MOLECULAR MECHANISMS ASSOCIATED WITH

CANINE CYCLIC HEMATOPOIESIS

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MOLECULAR MECHANISMS ASSOCIATED WITH CANINE CYCLIC HEMATOPOIESIS

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A Dissertation

Submitted to

the Graduate Faculty of

Auburn University

in Partial Fulfillment of the

Requirements for the

Degree of

Doctor of Philosophy

Auburn, Alabama
May 10, 2008

MOLECULAR MECHANISMS ASSOCIATED WITH

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Ronghua Meng, daughter of Zhaoxian Meng and Jilan Cui, was born October 24, 1973, in Ankang, Shaanxi province, People's Republic of China. She graduated from Ankang High School in 1990, and then attended Northwest Agricultural University in Yangling, Shaanxi province, for seven years. She graduated with a Bachelor of Science degree in Agronomy in 1994 and a Master of Science degree in Plant Genetics and Breeding in 1997, respectively. After working as a research assistant scientist at the Crop Institute, Beijing Academy of Agriculture and Forestry Sciences, she entered graduate school at Tuskegee University in January 2002 and graduated with a Master of Science degree in Plant Molecular Biology. She enrolled as a Ph.D. student in the Cellular and Molecular Biosciences program at Auburn University in January 2004, and then started her dissertation research in the Biomedical Sciences Graduate Program in the College of Veterinary Medicine in January 2005. She married Hui Gao in August 2000, and has one son, Yi (Kevin M.) Gao.

DISSERTATION ABSTRACT

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CANINE CYCLIC HEMATOPOIESIS

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Doctor of Philosophy, May 10, 2008
(M.S., Tuskegee University, 2004)
(M.S., Northwest Agricultural University, 1997)
(B.S., Northwest Agricultural University, 1994)

172 Typed Pages

Directed by Carl A. Pinkert

Canine cyclic hematopoiesis (CH) is an autosomal recessive disorder characterized by 12-14 day neutrophil cycles, a diluted coat color, and platelet storage pool disease. Canine CH is caused by a mutation in *AP3B1*, encoding the β3A subunit of adaptor protein complex – 3 (AP-3). AP-3 is a protein complex responsible for intracellular protein trafficking from the *trans*-Golgi network (TGN)/endosome to lysosomes and secretory lysosome-like granules. Canine CH is a model of human cyclic neutropenia/severe congenital neutropenia caused by mutations in the neutrophil elastase (NE) gene *ELA2*. In this dissertation, it was hypothesized that NE processing/trafficking was affected by the β3A gene mutation in CH dogs.

To study canine NE processing and trafficking, both polyclonal and monoclonal antibodies against two immunogenic peptides of canine NE were generated. The generated antibodies were characterized that recognize precursor (ELA269) or mature (ELA85) canine NE. These antibodies were used in western blot and immunocytochemistry analysis in this study. Results showed that neutrophils from the CH dog accumulated large amounts of NE precursor proteins. Granule-localized mature NE enzymes were significantly reduced in the CH dog. Corresponding to the accumulation of NE precursor proteins, protein expression of BiP/GRP78, a sensor of the unfolded protein response (UPR) and a chaperone which promotes folding and prevents aggregation of nascent proteins, was increased >5 fold in neutrophils from the CH dog. Up-regulation of BiP/GRP78 was demonstrated in *in vitro* cultured bone marrow mononuclear cells (BMMCs) stimulated with stem cell factor and granulocyte-colony stimulating factor in the CH dog. In addition, these cultured BMMCs from the CH dog showed increased apoptosis (20%) compared to normal dogs.

The induction of the UPR and apoptotic cell death of hematopoietic progenitor cells due to inefficient NE processing/trafficking represent a novel mechanism associated with neutropenia in CH dogs. Collectively, these studies further characterize the pathoetiology of canine CH and describe likely molecular mechanisms related with this disorder.

ACKNOWLEDGMENTS

I would like to thank my major advisor, Dr. Clinton D. Lothrop Jr., for offering an invaluable opportunity to study in this exciting research area and for his research guidance. I am grateful to Drs. Carl A. Pinkert, Glenn P. Niemeyer, Marie W. Wooten, Narendra K. Singh, Robert L. Judd, and outside reader Dr. Kevin Huggins, for serving on my graduate committee, for their advice on my research program, and their critical reading of my dissertation. I appreciate the contributions of the many people associated with the Biomedical Sciences Graduate Program, Dr. Robert Locy and the Cellular and Molecular Biosciences (CMB) Program, and the Scott-Ritchey Research Center that provided assistance, support and consideration through my graduate studies at Auburn University. Great appreciation goes to lab members: Nominanda Borbosa, David Upchurch, Steven Waters, Bridget Dean and Christopher Smith, and I thank Dr. Maria Toivio-Kinnucan for her electron microscopy analyses.

Special thanks go to my husband Hui, my son Kevin and all my family members in China, for the love, understanding, and support they provided during my graduate studies at Auburn University.

Style manual or journal used: (journal) Blood

Computer software used: Microsoft Word 2003, Adobe® Photoshop 6.0, Reference Manager 11, Microsoft® Excel 2002, Summit Software 4.3

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LIST OF ABBREVIATIONS

AP	adaptor protein complex
ATF6	activating transcription factor
BiP	proteins bound to immunoglobulin in pre-B cells
BMMCs	bone marrow mononuclear cells
BSA	bovine serum albumin
CCVs	clathrin-coated vesicles
CH	cyclic hematopoiesis
CN	cyclic neutropenia
DAB	3, 3'-diaminobenzidine
ER	endoplasmic reticulum
FBS	fetal bovine serum
FITC	fluorescein isothiocyanate
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
G-CSF	granulocyte-colony stimulating factor
GM-CSF	granulocyte-macrophage colony-stimulating factor
GRP78	glucose-regulated protein with molecular weight 78kD
HPS	Hermansky-Pudlak syndrome
HRP	horseradish peroxidase

Ig	immunoglobulin
IMDM	Iscove's modified Dulbecco's medium
IRE1	inositol-requiring enzyme 1
LAMP	lysosome-associated membrane protein
LPS	lypopolysaccharide
MPO	myeloperoxidase
NE	neutrophil elastase
PBS	phosphate buffered saline
PERK	double-strand RNA-activated protein kinase (PKR)-like ER kinase
PI	propidium iodide
PMN	polymorphonuclear neutrophil
RBL	rat basophilic leukemia cell line
SCF	stem cell factor
SCN	severe congenital neutropenia
SLPI	secretory leukocyte proteinase inhibitor
SPD	platelet storage pool deficiency
TBS	Tris-buffered saline
TEM	transmission electron microscopy
TGN	Tran-Golgi network
UPR	unfolded protein response

CHAPTER 1

LITERATURE REVIEW AND RESEARCH OBJECTIVES

1.1 Canine cyclic hematopoiesis

Canine cyclic hematopoiesis (CH), known as canine cyclic neutropenia or grey collie syndrome, is an autosomal recessive disorder occurring only in the collie breed (Lund *et al.*, 1967; Dale *et al.*, 1972a; Dale *et al.*, 1972b). Affected dogs can be identified at birth by a diluted coat color and small body size. The hallmark of CH in the dog is 12 to 14-day cycles of neutrophils, monocytes, lymphocytes, eosinophils, reticulocytes and platelets, albeit they oscillate in different pattern (Dale *et al.*, 1972a; Weiden *et al.*, 1974; Jones *et al.*, 1975b). The regular cycle of blood cells in CH dogs is not due to shortened survival of cells (t1/2), but a regulatory defect rooted from on/off proliferation and differentiation of pluripotent hematopoietic stem/progenitor cells (Dale *et al.*, 1972a; Weiden *et al.*, 1974; Dale & Graw, 1974; Dunn *et al.*, 1977). Among the different cell populations, neutrophils fluctuate predominantly and the regular neutropenic episodes primarily contribute to the clinical disease (Lund *et al.*, 1967; Chusid *et al.*, 1975).

1.1.1 Clinical symptoms of canine cyclic hematopoiesis

CH dogs are born weak compared to their littermates. Most of them die within the first week and puppies that survive usually succumb later due to repeated infections. The

CH dogs can live up to 4-7 years age with intensive medical care, However, lifelong infections will accompany the dog unless they are housed in a pathogen-free environment (Lund *et al.*, 1967; DiGiacomo *et al.*, 1983). The CH dogs are highly susceptible to microbial infections during the 3-4 day neutropenic periods. Clinical symptoms include fever, septicemia, enteritis, arthritis, anorexia, conjunctivitis, rhinitis, and other signs characteristic of gastrointestinal or respiratory infections (Lund *et al.*, 1967; DiGiacomo *et al.*, 1983). Anemia is a consistent symptom in grey collies. In addition, hyperglobulinemia is common in affected CH dogs and extensive amyloidosis occurs in spleen, liver, kidney after recurrent episodes of fever and infection (Cheville, 1968; DiGiacomo *et al.*, 1983). The high morbidity and mortality of CH dogs at younger age is attributed to the age-related changes in their tissues which impair various organ systems.

1.1.2 Treatments

Antibiotic therapy and fluid replacement are commonly used to treat the early signs of fever or dehydration in CH dogs. Other treatments that have been investigated include administration of endotoxin (Hammond *et al.*, 1978; Hammond *et al.*, 1979), lithium carbonate (Hammond & Dale, 1980; Hammond & Dale, 1982; Hammond *et al.*, 1987), or growth factors (Lothrop *et al.*, 1988; Hammond *et al.*, 1990; Pratt *et al.*, 1990; Mishu *et al.*, 1992; Dale *et al.*, 1995), and bone marrow transplantation (Dale & Graw, 1974; Jones *et al.*, 1975b).

1.1.2.1 Endotoxin or lithium carbonate administration

Endotoxins (lypopolysaccharide; LPS) are components of bacterial cell walls (Ulevitch & Tobias, 1995). Endotoxin derived from gram-negative bacteria can activate complement and has multiple effects on the hematopoietic system. Administration of endotoxin (5 μg/kg/day) eliminates the neutrophil cycle in CH dog and stabilizes the reticulocyte and platelet counts in the normal range. The continual administration of endotoxin also abrogates the cyclical pattern of hematopoietic progenitor cells in the bone marrow (Hammond *et al.*, 1978; Hammond *et al.*, 1979). However, endotoxin treatment is not without toxic side effects to the CH dog. Lithium carbonate, an agent known to stimulate leukocytosis in human beings and mice, abrogates the fluctuation of all cell types and prevents severe neutropenia in CH dogs (Hammond & Dale, 1980; Hammond & Dale, 1982; Hammond *et al.*, 1987). Corresponding to this effect, CH dogs treated with lithium no longer have fever or other clinical signs of infections. Endotoxin and lithium therapy altering neutrophil production in CH dogs is thought to be due to the direct influence on the flux of cells from the pluripotent stem cell pool into the committed stem cell compartment.

