RNA splicing, cytoskeletal structure, and cell division (Vaux and Strasser 1996).

Once activated, executioner caspases cause the breakdown of normal cellular compartmental barriers and despite the intracellular disassembly, the plasma membrane is left intact (Hetts 1998). It is important to realize that caspase-mediated cell demise can be inhibited at set points. The second step of executioner caspase activation can be inhibited by the inhibitor of apoptosis proteins (IAPs), so there is no autocatalytic cleavage (Sprick and Walczak 2004). XIAP, the best described homolog, interacts with caspase-3 and the initiator caspase, caspase-9 (Salvesen and Duckett 2002).

Inhibition of caspases still leads to cytochrome c release and to non-apoptotic cell death; therefore, another caspase-independent mechanism for commitment, via the mitochondria must exist. Amarante-Mendes, Finucane et al (1998) described this as cell death without activation of caspases, externalization of phosphatidylserine, nuclear condensation, or DNA fragmentation. Instead, cytoplasmic and nuclear vacuolization is a morphological characteristic (Amarante-Mendes, Finucane et al. 1998). However, caspase-8 inhibitors will prevent Fas-induced cytochrome c release (Green and Reed 1998). Also, Bcl-2 (an anti-apopototic protein) expression inhibits this death and preserves the clonogenic potential of the cells (Amarante-Mendes, Finucane et al. 1998), while Bax (a pro-apoptotic protein) still induces cytochrome c release and apoptosis even with caspase inhibition (Xiang, Chao et al. 1996; Jurgensmeier, Xie et al. 1998).

Knockouts targeting caspase-9 (and also caspases-3) have severe defects in the brain and undergo perinatal mortality (Zheng, Hunot et al. 1999). Caspase-8 deficient embryos die after 12 days due to ablation of cell death induction by TNFRs, Fas/Apo1, and DR3 (Varfolomeev, Schuchmann et al. 1998). These embryos have impaired heart muscle development and congested accumulation of erythrocytes contained in larger and smaller blood vessels.

Inhibitor of Apoptosis Proteins

Inhibitors of apoptosis proteins (IAPs) are a family of evolutionarily conserved intracellular proteins that suppress apoptosis whether induced by the TNFR-dependent (extrinsic) pathway or the mitochondrial-dependent (intrinsic) pathway. This could be because its targets are caspases that are common to both pathways (Reed 2000). Yet, some will inhibit initiator caspases in the mitochondria pathway, perhaps reflective of the later evolution of the death receptor (TNFR) pathway. By definition, all IAP proteins contain 1 to 3 copies of the zinc-binding fold, baculovirus inhibitor of apoptosis proteins repeat (BIR) domains, and also may contain CARDs (Reed 2000). Members of this protein family, such as XIAP,

cIAP1, and cIAP2, can inhibit initiator caspase-9, via the third BIR domain, in addition to already activated effector caspases-3 and -7, via the first and second BIR domains (Reed 2000).

Death by the Mediator

Mitochondria have been fittingly referred to as the understudy for the executioner in triggering apoptosis (Green and Reed 1998). Mitochondria, originally viewed as initiators of apoptosis are now recognized as amplifiers of caspase activity (Borner and Monney 1999; Lassus, Opitz-Araya et al. 2002). This is evident by the positive feedback loop seen following cytochrome c activation; cytochrome c is essential for caspase activation (Liu, Kim et al. 1996) while caspases cause cytochrome c release from intact mitochondria (Hengartner 1998; Lassus, Opitz-Araya et al. 2002). If this is true, this system would require "dampeners" to prevent unwarranted death such as the anti-apoptotic Bcl-2 proteins and the IAPs to protect OMM integrity and inhibit particular caspases, respectively.

Mitochondria kill a cell through three mechanisms: (1) disruption of electron transport chain activity, (2) alteration of reduction-oxidation (redox) potential, and (3) release of sequestered caspase-activating proteins.

An early feature of apoptosis at the mitochondria is disruption of the electron transport chain and oxidative phosphorylation that results in a reduced production of the ATP required for many processes, thus loss of ATP kills the cell. However, this occurs very late in the apoptotic process and is not likely to be the means of induction (Green and Reed 1998).

Multiple mechanisms engage mitochondrial involvement, leading to release of cytochrome c and other factors normally residing in the inter-mitochondrial space (Figure 15). (Wang 2001). (Acehan, Jiang et al. 2002).

Mitochondria are major producers of superoxide, where 1% to 5% of electrons traveling down the electron transport chain are lost and form O_2^- and other reactive oxygen species (ROS) (Green and Reed 1998). For this reason, a good hypothesis would be a decrease in the coupling efficiency of the respiratory chain leads to increased ROS production. But like disruption of the electron transport chain, this may be a late event (Green and Reed 1998). Furthermore, experiments (Jacobson and Raff 1995) have shown that ROS are not required for apoptosis induced by stimuli that do not necessarily generate ROS, as originally thought. ATP depletion and severe oxidative stress can push an apoptotic cell towards necrosis (Orrenius 2004). Bcl-2 can protect cells in anaerobic conditions through means that do not require inhibition of ROS production. However, ROS can still be generated in complete anaerobiosis; thus, a role for ROS must still be considered (Degli Esposti and McLennan 1998).

During apoptosis, the permeablized OMM nonselectively releases proteins, in addition to cytochrome c, that is also implicated in apoptosis. These apoptogenic proteins are involved in caspase-dependent and -independent cell deaths and aid in ensuring valid execution. Cytochrome c, Smac/Diablo, and apoptosis inducing factor (AIF) are the main proteins and

all require modifications within the mitochondria to become inducers of cell death; heme attachment to cytochrome c, flavin adenine dinucleotide (FAD) attachment to AIF and proteolytic processing of the N-terminal mitochondrial leader peptide for AIF and Smac/Diablo (Reed 2000; Wang 2001). This prohibits premature apoptosis from taking place (e.g. during biosynthesis) and functionally links apoptosis to a disturbance in the barrier function of the mitochondria (Reed 2000). Endonuclease G (EndoG), Omi/HtrA2 (protease), and caspases-2, -3, and –9 are also released from the mitochondria (Reed 2000; Wang 2001; Donovan and Cotter 2004).

Smac/Diablo, a 25-kD protein, is released from inside the inner mitochondrial membrane with cytochrome c (Du, Fang et al. 2000). The N-terminal of mature Smac/Diablo binds to IAPs and removes their inhibitory effects on caspases (Wang 2001). There is a direct competition between activated caspases and Smac/Diablo providing a feedback system. The presence of high amounts of IAPs terminates the activity of any caspases activated by newly formed apoptosomes, thus providing a safety net for transient or accidental release of cytochrome c (Wang 2001). Smac/Diablo is a larger molecule than cytochrome c and does not escape the confines of the mitochondria in this event (Chai, Du et al. 2000). However, if there is persistent and/or severe damage to the OMM, a large amount of Smac/Diablo is released to remove the inhibition of the IAPs (inhibitor of apoptosis proteins), for example the X-linked-inhibitor-of-apoptosis protein (XIAP) (Figure 15). In addition to the cytochrome c pathway, Smac/Diablo and IAPs regulate the death receptor pathway because the two pathways converge at caspase-3. Since XIAP does not inhibit Caspase-8 of the extrinsic pathway, apoptosis can still occur via cleavage of a pro-apoptotic Bcl-2 family member, the BH3-only Bid, by caspase-8. Activated Bid (tBid) inserts into the mitochondrial membrane and releases Smac/Diablo, cytochrome c, and AIF.

Apoptosis inducing factor (AIF), a 57-kD flavoprotein that resembles bacterial oxidoreductase, also resides in the intermembrane space of mitochondria and induces apoptosis in a caspase-independent manner (Daugas, Nochy et al. 2000; Wang 2001). Apoptosis induces AIF's translocation to the nucleus, condensation of chromatin, and DNA fragmentation (Susin, Lorenzo et al. 1999). The mechanisms are unclear how AIF brings about these changes since it itself has no measurable DNase activity (Susin, Daugas et al. 2000).