1.1.2.2 Growth factor treatments

The differentiation of hematopoietic progenitor cells towards a particular cell lineage is coordinated by the action of growth factors and cytokines in the extracellular matrix (ECM). Signaling mediated by stem cell factor (SCF) and its receptor c-*kit* plays a key role in maintaining self-renewal, differentiation, and growth of hematopoietic stem cells, mast cells, and melanocytes (Broudy, 1997). Long-term treatment of grey collies

with recombinant canine SCF (rc-SCF) abrogated the neutropenic periods in a dose-dependent manner (Dale *et al.*, 1995). Granulocyte-colony stimulating factor (G-CSF), a stimulus of granulopoiesis, induces expression of genes in neutrophilic lineage and hence the production of neutrophils (Berliner *et al.*, 1995). Administration of G-CSF prevented recurrent neutropenia in CH dogs (Lothrop *et al.*, 1988). The fluctuations in the other cell types were also blunted, and this phenomenon was sustained for months in dogs treated with either recombinant human or canine G-CSF (rhG-CSF or rcG-CSF) (Pratt *et al.*, 1990; Mishu *et al.*, 1992). Administration of recombinant granulocyte-macrophage colony-stimulating factor (rcGM-CSF), which is capable of inducing normal erythropoiesis, thrombocytosis and granulopoiesis, effectively increase eosinophil, monocyte and neutrophil counts in CH dogs. However, treatments with rcGM-CSF did not alter cyclic fluctuation in blood cell counts (Hammond *et al.*, 1990).

1.1.2.3 Bone marrow transplantation

Cyclic fluctuation of blood cells in grey collies can be completely cured by allogeneic bone marrow transplantation (Dale & Graw, 1974). Recipient dogs re-established normal granulopoiesis, and hence correct the periodic neutropenia. Conversely, when a normal dog was transplanted with bone marrow from a CH dog, it developed regular neutropenia and a cyclic rise and fall in numbers of platelets, reticulocytes and neutrophils (Weiden *et al.*, 1974; Jones *et al.*, 1975b). These experiments demonstrated that the cyclic fluctuation of all blood cells in CH dogs is caused by a regulatory defect in hematopoietic stem cells.

1.1.3 Genetic basis of canine cyclic hematopoiesis

1.1.3.1 The genes encoding growth factors

The hematologic abnormality and diluted coat color of the CH dog is phenotypically similar to *steel* mice, which have defects in pigmentation, germ cells and hematopoiesis with a mutation in the gene encoding SCF (Witte, 1990). The phenotype of the CH dog is also similar to *white-spotting* (W) mice (Chabot *et al.*, 1988) and human piebaldism (Syrris *et al.*, 2002), both having mutations in the SCF tyrosine kinase receptor c-*kit*. However, cloning and sequencing of SCF from CH dogs showed that there was no mutation present in the coding region of SCF gene in the dog (Dale *et al.*, 1995). Furthermore, CH dogs responded to rcSCF treatment, suggesting that the disease is not caused by the mutation in c-*kit* gene (Dale *et al.*, 1995). High dose G-CSF treatment has been known to stimulate neutrophil production in CH dogs and effectively eliminate cyclic hematopoiesis in these dogs, but *in vitro* grey dog bone marrow mononuclear cells required several fold higher G-CSF concentrations than normal dog cells to achieve half-maximal colony growth (Hammond *et al.*, 1990; Avalos *et al.*, 1994). Therefore, a defect in the G-CSF signal transduction pathways or the expression of G-CSF and its receptor had been proposed as a cause of canine cyclic hematopoiesis. However, it was determined that abnormal response to G-CSF in the progenitor cells from CH dogs is not associated with the mutation in the genes encoding either G-CSF or its receptor G-CSFR, as well as the alterations in early G-CSF/G-CSFR signaling (Avalos *et al.*, 1994).

1.1.3.2 *ELA2*, encoding neutrophil elastase

Cyclic neutropenia (CN) and severe congenital neutropenia (SCN) are two main forms of hereditary neutropenia in human beings (Berliner *et al.*, 2004). Human CN is characterized by periodically severe neutropenia and vulnerability to bacterial infections during the nadir of the cycle. Human CN, like canine CH, is a consequence of periodic failure of pluripotential stem cells, hence the cyclic oscillation of all blood elements (Guerry *et al.*, 1973; Krance *et al.*, 1982). Canine cyclic hematopoiesis is analogous to human CN and is a useful animal model for studying molecular mechanisms associated with human CN. However, human CN is frequently inherited as an autosomal dominant disorder, and is characterized by chronic, cyclical recurrence of severe neutropenia at 21-day intervals (Guerry *et al.*, 1973). Up to now, all identified human CN cases are caused by heterozygous point mutations of *ELA2*, encoding the hematopoietic serine protease neutrophil elastase (NE) (Horwitz *et al.*, 1999). Mutations in *ELA2* are also responsible for sporadic SCN and autosomal dominant types of SCN, indicating that NE is likely associated with the regulation of hematopoiesis (Horwitz *et al.*, 2007). However, canine cyclic hematopoiesis is not caused by the mutation in *ELA2*, although studies showed that NE enzymatic activity is significantly reduced in CH dog neutrophils (Benson *et al.*, 2003).

1.1.3.3 *AP3B1*, encoding β3A subunit of adaptor protein complex-3 (AP-3)

Phenotypically, canine cyclic hematopoiesis resembles another rare human disorder Hermansky-Pudlak syndrome type -2 (HPS-2), which is a member of a group of disorders characterized by the combination of oculocutaneous albinism and prolonged bleeding (Di

Pietro & Dell'Angelica, 2005). HPS is classified from HPS-1 to HPS-7, each of which is caused by mutations in different genes. Interestingly, all the genes mutated in HPS subtypes are encoding proteins which are involved in the biogenesis/function of lysosome or lysosome-related organelles, such as melanosomes in melanocytes, platelet dense granules in platelets, and primary granules of granulocytes (Di Pietro & Dell'Angelica, 2005). Unlike the other HPS subtypes, HPS-2 also manifests congenital neutropenia and mild immunodeficiency. HPS-2 is caused by a mutation in *AP3B1*, encoding β3A subunit of adaptor protein complex -3 (AP-3) (Dell' Angelica *et al.*, 1999b). By cloning dog *AP3B1* cDNA and sequencing the CH dog genome, Benson et al. identified that the affected dog contains one coding alteration, where an extra adenosine is inserted into a nine adenosine tract in exon 20 of *AP3B1*, causing a frameshift and premature translation termination (Benson *et al.*, 2003). Therefore, the genetic basis of canine cyclic hematopoiesis is this insertional mutation in *AP3B1*. Since CH dogs also have a significantly reduced amount of NE and the *ELA2* mutations are the genetic basis of human CN and some cases of SCN, Benson et al. hypothesized that elastase might be a cargo protein of AP-3. Yeast two hybrid experiments demonstrated the *in vitro* interaction between mature human elastase and the μ3A subunit of AP-3. Therefore, the mutation of *AP3B1* disrupts the stability of AP-3 and hence interrupts the granular targeting of NE, resulting in reduced accumulation of elastase in granules. However, analysis of CH and CH carrier dogs demonstrated that heterozygous CH carrier dogs produce a homogeneous population of normal *AP3B1* transcripts, while homozygous affected dogs produce a heterogeneous population of *AP3B1* mRNA containing wild-type transcripts with nine adenines (Benson *et al.*, 2004). This paradoxical expression of *AP3B1* gene in CH

heterozygotes and normal β3A mRNA in affected dogs is due to transcriptional slippage of RNA polymerase in similar repetitive sequences (Benson *et al.*, 2004). The generation of normal *AP3B1* transcripts in CH dogs may complicate the understanding of mechanisms associated with this disorder.

1.2 Development of neutrophils and the storage of proteins in neutrophil granules

Blood is composed of different types of fully differentiated, mature cells (white blood cells, red blood cell, platelets) which are derived from one common progenitor cell, the pluripotent stem cell, in the bone marrow (Babior & Golde, 1996). Neutrophil maturation can be divided into following stages: myeloblast, promyelocyte, myelocyte, metamyelocyte, band cells and segmented neutrophil (polymorphonuclear neutrophil; PMN). The myeloblast is a committed lineage progenitor cell with a large oval nucleus, sizable nucleoli, and few or no granules. Mitotic cell divisions occur during the first three stages of neutrophil maturation, and the cells after the myelocyte stage no longer undergo mitosis. It takes an average of two weeks for a neutrophil to develop from a myeloblast in human beings and seven days in dogs.

1.2.1 Neutrophil granules

Proliferation and differentiation from the myeloblast stage to the segmented neutrophil is accompanied by the expression of genes encoding the proteins which are involved in host defense, and the packing of these proteins into subcellular compartments – granules. It is believed that granules are generated by aggregation of immature transport vesicles that bud off from the *trans*-Golgi network (TGN), which is a final sorting station

for proteins to be routed to either constitutively secreted pathway or regulated secreted pathway (stored in granules) (Sossin *et al.*, 1990; Rothman & Orci, 1992). Generally, neutrophil granules are classified into three types according to their size, morphology, electron density and the presence of marker proteins (Borregaard & Cowland, 1997). Large peroxidase-positive granules, known as azurophil/primary granules, which are formed during the promyelocyte stage, and persistent in mature neutrophils (Smolen & Boxer, 1996). Primary granules are characterized by their content of hydrolytic and bactericidal proteins. Primary granules can be further divided into large defensin-rich azurophil granules with a peripheral distribution of MPO, and small defensin-poor azurophil granules with a uniform distribution of MPO (Egesten *et al.*, 1994; Bainton, 1999). Peroxidase-negative granules include the specific/secondary granules and tertiary/gelatinase granules. Specific granules appear in the myelocyte/metamyelocyte stage, and tertiary/gelatinase granules form during the band cell stage. Specific granules contain several potent antimicrobial peptides such as lactoferrin, lysosome, and three metalloproteases. Lactoferrin is a common marker used to identify specific granules. Tertiary granules mainly contain acetyltransferase, β2-microglobulin, lysozyme and gelatinase; gelatinase is a marker protein for tertiary granules.

1.2.2 Primary/azurophil granule

Like lysosomes, primary granules contain granulophysin (CD63) in their membrane (Cham *et al.*, 1994). However, they do not express the lysosome-associated membrane proteins (LAMP) -1 and LAMP-2 which are commonly found in lysosomes, and the sorting of proteins into primary granules uses a pathway which is different from protein

lysosomal targeting (Dahlgren *et al.*, 1995). Therefore, primary granules are considered lysosome-like secretory granules which store and release bioactive agents upon pathogen stimulation.

The neutrophil primary granule lumen is the storage site for a group of antimicrobial polypeptides and digestive enzymes. These bioactive agents include myeloperoxidase (MPO), defensin, azurocidin, bacterial/permeability-increasing protein (BPI), NE, proteinase 3, and cathepsin G. Among these agents, MPO and NE are two important cytolytic proteins in the neutrophil microbicidal system. MPO is a 150kD heme enzyme that uses the superoxide ion and hydrogen peroxide generated by the neutrophil oxidative burst to produce hypochlorous acid and other reactive oxidants. MPO is also a commonly used marker protein for identification of primary granules.