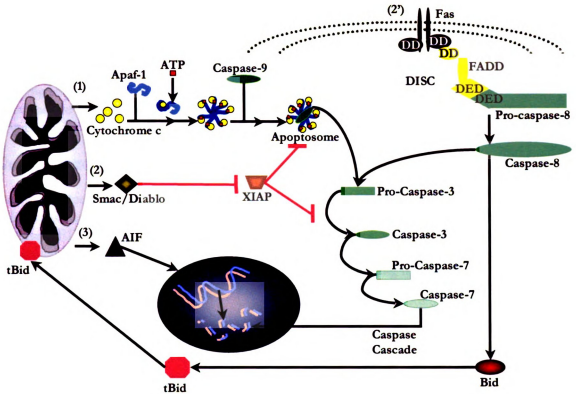

Figure 15. Mitochondria are amplifiers and mediators of apoptosis, rather than initiators. (1) Cytochrome c leaks out of the intermembrane space into the cytosol where it binds to and increases nucleotide-binding affinity of Apaf-1 by 10-fold. ATP (or dATP) binding to Apaf-1/cytochrome c induces a shapa change and oligomerization into a multimeric wheel-like structure to which caspase-9 binds to. This functional complex is called an apoptosome. The apoptosome cleaves pro-caspase-3 and releases active caspase-3, which will cleave pro-caspase-7 and release caspase-7 in the caspase cascade, leading to nuclear fragmentation and apoptosis. (2) Smac/Diablo is released when the functional barrier is disrupted. In the cytosol, Smac/Diablo's N-terminal binds to the BIR domain of IAPs (e.g. XIAP) and removes the inhibitory effects of XIAP on caspase-3 and -9. (2') In the presence of high IAPs, Smac/Diablo cannot bind enough IAP and the inhibitory affects remain. The Fas-mediated pathway can still initiate apoptosis, however, because of caspase-8 cleaving and activating cystolic Bid, a pro-apoptotic Bcl-2 family member, causing its translocation and insertion into the outer mitochondrial membrane (OMM). Insertion of truncated Bid (tBid) leads to OMM permeabilization. Caspase-8 is insensitive to XIAP (Riedl, Renatus et al. 2001). (3) AIF is released and induces nuclear condensation, independent of any caspase activation.

EXTRINSIC PATHWAY PROTEINS

Death Domains

Many proteins involved in death pathways have so-called death domains (DD). Oligomerization of one protein's DD with another's facilitates execution of apoptosis by bringing required molecules into close proximity with a receptor or protease, for example. This leads to initiator caspase activation followed by activation of downstream effector caspases.

Death Effector Domains

Death effector domains (DED) are found in the initiator caspases, caspase-8 and caspase-10, in humans (Reed 2000). Cleavage of these pro-caspases requires interaction of the two pro-caspase DEDs with the DED on the adapter protein, Fas-associated death domain (FADD), also called MORT1. This adapter protein is absolutely crucial in caspase activation because it contains *both* a DD and DED (Zhang, Cado et al. 1998), linking it to the receptor (via DD) to the pro-caspases (via DED). Additional DED-containing modulators are described in the review by Reed (2000).

Caspase-Associated Recruitment Domains

Caspase-associated recruitment domains (CARD) are found on the N-terminals of pro-caspases for caspase-1, -2, -4, -5, and -9 in humans and caspase-1, -2, -9, -11, and -12 in mice (Reed 2000). They are similar in structure as the DD and DED. Ablation of CARD-carrying caspases has significant effects on disease, including, reduced tissue loss after stroke, increased resistance to endotoxin-induced sepsis, slowing of Huntington's disease progression (Bergeron, Perez et al. 1998; Ona, Li et al. 1999). Importantly, Apaf-1 contains a CARD that mediates interaction with the CARD on pro-caspase-9 to initiate caspases by the induced proximity model.

Ligand binding to the receptor induces a shape change on the intracellular portion of the receptor to expose the DD and facilitate oligomerization with the DD of an adapter protein, and possibly binds additional downstream adapter proteins. Pro-caspase-8 binding to the adapter protein FADD/MORT1 via their DEDs completes the formation of the DISC, to which additional pro-caspases can bind and autocatalytically cleave and activate caspases (Boldin, Goncharov et al. 1996, Muzio, 1996 #100). Therefore, FADD/MORT1 indirectly links pro-caspase-8 to the cystolic domains of TNF family of death receptors [e.g. Fas (Apo1/CD95)].

Numerous experiments have been performed to characterize the functions, interactions, and regulations of members of the Bcl-2 family. Gene disruptions experiments have shown that Bcl-2 is widely expressed during embryogenesis and becomes more restricted postnatally (Chao and Korsmeyer 1998). Newborn *bcl-2*$^{-/-}$ or *bcl-x*$_L$$^{-/-}$ animals have extensive cell death and usually die within a few weeks. Mice *bcl-2*$^{-/-}$ knockouts are born viable where *bcl-x*$^{-/-}$

animals die at around embryonic day 13 (Chao and Korsmeyer 1998). The *bcl-2* $^{-/-}$ mice turn gray at 5 to 6 weeks reflecting decreased melanocyte survival and have polycystic kidney disease-like changes in the renal tubules (Nakayama, Negishi et al. 1994). These mice appeared to have a normal nervous system unlike Bcl-x ablated mice. Mice lacking Bcl-x have substantial death of hematopoietic cells and postmitotic immature neurons in brain, spinal cord, and dorsal root ganglia (Motoyama, Wang et al. 1995).