1.2.3 Neutrophil elastase

In human beings, mature NE is a 218 residue, single chain glycosylated serine protease (Sinha *et al.*, 1987). NE precursor polypeptides are synthesized in promyelocyte and promonocyte stage cells, and the mature, active forms of the protein are mainly stored in neutrophil primary granules and monocyte lysosomes (Unanue, 1976; Cramer *et al.*, 1989). NE is capable of functioning at neutral pH and destroying a broad spectrum of substrates, including elastin, collagen, the protein component of proteoglycan, fibronectin, laminin, and cytokines such as G-CSF (Pham, 2006). When microorganisms are engulfed by neutrophils, NE is released from the granules into vacuoles for proteolysis. In addition, a great amount of NE is secreted out of the cells constitutively or by a regulated mechanism (Garwicz *et al.*, 2005). Extracellular NE is tightly bound by its endogenous

inhibitors – α_1 antitrypsin, secretory leukocyte proteinase inhibitor (SLPI) and elafin to prevent damage NE would cause to exposed tissues (Korkmaz *et al.*, 2005). Human beings with a mutation of α_1-antitrypsin develop pulmonary emphysema due to the migration of neutrophil into the lung in response to inflammation and the excessive cleavage of the elastic tissues by secreted NE (Lee *et al.*, 1981).

As a typical serine protease, NE precursor polypeptides undergo extensive processing and modification before storage as an active enzyme in the primary granules. Removal of a signal peptide at the amino-terminus (N-terminus) generates a zymogen containing an N-terminal dipeptide and a carboxyl-terminal (C-terminal) prodomain which are not found in the mature enzyme (Gullberg *et al.*, 1997; Gullberg *et al.*, 1999). The presence of the N-terminal dipeptide in the preprotein prevents premature enzymatic activation, and it most be removed to achieve a mature conformation. In addition, NE precursors undergo extensive glycosylation with complex oligosaccharides side chains in the medial cisternae of the Golgi complex (Lindmark *et al.*, 1990). This glycosylated zymogen is constitutively secreted out of the cell in an enzymatically inactive form (Gullberg *et al.*, 1997; Garwicz *et al.*, 2005). Some of the zymogen are processed in a pre-granular compartment to remove the N-terminal dipeptide, and then sorted into granules (Gullberg *et al.*, 1997). The cleavage of the elastase C-terminal peptide might be parallel or even precede the cleavage of N-terminal peptide (Gullberg *et al.*, 1995). However, the exact compartment and timing of removal of C-terminal peptide has not been determined.

The function of the C-terminal peptide has been studied using a rat basophilic leukemia cell line, RBL cells, transfected with a cDNA encoding NE with a C-terminal

deletion. The absence of the C-terminaus did not prevent the processing or granular targeting of NE native proteins. Therefore, the C-terminus is not absolutely required for the sorting of NE into granules (Gullberg *et al.*, 1995). However, sorting of NE through a pathway involving the plasma membrane required the presence of the C-terminus. RBL cells expressing NE with the C-terminal peptide removed bypass plasma membrane trafficking (Tapper *et al.*, 2006). Therefore, NE C-terminal region is likely critical for NE translocation through the plasma membrane and then transport back into the cell.

Human *ELA2*, the gene encoding neutrophil elastase, is localized on the short arm of chromosome 19 and clustered with the genes encoding proteinase 3 and azurocidin. The expression of *ELA2* is mainly in promyelocyte and myelocyte stage cells (Fouret *et al.*, 1989). Mature neutrophils do not contain NE mRNA transcripts, but NE proteins are persistent throughout neutrophil maturation. *ELA2* has five exons, with exon1 encoding signal sequence which directs NE protein synthesis to the rough ER (Horwitz *et al.*, 2007). Heterozygous point mutations in neutrophil elastase cause all cases of human CN and some cases of SCN (Horwitz *et al.*, 2007). *ELA2* mutations scattered through exon 2 to exon 5 in CN and SCN, resulting in amino acid substitutions, nonsense (deletion), or frameshift mutations. The most common *ELA2* mutations in SCN are located in exon 5, but no mutation is located in the coding region of NE C-terminus. Interestingly, most *ELA2* mutations do not affect the intrinsic elastase enzymatic activity (Li & Horwitz, 2001). It has been suggested that secreted elastase with altered degradation ability might contribute to the apoptosis of bone marrow precursors and result in reduced production of neutrophils (Aprikyan *et al.*, 2001). NE enzymatic activity is found significantly reduced in PMNs from CH dogs (Benson *et al.*, 2003). PMNs from HPS-2 patients contain

decreased level of NE proteins (Fontana *et al.*, 2006). Both disorders are caused by homozygous mutations in *AP3B1* gene (Dell' Angelica *et al.*, 1999b; Benson *et al.*, 2003). It is hypothesized that in these disorders, the processing and sorting of NE into granules during granulopoiesis is not very efficient due to the mutation in *AP3B1*.

1.3 Adaptor-related protein complexes and intracellular protein trafficking

Protein sorting and shuttling is critical for the generation of the lysosome and lysosomal-like organelles, which have unique functions depending on the cell type. In eukaryotes, a highly regulated system has been evolved and established to ensure correct trafficking of macromolecules between donor and acceptor compartments (Sossin *et al.*, 1990; Gullberg *et al.*, 1997; Gullberg *et al.*, 1999; Robinson & Bonifacino, 2001; Nakatsu & Ohno, 2003; Robinson, 2004).

1.3.1 Transport vesicles and adaptor protein complexes

Intracellular protein transport is mainly mediated by membrane-bound vesicles which are "pinched off" from a cargo concentrated patch on a donor membrane to target and fuse with an acceptor membrane of organelles of the secretory and endocytic pathways (Simpson *et al.*, 1996). One of the best-characterized vesicles are the clathrin-coated vesicles (CCVs), which are composed of an outer layer – clathrin coat, inner-membrane layer with the cargo, and a middle layer linking the clathrin layer with the inner layer. The middle layer consists of various clathrin-adaptor molecules and other proteins that have accessory/regulatory roles in CCV assembly (Edeling *et al.*, 2006). At least 20 clathrin adaptors have been identified, and the first described were the two

adaptor-related protein complexes (AP-1 and AP-2), each of which mediates a distinct membrane trafficking step associated with clathrin (Robinson & Bonifacino, 2001). Through homology search of cDNA libraries as well as genome databases, another two AP complexes AP-3 and AP-4 were identified (Dell' Angelica *et al.*, 1999a; Dell' Angelica *et al.*, 1999b). All the identified AP complexes are composed of four subunits: two large subunits (β1-4 subunit and γ/α/δ/ε subunit in AP-1/2/3/4) of 100-130kD, one medium-sized subunit (μ1-4) of ~50kD and one small subunit (σ1-4) of ~20kD. The four subunits in one complex are homologous to the corresponding subunits in the other three protein complexes.

In the secretory and endocytic pathway, AP complexes recognize cargo proteins by directly binding to the protein sorting signal in the target protein. Usually the sorting signal consists of a short amino acid stretch located within the cytoplasmic region of membrane proteins (Nakatsu & Ohno, 2003). The well-characterized sorting signals of AP complexes are tyrosine or di-leucine based signals. The cargo selection of the four AP complexes varies depending on the different intracellular localization of the APs (Robinson, 2004). AP-1 is found on the TGN and endosomes, while AP-2 is localized at the plasma membrane. AP-1 is likely responsible for protein trafficking from the TGN to endosome and/or from endosome to the TGN. AP-2 is mainly involved in protein endocytosis (from plasma membrane to endosome). AP-3 is found at TGN/endosomal membranes and mainly responsible for cargo transport from the TGN/endosome to lysosome and lysosome-like granules. AP-4 is mostly associated with the TGN (Edeling *et al.*, 2006). AP-1, AP-2 and AP-3 are associated with assembly of CCVs, while AP-4 is associated with a non-clathrin coat (Nakatsu & Ohno, 2003).

1.3.2 Adaptor-related protein complex – 3 (AP-3)

AP-3 has two forms: AP-3A and AP-3B. AP-3A is composed of subunit β3A, μ3A, σ3 and δ, all of which are ubiquitously expressed. AP-3B is a neural specific adaptor protein complex, which recruits σ3 and δ subunit as in AP-3A, and β3B, μ3B which are exclusively expressed in neurons (Robinson & Bonifacino, 2001). AP-3 is involved in trans-membrane protein trafficking into lysosome and lysosome-related organelles from the TGN/endosome compartments. In mice *AP3B1* null mutants, deficiency of AP-3 results in accumulation of lysosomal membrane protein CD63, LAMP-1 and LAMP-2, as well as melanosomal membrane protein tyrosinase on the cell surface (Wang *et al.*, 2000). AP-3 – deficient cells greatly increase recycling of endocytosed LAMP-1 and CD63 back to the cell surface (Peden *et al.*, 2004).

The structure of AP-3 resembles the other three adaptor protein complexes AP-1, AP-2 and AP-4. The core structure of AP-3 consists of N-terminal domains (trunk domain) of the two large subunits β3A and δ, the medium subunit μ3A and the small subunit σ3A. The C-terminal domains of the two large subunits project as "ears", connected to the "head" of the complex by flexible hinges. It has been shown that δ subunit interacts with σ3A subunit, β3A interact with μ3A subunit, and the two large subunits interact with each other (Robinson & Bonifacino, 2001; Edeling *et al.*, 2006). The four subunits of AP-3 perform different functions. The δ subunit mediates binding to the target membrane, and the β3A subunit recruit clathrin through the clathrin binding sequence. The μ3A are responsible for cargo recognition through tyrosine-based sorting signals, and the small subunit σ3A is likely involved in the stabilization of the complex (Nakatsu & Ohno, 2003).

1.3.3 Mouse mutants *pearl* and *mocha*, and Hermansky-Pudlak syndrome type 2 are caused by mutations in the gene encoding AP-3 subunit

The understanding of AP-3 function is derived from studies of mouse mutants and human beings which have gene mutations in one of the four subunits of AP-3. The mouse mutant *pearl* has a mutation in the *AP3B1*, encoding β3A subunit of AP-3 (Feng *et al.*, 1999). *Pearl* mouse is characterized by hypopigmentation due to reduced quantity and morphologically abnormal melanosomes, as well as prolonged bleeding due to deficiencies of platelet dense granules (Zhen *et al.*, 1999). The *pearl* trait is inherited in an autosomal recessive pattern and *pearl* mouse is a rodent model for HPS-2. The nucleotide alterations in *AP3B1* gene cause a deficiency of β3A subunit in all *pearl* mouse cells and tissues. The expression of other three AP-3 subunits is also significantly decreased in *pearl* mouse. However, there is no neutropenia or immunodeficiency in *pearl* mouse (Feng *et al.*, 1999). *Mocha* mouse has a mutation in δ subunit of AP-3. Therefore, *mocha* mouse has similar phenotypes as seen in *pearl* mouse, such as coat color dilution and prolonged bleeding. However, neuronal disorders including deafness, balance problems and seizures are observed in the *mocha* mouse, but not in the *pearl* mouse. This is caused by the depletion of both AP-3A and AP-3B isoforms, since the δ subunit is a common component of both AP-3 complexes (Kantheri *et al.*, 1998).

The *AP3B1* mutation is the genetic basis of a rare human disorder – HPS-2, an autosomal recessive disorder characterized by oculocutaneous albinism, platelet storage pool deficiency, and mild immunodeficiency (Dell' Angelica *et al.*, 1999b). The AP3 β3A, μ3A subunits are almost completely absent in HPS-2 patients, while the expression of the other two subunits is either moderately or severely decreased (Dell' Angelica *et al.*, 1999b;

Fontana *et al.*, 2006). HPS-2 patients display an increased surface expression of lysosomal membrane protein CD-63, LAMP-1 and LAMP-2, but not nonlysosomal proteins, in AP-3 – deficient fibroblasts (Dell' Angelica *et al.*, 1999b). The expression, distribution and internalization of non-lysosomal membrane proteins is indistinguishable in normal individuals and HPS-2 patients, indicating a specific role of AP-3 in lysosomal integral membrane protein trafficking. Neutrophils from HPS-2 patients show increased surface expression of CD63 and reduced NE levels. In addition, NK cells from HPS-2 patients have defective cytolytic activity and reduced perforin content in the lysosomes (Fontana *et al.*, 2006). These findings suggest that AP-3 deficiency in HPS-2 might be associated with intracellular sorting and trafficking defects of these soluble proteins.

1.4 Biosynthesis of secretory proteins, unfolded protein response and apoptosis of hematopoietic progenitor cells in hematologic disorders

The biosynthesis of proteins entering the secretory pathway is initiated on the rough endoplasmic reticulum (ER). Newly made polypeptides enter or cross the ER membrane during their synthesis. Secretory proteins move from the ER lumen through the *cis*-Golgi reticulum to the Golgi complex. Proteins destined to be secreted move by cisternal progression from *cis*-Golgi to the *trans*-Golgi, and then into the *trans*-Golgi reticulum (*trans*-Golgi network, TGN). From there, a secretory protein is sorted into either the constitutively secretion, or the regulated secretion pathway. Proteins entering constitutive secretion are sorted into transport vesicles that immediately move to and fuse with the plasma membrane, and release their contents by exocytosis. For proteins entering regulated secretion, they are sorted into secretory vesicles that are stored inside the cell.

These proteins are released through exocytosis after the cells are exposed to specific external stimuli (Sossin *et al.*, 1990; Rothman & Orci, 1992; Gullberg *et al.*, 1999; Lodish *et al.*, 2000).

To exit from the ER into the Golgi complex, a protein has to be modified and properly folded in the ER lumen. The movement of a polypeptide from the ER to the Golgi will be blocked if there is a mutation in the protein causing a folding problem. The folding of the polypeptide in the ER is closely coupled with their processing and modification. For example, improperly glycosylated polypeptides will not be able to interact with calnexin and calreticulin, and rearrange their disulfide bonds. Polypeptides which are not properly folded fail to leave the ER and will be bound by ER chaperones to be degraded, this is called quality control. The accumulation of unfolded/mis-folded polypeptides in the ER activates a stress signaling pathway known as the unfolded protein response (UPR) (Kaufman, 2002; Zhang & Kaufman, 2006a).

To prevent polypeptide misfolding and aggregation and ER stress, a group of ER chaperones bind to nascent peptides and promote the folding of these peptides in the ER (Ni & Lee, 2007). Accumulation of unfolded/misfolded protein is sufficient to induce the expression of these ER chaperones. The most abundant chaperone in the ER lumen is BiP/GRP78, which is known as a sensor of ER stress. In 1980s, it was first recognized that a glucose-regulated protein with molecular weight 78kD (GRP78) in fibroblasts was the same protein which bound to immunoglobulin in pre-B cells (BiP) (Lee *et al.*, 1983; Hass & Wabl, 1983; Lee *et al.*, 1984). Subsequently, it was observed that the BiP/GRP78 was induced when an unfolded mutant of the influenza hemagglutinin was over-expressed in the cell. BiP/GRP78 plays a central role in regulating the ER stress

transducers – inositol-requiring enzyme 1 (IRE1), double-strand RNA-activated protein kinase (PKR)-like ER kinase (PERK) and activating transcription factor (ATF6) (Okada *et al.*, 2002; Zhang & Kaufman, 2004; Zhang & Kaufman, 2006a; Zhang & Kaufman, 2006b). IRE1, PERK and ATF6 contain a transmembrane domain and an N-terminal domain that resides in the ER lumen. The binding of BiP/GRP78 to their N-terminal domain prevents the activation of IRE1, PERK and ATF6. When the ER is jammed with improperly folded proteins, BiP/GRP78 binds to these proteins and there is a decrease in the amount of free BiP/GRP78, which allows the activation of IRE1 and PERK, as well as the release and transport of ATF6 into the Golgi complex. The activation of these ER stress transducers eventually induces a cellular response – adaptive or apoptotic cell death. Therefore, BiP/GRP78 regulates the activation of these UPR transducers and provides a link between unfolded proteins and cellular response to ER stress.

During myelopoiesis, sorting and shuttling of proteins into appropriate compartments is critical for neutrophil maturation. Disorders associated with neutropenia, such as human CN and SCN, have been suggested to result from impaired survival of bone marrow hematopoietic progenitor cells (Aprikyan *et al.*, 2001; Carlsson *et al.*, 2004). The increased apoptosis of these neutrophil precursor cells occurs in the post-mitotic compartment during PMN maturation but before being released into the blood (Mackey *et al.*, 2003). Differentiating HL-60 cells show accelerated apoptosis when they are transduced with NE mutant that causes the most severe SCN. The premature death of HL-60 cells is induced by aberrantly processing and targeting of this NE mutant (Massullo *et al.*, 2005). Using a monoblast-like cell line U937 and PMNs from SCN patients, Köllner et al. studied the processing and localization NE mutants in these cells.

Their data indicated that intracellular NE protein trafficking is disrupted, resulting in cytoplasmic accumulation of non-functional NE proteins. The abnormal accumulation of NE proteins induces the expression of BiP/GRP78, indicating a cellular stress and UPR induction (Kollner *et al.*, 2006). Accelerated apoptotic cell death was also observed in differentiating U937 cells containing NE mutants and primary human promyelocytes obtained from SCN patients (Kollner *et al.*, 2006; Grenda *et al.*, 2007).

Induction of the UPR associated with apoptosis of myelopoietic progenitor cells represents a novel mechanism for understanding the pathogenesis of human CN and SCN. The mutation in CH dogs is different from these human disorders, although they have similar clinical manifestations. Reduced storage of NE active enzymes in neutrophils from CH dogs suggested that there may be a protein trafficking defect similar to that seen in human CN and SCN.

1.5 Objectives

The CH dog has long been used as an animal model for studying human CN and SCN. Study of CH in the dog will further our understanding of the regulation of hematopoiesis, intracellular trafficking of secretory proteins and the molecular mechanisms associated with these hematologic disorders. Canine CH is caused by a mutation in the β3A subunit of AP-3 which is involved in lysosomal membrane protein trafficking. Human CN, sporadic or autosomal dominant forms of SCN are caused by heterozygous point mutations in NE. Yeast two-hybrid experiments showed that NE without the C-terminus interacts with the μ3A subunit of AP-3 complex through a tyrosine-based sorting signal. NE mutants lacking the μ3A sorting signal trafficked to the

plasma membrane instead of granules (Benson *et al.*, 2003). However, AP-3 is mainly responsible for the trafficking of integral membrane proteins from TGN to lysosome or lysosome-like organelles. Although it is suggested that NE might adopt a transmembrane conformation, there is lack of direct evidence to prove the existence of this configuration (Benson *et al.*, 2003; Horwitz *et al.*, 2007). Therefore, the sorting and localization of NE remains a central unanswered question which is crucial to our understanding of the basic mechanism regulating myelopoiesis.

NE, a typical serine protease, undergoes extensive modification and processing before it is stored as a mature enzyme in neutrophil granules. The expression of *ELA2*, encoding NE, is tightly regulated during myelopoiesis, indicating NE is critical for neutrophil maturation. NE transcripts are maximally detected at the promyelocyte stage, and NE protein is primarily synthesized at this time (Fouret *et al.*, 1989; Garwicz *et al.*, 2005). *In vitro* bone marrow culture experiments showed that the inactive NE precursor proteins are constitutively secreted out of cell, while the active mature protein is stored in granules which are subsequently secreted upon stimulation (Gullberg *et al.*, 1997; Garwicz *et al.*, 2005). Except for the storage of mature NE in primary granules, NE precursor proteins are found in segmented neutrophils (Horwitz *et al.*, 2007). Whether these stored NE precursor proteins are part of constitutively secreted zymogen is not known.

Mature, active NE is stored mainly in neutrophil primary granules. Consistent with their functional defects, neutrophils from CH dog contain significantly reduced NE enzymatic activity, although the *ELA2* gene is intact in the dog (Chusid *et al.*, 1975; Benson *et al.*, 2003). Also, neutrophils from HPS-2 patients are deficient of NE proteins

(Fontana *et al.*, 2006). Therefore, intracellular trafficking of NE protein is possibly affected due to the *AP3B1* mutation in HPS-2 patients and in CH dogs. However, the storage and distribution of NE precursor proteins has not been determined in neutrophils from these hematologic disorders.

Accumulative data illustrate an increased apoptotic cell death rate of proliferating myelopoietic progenitor cells as a common mechanism of reduced generation of neutrophils in peripheral blood (Mackey *et al.*, 2003; Carlsson *et al.*, 2004; Massullo *et al.*, 2005; Kollner *et al.*, 2006; Grenda *et al.*, 2007). The induction of cellular stress and the UPR due to abnormal processing and accumulation of NE mutants in the cytoplasm has been proposed as a novel mechanism of pathogenesis for human CN and SCN (Kollner *et al.*, 2006; Grenda *et al.*, 2007). These observations are from the study of human hematopoietic disorders associated with *ELA2* mutations. The over-archery goal of this dissertation research was to characterize the interaction of NE and AP-3 in normal and CH dogs to better define the molecular mechanisms for canine CH. To accomplish this goal, I developed canine specific NE antibodies that allowed me to determine the subcellular localization of NE isoforms (mature and precursor), to understand the relation between NE trafficking and the *AP3B1* mutation, and to determine the molecular basis of cyclic fluctuation of blood cells in CH dogs. The objectives of my dissertation research are arranged into the following 5 sections:

1. Development of monoclonal and polyclonal antibodies generated against antigenic peptides from canine NE will be undertaken. These antibodies specifically recognized two isoforms (mature and precursor) of canine NE.
2. Characterization of NE precursor and mature forms in neutrophils from CH and

normal dogs will be performed.

3. Characterization of NE expression will be confirmed for *in vitro* cultured bone marrow mononuclear cells (BMMCs) from CH and normal dogs. This will provide substantial evidence of NE trafficking defect in CH dogs.
4. Determination whether the UPR is induced in *in vitro* cultured BMMCs from CH and normal dogs will be completed.
5. Determination of UPR induction which leads to increased apoptosis of myeloid precursor cells in CH dogs will be accomplished.