There is evidence to support a two-class model for BH3 domains: 1) Bid-like domains that activate, and 2) Bad-like domains that sensitize (Letai, Bassik et al. 2002). Bid and Bim with a α-helical BH3 domain are capable of activating and mediating Bax and Bak oligomerization, while Bad and Bik preferentially interact with the pockets of the anti-apoptotic members like Bcl-2 and Bcl-x_L, which displaces Bid-like BH3 domains. Most of the BH3-only proteins act as trans-dominant inhibitors of anti-apoptotic members and are unable to homodimerize (Reed 2000). Of those listed in Table 1, only Bik and Blk are similar to each other and all others are unrelated to any known protein (Adams and Cory 1998).

The function of either Bax or Bak is required to initiate most forms of apoptosis. Independently, *Bax* inactivation has only slight effects on apoptosis with modest phenotypic abnormalities and *Bak* inactivation shows no effects (Lindsten, Ross et al. 2000) and in both models underwent Bim- and Bad-induced apoptosis (Zong, Lindsten et al. 2001). However, *Bax*$^{-/-}$*Bak*$^{-/-}$ double-knockouts showed dramatic impairments of apoptosis (preservation of interdigital webs, an imperforated vaginal canal, and accumulations of excess cells within the central nervous and hematopoietic systems) and die perinatally (Lindsten, Ross et al. 2000). *Bax*$^{-/-}$ mice have apparent normal external development, however, they exhibit hyperplasia of thymocytes and B cells and the gonads, in both ovaries and testis, as well as hypoplasia accompanied by multinucleated giant cells and dysplastic cells (Knudson, Tung et al. 1995). Overexpression of Bax accelerated cytokine deprivation-induced cell death in a IL-3-dependent cell line (Oltvai, Milliman et al. 1993). Constitutively active forms of Bim and Bad fail to induce apoptosis in the mice without Bax or Bak (Zong, Lindsten et al. 2001).

In a recombinant Bcl-2 protein experiment, Schendel, Xie et al (1997) found that Bcl-2 forms distinct ion-conducting, cation-selective channels. Such pore forming abilities are dependent on a low pH and acidic lipid membranes to permit proper orientation. Acidic pH has not only been shown to induce pore formation, but it stabilizes hetero- or homodimerized Bcl-x_L to Bcl-2, Bax, and Bid (Xie, Schendel et al. 1998). The perpendicular insertion of an estimated minimum of four dimerized or oligomerized hydrophobic α-helices into the lipid bilayer provide the make up of the channel's core. This channel is in a closed state *in vitro* at neutral pH; however pH-dependent mechanisms for channel formation are unlikely because low *in vivo* pH does not induce channel formation but only promotes association of pore-forming fragments with the mitochondrial membrane (Schendel, Xie et al. 1997).

Both Bcl-2 and Bax insert into experimental lipid membranes in a pH-dependent fashion with maximum activity at pH of 4.0 (Schlesinger, Gross et al. 1997). Bax channels have a minimal conductance of 22pS with at least three subconductance levels (Schlesinger, Gross et al. 1997) that decrease with an increase in pH (0.731 nS at pH 4.0 to 0.329 nS pH 7.0) and mild chloride anion selectivity in a predominantly open state. In contrast, Bcl-2 or Bcl-x_L channels are slightly potassium cation selective (Schendel, Xie et al. 1997) and have a prominent conductance of 80pS (Chao and Korsmeyer 1998), although 20pS has been reported in lipid membrane experiments (Schendel, Xie et al. 1997). Despite these moderate differences in ion selectivity of the Bcl-2 and Bax channels, other characteristics such as conductance, voltage dependence, and rectification are exhibited making either protein a candidate for exerting regulatory or functional components on the PT pore and cytochrome c release (Schlesinger, Gross et al. 1997).

PT is always followed by $\Delta\Psi_m$, but $\Delta\Psi_m$ is not always caused by PT, and cytochrome c may still be released without $\Delta\Psi_m$ (Bernardi, Scorrano et al. 1999; Kroemer and Reed 2000). This may be possible due to the rapid opening and closing of the channel, at a low conductance state, to allow for a repeated, respiratory-driven re-establishment of $\Delta\Psi_m$. This explains why OMM disruption and cytochrome c release precede $\Delta\Psi_m$ collapse (Green and Reed 1998). Evidence exists, however, for PT to induce apoptotic $\Delta\Psi_m$ dissipation (Zamzami, Marchetti et al. 1996).

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