CHAPTER 2

GENERATION AND USE OF ANTIBODIES TO CANINE NEUTROPHIL ELASTASE

ABSTRACT

Neutrophil elastase, a major component of the neutrophil primary granule, plays a fundamental role in host defense against microbial invasion. Recent studies demonstrated neutrophil elastase involvement in the regulation of granulopoiesis. Mutations in the neutrophil elastase gene, *ELA2*, are responsible for human cyclic neutropenia and severe congenital neutropenia. Neutrophils from cyclic hematopoietic dogs contain significantly decreased neutrophil elastase activity, albeit they have an intact elastase gene. Canine cyclic hematopoiesis is caused by a mutation in *AP3B1*; encoding the β3A subunit of AP-3 with functions in intracellular trafficking of nascent proteins. Preliminary studies demonstrated that polyclonal antibodies to human elastase reacted with multiple cross reacting proteins in dogs and were not reliable for specific detection of canine elastase. Antibodies against immunogenic peptides of canine neutrophil elastase were generated to specifically detect canine neutrophil elastase and characterize molecular mechanisms associated with canine cyclic hematopoiesis and further our understanding of this disorder in human, western immunoblotting and immunocytochemistry demonstrated that one antibody (mELA85, pELA85) specifically recognized the mature form of neutrophil

elastase, and a second antibody (mELA269, pELA269) exclusively reacted with an elastase precursor protein. These antibodies are useful reagents for studying the biosynthesis, processing and trafficking of neutrophil elastase during normal myelopoiesis and for the comprehensive study of pathophysiology of hematologic disorders associated with this protease in both humans and dogs.

2.1 Introduction

Neutrophils arise from the orderly progression of distinct precursor cells in the bone marrow that are microscopically classified into the following stages: myeloblast, promyelocyte, myelocyte, metamyelocyte, band cell, and segmented neutrophil (PMN, polymorphonuclear neutrophil leukocyte) (Bainton, 1996). A typical PMN contains at least 4 types of cytoplasmic granules, including azurophilic/primary, specific/secondary, and gelatinase/tertiary granules, as well as secretory vesicles. The formation and granule content are dependent on biosynthesis of proteins which are expressed at given stages in differentiating myeloid precursors (Borregaard & Cowland, 1997; Gullberg *et al.*, 1997; Cowland & Borregaard, 1999; Garwicz *et al.*, 2005). Therefore, the expression of genes encoding granular proteins is rigidly regulated to ensure the correct sorting and packaging of granular proteins. Neutrophil elastase, encoded by *ELA2*, is transcriptionally activated in early myeloid development and expressed maximally at the early promyelocyte stage (Fouret *et al.*, 1989). The production of neutrophil elastase is associated with the formation of neutrophil primary granules, storage depots for active elastase enzyme as well as other macromolecules, such as myeloperoxidase, defensins, proteinase 3 and

cathepsin G (Cramer *et al.*, 1989; Fouret *et al.*, 1989; Yoshimura & Crystal, 1992; Egesten *et al.*, 1994).

In human beings, neutrophil elastase is produced as a precursor molecule with the N-terminus containing a 27-residue "pre" signal peptide followed by a "pro_N" dipeptide (Gullberg *et al.*, 1995). Cleavage of the signal peptide generates a zymogen molecule which exposes the 2-amino-acid "pro_N" dipeptide N-terminally. The presence of "pro_N" dipeptide prevents premature activation during intracellular trafficking of the zymogen. A complex N-linked oligosaccharide side chain is added to the protein when the zymogen is processed and folded in the medial and trans- of the Golgi complex. Most of the glycosylated zymogen is constitutively secreted out of the cell in an enzymatically inactive form (Lindmark *et al.*, 1990; Garwicz *et al.*, 2005). Some of the zymogen are processed in a pre-granular compartment to remove the N-terminal dipeptide, and then sorted into lysosome-like granules (neutrophil primary granules) (Gullberg *et al.*, 1997). Removal of NE C-terminus is either parallel or precedes cleavage of the N-terminal dipeptide (Gullberg *et al.*, 1995).

As a typical neutral serine protease, neutrophil elastase is capable of digesting a wide spectrum of substrates, including elastin, collagen, proteoglycan, cytokines and complements (Hunter *et al.*, 2003; Chua & Laurent, 2006). Recently, neutrophil elastase has been implicated in the regulation of granulopoiesis (El Ouriaghli *et al.*, 2003; Lane & Ley, 2003; Garwicz *et al.*, 2005). Wide spread point mutations in the *ELA2* gene, encoding neutrophil elastase, are responsible for all cases of cyclic neutropenia (CN) and approximately 50% of the cases of autosomal dominant severe congenital neutropenia (SCN) in humans (Horwitz *et al.*, 1999; Horwitz *et al.*, 2004; Carlsson *et al.*, 2006;

Horwitz *et al.*, 2007). Expression of neutrophil elastase mutants in RBL-1 cells indicated that all mutated elastase genes express as efficiently as wild-type (Li & Horwitz, 2001). Most mutants have reduced levels of proteolytic activity, but none of the mutations result in complete loss of enzymatic activity. In dogs, active neutrophil elastase is a single chain protein, and is mainly stored in primary granules like in human neutrophils (Delshammar & Ohlsson, 1976; O'Donnell & Andersen, 1982). Canine cyclic hematopoiesis (CH), an autosomal recessive disorder which has long been studied as a model of human CN, is characterized by periodic fluctuations of all blood cells (Lund *et al.*, 1967; Dale *et al.*, 1972a). This disorder is caused by an insertional mutation in exon 20 of *AP3B1*, encoding adaptin β3A, which is one of four subunits of adaptor protein complex -3 (AP-3) (Benson *et al.*, 2003). Neutrophils isolated from CH dogs contain significantly reduced elastase enzymatic activity. To further characterize neutrophil elastase in the dog and better determine the interrelation between elastase and AP-3, antibodies against two immunogenic peptides of canine neutrophil elastase were generated. In this report, the specificity of these two antibodies to canine neutrophil elastase on western immunoblotting and immunocytochemistry were determined using dog cells. These antibodies are useful tools for further study of hematologic disorders associated with neutrophil elastase in the dog, and for investigation on biosynthesis and processing of neutrophil elastase during myelopoiesis

2.2 Materials and methods

2.2.1 Antibodies

Two peptides with amino acid sequence LGAHDLGERESTRQ (#85-98), RAPPPAPHRPRPTQ (#269-282) from canine neutrophil elastase were synthesized by Perkin-Elmer Sciex API 100 and the sequence was analyzed by mass spectroscopy. The two peptides and polyclonal antibodies to these two peptides were produced by Zymed Laboratories Inc. (Invitrogen Corporation, San Francisco, CA). Rabbit antiserum was obtained for western immunoblotting. Monoclonal antibodies to the two antigenic peptides were generated by the Auburn University Hybridoma Facility. Mouse ascites fluid was subjected to purification using Ab SpinTrap (GE Healthcare, Piscataway, NJ) to obtain monoclonal antibodies.

Four clones of monoclonal antibody (7G1-3H2, 7G1-1D9, 10A6 and 1C9) to canine myeloperoxidase (MPO) and purified canine MPO were kind gifts of Dr. William Vernau (University of California, Davis, CA). Clone 7G1-3H2 and 7G1-1D9 were determined recognize canine MPO on western immunoblot. Rabbit polyclonal antibodies to human neutrophil elastase were purchased from Calbiochem (EMD Biosciences, San Diego, CA), EPC (Owensville, MO). Sheep polyclonal antibody to human neutrophil elastase was obtained from Serotec (Raleigh, NC). Monoclonal antibody to GAPDH (ab8245, Abcam Inc., Cambridge, MA) was used as protein loading control. HRP-conjugated goat anti-mouse IgG (sc-2031) or goat anti-rabbit IgG (sc-2301) (Santa Cruz Biotechnology, Inc. Santa Cruz, CA) were used as secondary antibodies.

2.2.2 Experimental animals, blood and bone marrow sample collection

Dogs were housed in AAALAC, Intl. accredited facilities and the experimental protocols were approved by the institutional IACUC (OLAW assurance #: A-3152-01). Dogs were administered routine vaccinations and used only when free of concurrent infections.

Peripheral blood was collected from dogs into a EDTA-containing BD Vacutainer® (BD, Franklin Lakes, NJ) tube. Polymorphonuclear leukocytes (PMN) were isolated by Ficoll-Hypaque (Amersham Biosciences, Piscataway, NJ) density gradient sedimentation and erythrocytes were removed by Dextran T-500 (Sigma) sedimentation. Residual erythrocytes were hypotonically lysed in 0.3% NaCl. The purity of neutrophils from each sample was greater than 95% based on evaluation of cytocentrifuge cell preparations (Shandon Cytospin III).

Bone marrow from dogs was collected in a 12ml syringe containing 3ml Iscove's modified Dulbecco's medium (IMDM) (Invitrogen Corporation, Carlsbad, CA) and 150 units of Heparin (Sigma). Following centrifugation on Ficoll-Hypaque gradients, bone marrow mononuclear cells were collected, washed three times in phosphate buffered saline (PBS, Invitrogen) and resuspended in IMDM containing 10% heat-inactivated fetal bovine serum (FBS; Hyclone, Logan, UT) and 100U/ml penicillin and 100 µg/µl streptomycin (Invitrogen).

2.2.3 Culture of bone marrow mononuclear cells

Freshly isolated bone marrow mononuclear cells (BMMCs) were cultured in IMDM containing 10% FBS and 100U/ml penicillin, 100µg/µl streptomycin (Invitrogen),

25ng/ml canine stem cell factor (SCF; R&D Systems, Inc. Minneapolis, MN), and 10ng/ml canine granulocyte colony stimulating factor (G-CSF; Stem Cell Technologies, Inc.) at 37°C in a CO_2 water-jacketed incubator (Thermo Electron Corporation, Waltham, MA). Cells were collected at seven successive time points: 0, 6, 18, 30, 42, 54 and 66 hour. The cells were washed 3 times in cold PBS to remove culture medium, and then cell lysates were prepared for immunoblot analysis.

2.2.4 Subcellular fractionation

Subcellular fractionation experiment was performed as described with minor modifications (Kjeldsen *et al.*, 1999). Briefly, freshly isolated neutrophils were pelleted and resuspended at 0.5-1.0×10^8 cell/ml in disruption buffer (100mM KCl, 3mM NaCl, 1mM $ATPNa_2$, 3.5mM $MgCl_2$, 10mM Piperazine, N, N'-bis-2 ethanesulfonic acid, pH7.2) and then pressurized at 400psi on ice in a nitrogen bomb (Parr Instrument, Moline, IL). Cavitates were collected in disruption buffer with 1.5mM EGTA, and then centrifuged to pellet nuclei and intact cells. The supernatant was applied on a three-layer discontinuous Percoll gradient (Amersham Biosciences, Piscataway, NJ;1.050/1.090/1.120g/ml), then centrifuged at 37,000×g for 30 minutes at 4°C in a SM-24, fixed angle rotor in a Sorvall RC-5C centrifuge (Sorvall Instruments, Duluth, GA). Fractions were collected as 1ml aliquots by carefully pipetting. Percoll was removed from each fraction by centrifugation at 100,000×g for 90 minutes at 4°C in a 70.1Ti, fixed angle rotor in a Beckman L8-M ultracentrifuge (Beckman Instrument, Inc. Palo Alto, CA).

2.2.5 Cell lysate preparation and immunoblot analysis

Cells were washed in PBS and resuspended in 1×RIPA lysis buffer (Upstate, Lake Placid, NY) containing 1× protease inhibitor (Roche Applied Science, Mannheim, Germany). The cell suspension was further homogenized by passage through a 21 gauge needle and centrifuged at 15,000×g to obtain cell lysate. Samples were denatured at 95°C for 5 minutes for immuno-blot analysis. Proteins were separated in a 4-20% linear gradient gel (Bio-Rad Laboratories, Hercules, CA) using a Ready Gel Precast Gel System (Bio-Rad Laboratories), and transferred in Towbin buffer [25 mM Tris, 192 mM glycine, 20% (v/v) methanol, pH 8.3] to a nitrocellulose membrane (Bio-Rad Laboratories) under a high intensity transfer condition (100V, 1 hour). After initial blocking with StartingBlock^{TM} Tris-buffered saline (TBS) blocking buffer (Pierce Biotechnology, Rockford, IL), blots were incubated with primary antibodies overnight at 4°C, and then washed in wash buffer before incubation with HRP-conjugated secondary antibodies. After 1 hour incubation, blots were washed again and were incubated with substrate (Pierce Biotechnology, Rockford, IL) for 5 minutes and then visualized by exposure to a Kodak BioMax XAR film (Fisher Scientific, Pittsburgh, PA).

The specificity of the antibodies to canine neutrophil elastase was determined by incubating the primary antibody with the immunogenic peptides prior to blotting. The mixture was then centrifuged for 15 minutes at 15,000rpm to remove immune complexes, and then diluted in blocking buffer before applying to blots.

2.2.6 Immuno-electron microscopy

2.2.6.1 Sample preparation

Buffy coat samples from peripheral blood were prepared as described (Fittschen *et al.*, 1983). Briefly, EDTA-treated blood was centrifuged for 3 minutes at 3000 rpm at 4°C in Kimax borosilicate glass tubes (6 × 50 mm O.D. x L; Fisher Scientific, Suwanee, GA). The plasma layer was removed and cold fixative (4% paraformaldehyde + 0.05% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4) was gently added to the top of the tube. After fixation, the buffy coats were gently released from the tubes and minced with a razor blade in the same fixative solution at 4°C. Subsequently, the buffy coat pieces were rinsed in the cacodylate buffer. The buffy coat pieces were washed with the 0.05 M Tris buffer containing 5% sucrose (Bainton, 1999), and postfixed in 1% osmium tetroxide in Millonig's phosphate buffer (0.1 M, pH 7.3) for 1 hour. The samples were then dehydrated in an ascending series of ethanol : propylene oxide ratios, and embedded in Durcupan ACM plastic (Electron Microscopy Sciences, Ft. Washington, PA).

2.2.6.2 Immunolabeling of PMN

The grids were incubated with 0.05 M glycine in PBS (10mM phosphate buffer, 150 mM NaCl, pH 7.4) for 15 minutes, and then blocked with 5% BSA and 0.1% cold water fish gelatin (CWFG) in PBS for 30 minutes. Before and after the incubation with primary antibody, the grids were washed with incubation buffer (IB: 150 µl of 10% BSA in 10 ml PBS), then incubated with secondary antibody (15 nm gold conjugated goat anti-mouse IgG/IgM for 2 hours. The grids were washed in IB again and post-fixed in 2% glutaraldehyde in PBS for 5 minutes, then washed with PBS and distilled water. The

ultrathin sections for routine TEM were stained with uranyl acetate and lead citrate. All specimens were examined using a Philips 301 TEM (FEI Co., Hillsboro, OR) operating at 60 kV.

2.2.7 Double immunofluorescence labeling and confocal microscopy

Cytospin preparations of fresh isolated neutrophils were fixed with 4% paraformaldehyde. Following fixation, cells were permeabilized with 1% Triton X-100 in PBS with 1% bovine serum albumin (BSA, Invitrogen). Nonspecific binding was blocked for 30 minutes with 5% goat serum (Jackson ImmunoResearch Laboratories, West Grove, PA) containing 2.5% BSA. Cytospins were incubated with mELA85 or a mouse IgG1 negative control (MCA928; AbD Serotec, Raleigh, NC) overnight at 4°C. Incubation with Alexa Fluor®488-conjugated goat anti-mouse IgG (Invitrogen) was performed at room temperature for 1 hour. Cytospins were blocked with normal mouse serum (1:20 in PBS) and then Fab-fragment goat anti-mouse IgG (H+L) (20µg/ml in PBS) (Jackson ImmunoResearch Laboratories) for 1 hour, respectively. Cytospins were then incubated with a second monoclonal antibody to myeloperoxidase (MPO) overnight at 4°C and finally incubated with Alexa Fluor®574 -conjugated goat anti-mouse IgG (Invitrogen) for 1 hour at room temperature. Cytospins were washed with PBS three times between each incubation step.

Finally, cytospins were mounted with Vectashield® Mounting Medium (Vector Laboratories Inc., Burlingame, CA). Immunofluorescence was observed under a BioRad MRC-1024 laser scanning confocal microscope (Bio-Rad Laboratories).

2.3 Results

2.3.1 Generation of monoclonal antibodies against two antigenic peptides from canine neutrophil elastase

Initially, three commercially available antibodies to human NE were used for western immunoblot analysis of canine neutrophils. The antibodies recognized a protein band approximately 28-29kD, which was similar to the MW of NE (Figure 2.1). Canine neutrophils lysates were then fractionated under nitrogen cavitation, and 8 fractions were collected. Fraction 6 and 7 was determined to have maximal elastase enzyme activity (Figure 3.6). However, when the same fractions were subjected to western immunoblotting, the protein band which was recognized by these antibodies was distributed primarily in fraction 1 and 2, which were not the fraction containing primary granules with maximal NE enzyme activity (Figure 2.2, Table 3.1). Therefore, these commercially available antibodies were demonstrated not to be cross-reacted with canine NE.

Canine NE precursor protein (preprotein) was determined to be a single chain polypeptide composed of 282 amino acids. To generate antibodies which would likely specifically react with canine NE, the complete amino acid sequence was retrieved from NCBI canine genome database (Figure 2.3). Two peptides were predicted to be immunogenic based on hydrophobicity plots, favorable secondary structure and peptide location. The position of one immunogenic peptide was #85-98, located in the middle of the protein sequence. The generated antibody to peptide #85-98 was defined ELA85. The position of another peptide was #269-282, located on the C-terminus of the preprotein. The antibody to peptide #269-282 was defined ELA269. The two peptides were

synthesized by Zymed Laboratories, and two polyclonal antibodies (pELA85, pELA269) and two monoclonal antibodies (mELA85, mELA269) were generated by Zymed Laboratories and the Auburn University Hybridoma Facility, respectively.

2.3.2 Use of polyclonal antibody pELA85 and monoclonal antibody mELA85 to detect neutrophil elastase in neutrophil lysates and neutrophil subcellular fractions

Western blot analysis of neutrophil whole cell lysates incubated with pELA85 yielded multiple bands, making it difficulty to distinguish specific and non-specific binding. To further test pELA85, fresh isolated neutrophils were homogenized in a nitrogen bomb and fractionated by discontinuous Percoll gradient sedimentation. Western blotting of these fractions incubated with pELA85 indicated that one band with a MW close to canine neutrophil elastase was detected in fraction 6, 7 and 8, with maximum concentration obtained in fraction 7 (Figure 2.4A). Fraction 7 mainly contains primary granules, as demonstrated by predominant localization of MPO in fraction 7 (Figure 2.4C). Incubation of pELA85 with immunogenic peptides completely abrogated the binding of the antibody to the antigen, confirming that this band was canine neutrophil elastase (Figure 2.4A, B). The multiple bands on PMN whole cell lysates, using pELA 85, were possibly due to the presence of a protein band in subcellular fraction 1 and 2 with a similar MW to elastase. Fraction 1 and 2 mostly contained cytosol. Western blotting of whole neutrophil lysates with mELA85 showed that a protein band with MW around 28-29kD was specifically recognized by the antibody (Figure 2.5). Incubation of the antibody with corresponding immunizing peptide inhibited the binding of the antibody to the protein, indicating this protein was canine neutrophil elastase.

2.3.3 Application of pELA269 and mELA269 to bone marrow mononuclear cells

Antibodies pELA269 and mELA269 were generated to an amino acid sequence in the C-terminus of the elastase precursor protein. The C-terminus is removed during elastase processing, and is not critical for activation and storage of mature elastase enzyme (Gullberg *et al.*, 1995). The processing of canine NE was similar to human NE as it was demonstrated using mELA269 on western blotting. Dog neutrophils were found to contain a trace amount of elastase preprotein, the majority stored in PMNs is mature elastase without C-terminus (Figure 2.6). The antibody mELA269 and pELA269 both specifically recognized two protein bands with MW ~33kD and ~35kD in bone marrow mononuclear cells (BMMCs) (Figure 2.7A, 2.8A). According to the protein sequence, the band with MW ~33kD is probably NE preprotein, while the band with MW ~35kD is possible the modified NE preprotein. Incubation of the antibody with the corresponding peptides completely inhibited the binding of the antibody to the antigen (Figure 2.7B, 2.8B). This demonstrated the specificity of antibodies mELA269 and pELA269 to NE preprotein. Similar to pELA85, pELA269 yielded multiple non-specific binding on the blots which limited its use in other applications.

Since mELA269 specifically recognized elastase preprotein, it was important to determine the time point that the protein is produced *in vitro* during myelopoiesis. BMMCs were isolated and cultured with stem cell factor (SCF) and granulocyte-colony stimulating factor (G-CSF), and then collected at seven time points (0, 6, 18, 30, 42, 54, and 66 hour). As expected, mELA269 specifically reacted with elastase preprotein in cells collected at all the time points (Figure 2.9). The preprotein was present in BMMCs

and induced to express after 6 hours in culture. The modified preprotein expressed after 18 hours in culture and gradually increased its expression in the subsequent time points.

2.3.4 Use of mELA85 to detect neutrophil elastase by immunocytochemistry using confocal microscopy and electron microscopy

Using confocal microscopy, canine NE was specifically recognized by mELA85 (Figure 2.10A). Elastase was co-localized with a neutrophil primary granule marker – myeloperoxidase (MPO) (Figure 2.10B, C), indicating that processed elastase was exclusively stored in neutrophil primary granules in the dog. The results of electron microscopy indicated that mELA85 also recognized elastase in paraformaldehyde fixed buffy coat cells (mainly contain blood neutrophils) (Figure 2.11). Immunogold label was found in granules, although the type of the granules was not determined by marker proteins.

2.3.5 Staining of segmented neutrophils with mELA269

Western immunoblotting of neutrophils from the dog with mELA269 indicated that a small amount of elastase preprotein was found in mature PMNs (Figure 2.6). Double-immunolabeling with mELA269 and monoclonal antibody to canine MPO suggested that segmented neutrophils from dog contained weak staining of this NE preprotein (Figure 2.12A). In addition, this preprotein was not co-localized with MPO in PMN primary granule (Figure 2.12C). The staining of nucleus with PI showed that elastase preprotein was not localized in PMN nucleus (Figure 2.13). Therefore, this residual elastase precursor protein was localized either in the cytoplasm, ER, the Golgi,

or on the plasma membrane. Since permeabilized neutrophils were stained with antibodies, it was difficult to determine where exactly this elastase precursor protein was localized.

2.4 Conclusion and discussion

Neutrophil elastase (NE), a major component of neutrophil primary granules, is produced as a preprotein and subjected to extensive modification and processing before it is stored as an active protease in neutrophils (Lindmark *et al.*, 1990; Gullberg *et al.*, 1997). As a typical serine protease, the processing of NE includes multiple steps but the exact compartmental localization of specific modifications has not been defined. In addition to the role of host defense, NE has recently been found to play a central role in the regulation of granulopoiesis. NE is both constitutively secreted in an inactive form during *in vitro* myeloid differentiation and stored intracellularly in an active form (Lindmark *et al.*, 1990; Garwicz *et al.*, 2005). All cases of human CN and ~50% cases of SCN are caused by heterozygous point mutations in the *ELA2* gene, and the enzymatic activity of NE is found significantly reduced in neutrophils from Hermansky-Pudlak syndrome type 2 (HPS-2) and CH dogs, indicating the important function of elastase as a hematopoietic regulator (Benson *et al.*, 2003; Horwitz *et al.*, 2004; Fontana *et al.*, 2006; Horwitz *et al.*, 2007).

To characterize the role of NE in the pathogenesis of cyclic hematopoiesis in the dog, the sequence difference between the canine elastase preprotein and processed forms was exploited. Polyclonal and monoclonal antibodies were generated that specifically recognized two forms of canine NE. Antibodies mELA85 and pELA85 were generated

against amino acid sequence 85-98. These antibodies react with mature canine NE with a MW ~28kD. Antibodies mELA269 and pElA 269 are generated against amino acid sequence 269-282, which are specific to elastase prepotein with a MW ~33kD. Although immunogenic peptide 85-98 is present in both preprotein and processed elastase, mELA85 and pELA85 are not able to recognize elastase preprotein. It is possible that the positional influences mask peptide localization and the antigen site is not present in the immature proteins. Alternatively, the NE preprotein modifications block antigenicity of the immunogenic peptide 85-98.

Use of mELA269 indicates that elastase preprotein is promptly produced when BMMCs are induced by SCF and G-CSF *in vitro*. Further, modification of NE preprotein appears to intimately follow the expression of preprotein in myeloid precursors. Previous studies describe that the folding, activation, and granular targeting of elastase is independent of C-terminus. Deglycosylation/removal of C-terminus might occur after elastase is sorted into granules (Lindmark *et al.*, 1990; Gullberg *et al.*, 1995). These results indicate that canine neutrophils still contain small amount of modified and non-modified elastase preproteins, albeit it is not at concentrations comparable to mature elastase with a MW ~28kD (Figure 2.6). These elastase preproteins are not co-localized with MPO in primary granules. Therefore, it is likely that the deglycosylation/removal of C-terminus of elastase is occurred before sorting into granules. Using rat basophilic/mast cell line RBL, previous studies suggest that a population of neutrophil elastase precursors is transported to plasma membrane first, and then trafficking back to secretory lysosomes by endocytosis. Removal of elastase C-terminus resulted in bypass of plasma membrane trafficking (Tapper *et al.*, 2006). In agreement with this finding, these results indicate that

the translocation of elastase might include two routes. One route is direct deposit of elastase into granules from the TGN, which does not require the presence of C-terminal peptide. This is likely the route that elastase is sorted into regulated secretory pathway. The second route is internalization of elastase after their transport to plasma membrane. Elastase C-terminal peptide is critical to the success of the second route. However, the timing and the mechanism of the divergence between the sorting of elastase into constitutive and regulated secretion pathway, and the role of NE C-terminus in this process remain to be investigated.

These results demonstrate that a modified NE preprotein with MW ~35kD is gradually increased during granulopoiesis. How these modified proteins are further processed into catalytically active enzymes can be further studied when BMMCs are cultured for a long term with induction by growth factors SCF and G-CSF. Alternatively, culture of purified canine hematopoietic progenitor cells – $CD34^+$ cells would allow a more fundamental investigation of biosynthesis and modification of elastase precursor proteins (Niemeyer *et al.*, 2001).

The antibodies to immunogenic peptides of canine NE have been demonstrated to be useful for western immunoblotting and immunocytochemistry using confocal microscopy and electron microscopy. The ability to recognize preprotein and mature forms of elastase will greatly facilitate the study of the intracellular compartmentalization, and the processing and modification of canine NE. Since NE is involved in multiple hematologic disorders, the understanding of this process will help us better characterize molecular mechanisms associated with congenital neutropenia in human and dogs.

Figure 2.1. Western blot analysis of canine PMNs. Rabbit polyclonal antibody to human neutrophil elastase (#481001, Calbiochem) was used as primary antibody. Secondary antibody was horseradish peroxidase (HRP) -conjugated goat anti-rabbit IgG. The primary antibody recognized one protein band with MW ~28kD in canine neutrophils. Lane 1 to 4: canine PMN cell lysate.

Figure 2.1

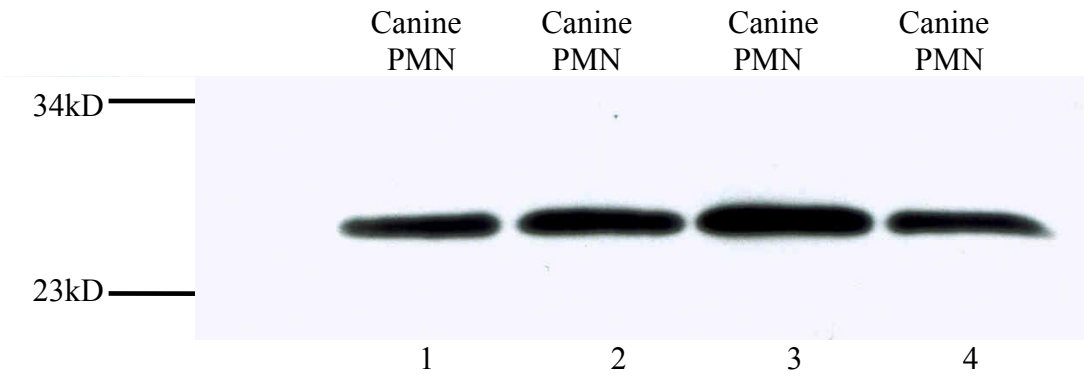


Figure 2.2. Western blot analysis of neutrophil subcellular fractions. Neutrophils were isolated, homogenized by nitrogen cavitation, and fractionated into 8 subcellular fractions using discontinuous Percoll gradients. Primary and secondary antibodies were the same as in figure 2.1. Lane 1: human leukocyte elastase; Lane 2 to 9: canine PMN subcellular fraction 1 to fraction 8. The protein recognized by the antibody was found in PMN fraction 1 and 2, which primarily contain PMN cytosolic proteins.

Figure 2.2

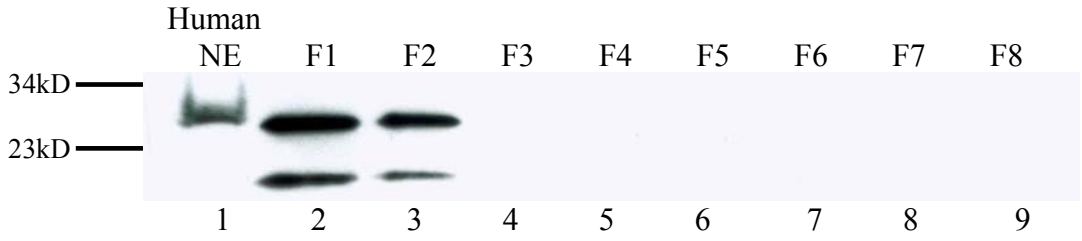


Figure 2.3. Amino acid sequence of canine neutrophil elastase (NE) [*Canis familiaris*]. Highlighted sequences are the predicted immunogenic peptide sequences which were used to generate polyclonal and monoclonal antibodies. The antibody generated to peptide located in amino acid sequence 85-98 was ELA85. The antibody generated to peptide located at the C-terminus was ELA269.

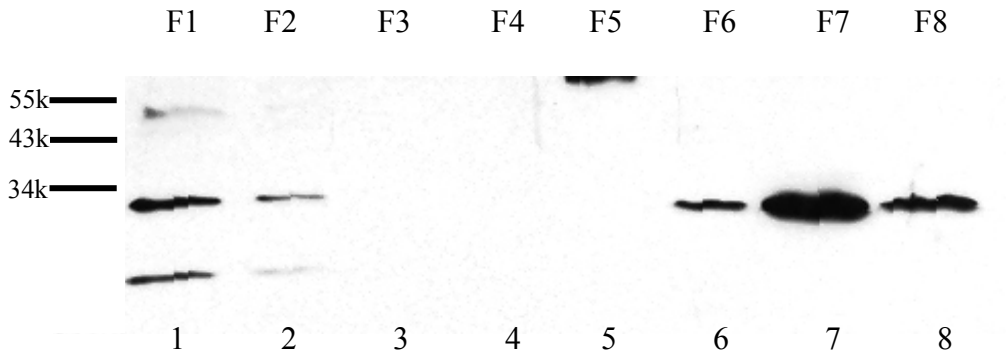
Figure 2.3

MTARRVPAGPALGPLLLLATLLPGPALASEIVGGRPAQPHAWPFMVSLQRRGGHF
CGGTLIAPNFVMSAAHCVDGLNFRSVVVVLGAHDLGERESTRQLFAVQRVFENG
FDPVRLVNDIVLLQLNGSATINANVQVARLPAQNQGVGNGVQCLAMGWGQLGT
AQPPPRILQELNVTVVTTLCRRSNVCTLVPRRRAGICFGDSGGPLVCNGLIQGIDSF
IRGSCASGFFPDAFAPVAQFVDWINSIIRRPPALPPARPGQQDPERGAARAPPPAPH
RPRPTQ

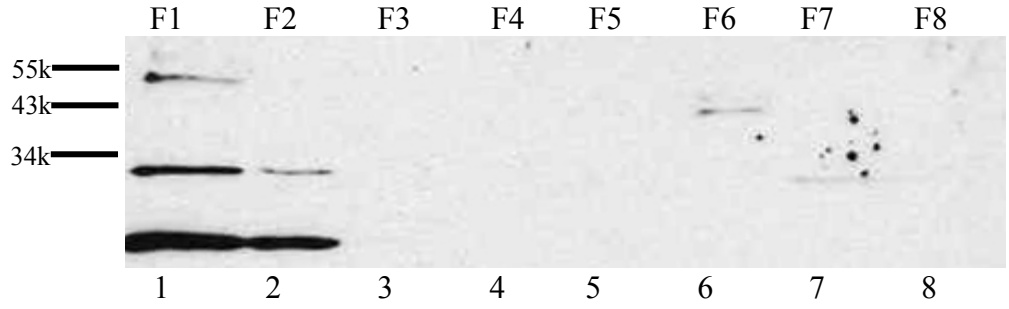
Figure 2.4. Western blot analysis of canine PMN subcellular fractions. Neutrophils were isolated, homogenized by nitrogen cavitation, and fractionated into 8 subcellular fractions using discontinuous Percoll gradients. Equivalent amounts of protein (5μg) were loaded in each lane. A. Antibody pELA85 was used as primary antibody on western blot. Lane 1 to 8: PMN subcellular fraction 1 to fraction 8. Maximal concentration of a band with MW ~ 28kD was found in fraction 7. B. pELA85 was first incubated with antigenic peptide #85-98, and then used on western blot. Lane 1 to Lane 8: PMN subcellular fraction 1 to fraction 8. Incubation with the peptide inhibited the recognition of pELA85 to the protein band. The protein with MW ~28kD should be neutrophil elastase mature protein. C. Monoclonal antibody to canine myeloperoxidase (MPO) was used as primary antibody. Lane 1: canine MPO. MPO is a marker protein of neutrophil primary granules. Lane 2 to 9: PMN subcellular fraction 1 to 8. MPO in the neutrophils was maximally concentrated in fraction 7.

Figure 2.4

A



B



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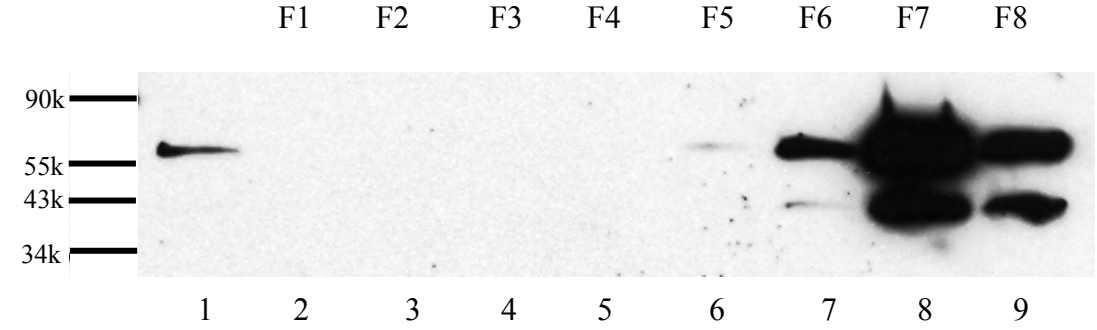


Figure 2.5. Western blot analysis of canine PMNs. Monoclonal antibody mELA85 was used as primary antibody. Secondary antibody was HRP-conjugated goat anti-mouse IgG. mELA specifically recognized a protein band with MW ~28kD. Lane 1 to 4: canine PMN whole cell lysates.

Figure 2.5

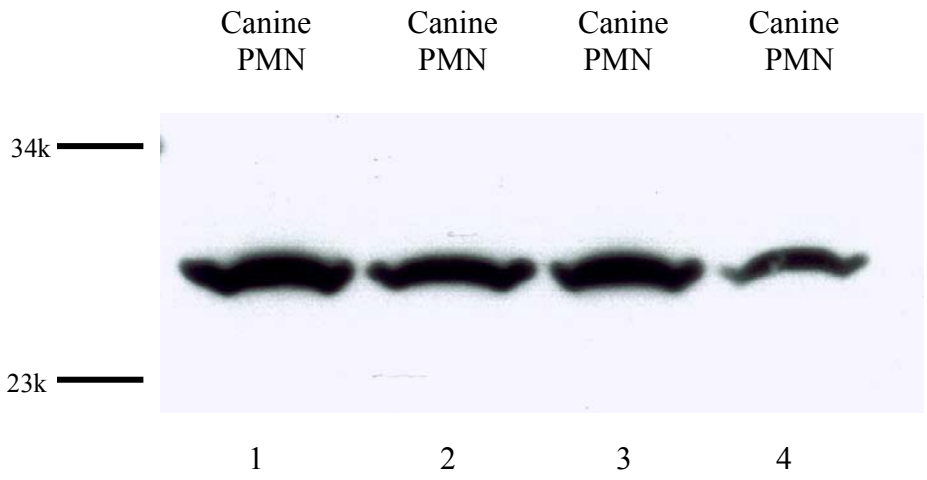


Figure 2.6. Western blot analysis of canine PMNs. Lane 1 and 2: mELA269 was used as primary antibody. Lane 3 and 4: mELA85 was used as primary antibody. Secondary antibody was HRP-conjugated goat anti-mouse IgG. mELA269 and mELA85 recognized two isoforms of neutrophil elastase (NE). mELA269 recognized two NE precursor proteins with MW ~33 and ~35kD, respectively. mELA85 recognized mature NE protein with MW ~28kD.

Figure 2.6

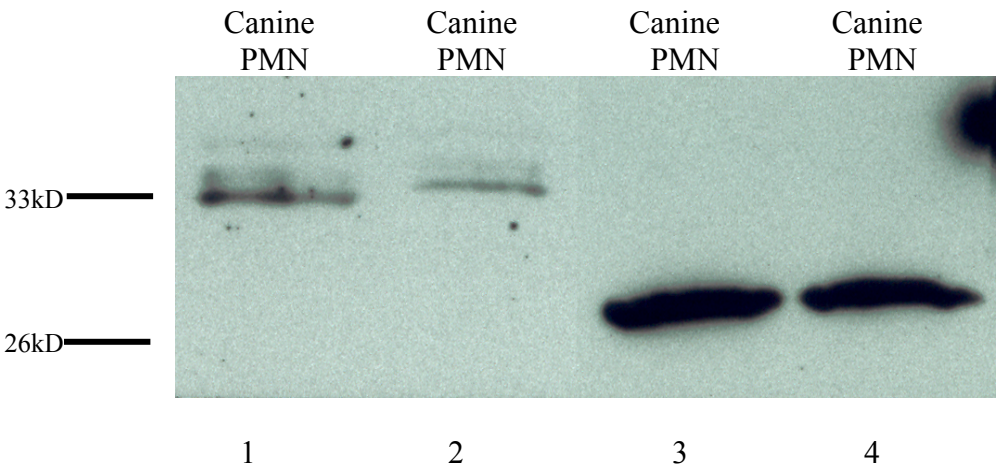
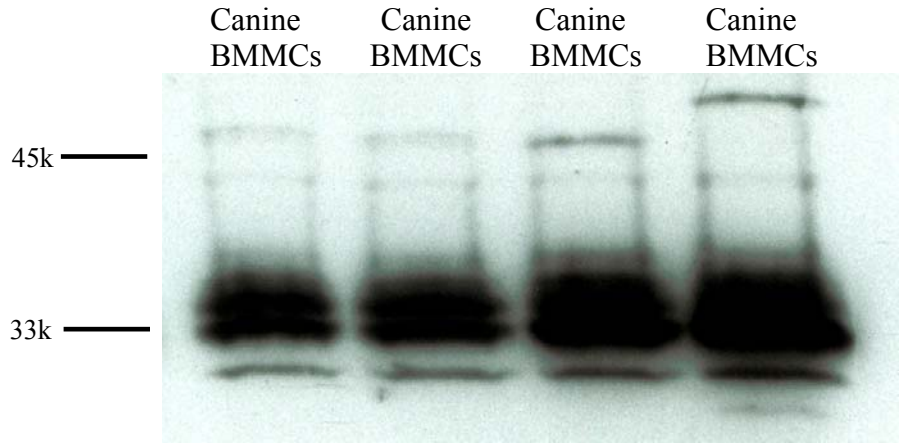


Figure 2.7. Western blot analysis of canine bone marrow mononuclear cells (BMMCs). A. pELA269 was used as primary antibody. B. pELA269 was incubated with peptide #269-282 and then used as primary antibody. Secondary antibody was horseradish peroxidase (HRP) -conjugated goat anti-mouse IgG. The incubation with peptide inhibited the binding of pELA269 to the two protein bands with MW ~33 and ~35kD. Lane 1 to 4: canine BMMCs cell lysates.

Figure 2.7

A


Canine BMMCs
Canine BMMCs
Canine BMMCs
Canine BMMCs
45k
33k

B

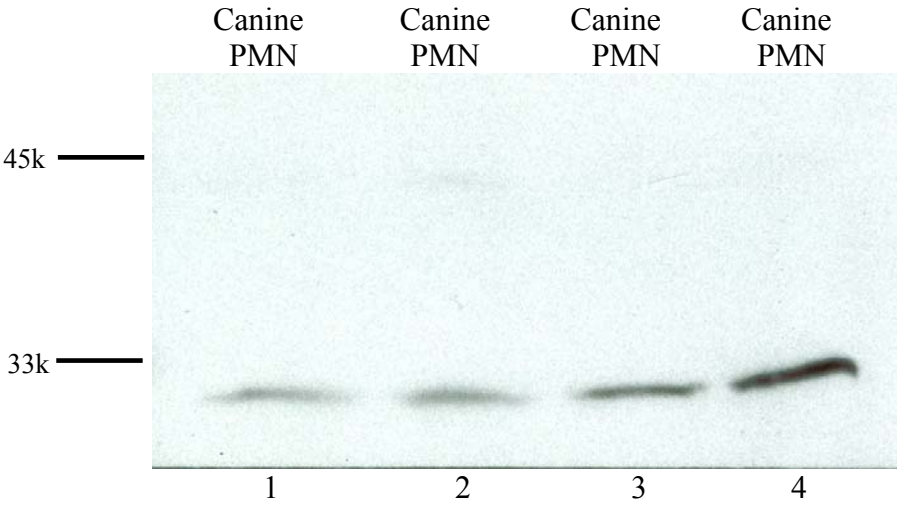
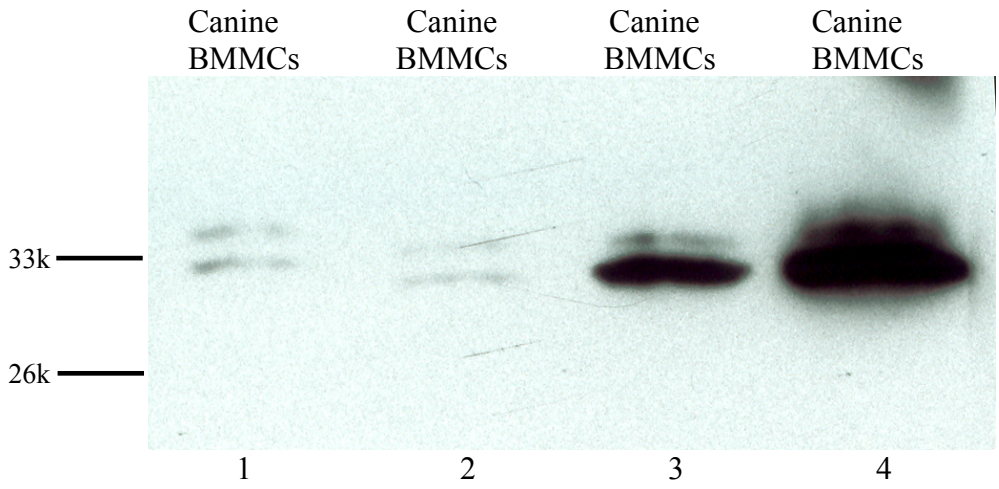

Canine PMN
Canine PMN
Canine PMN
Canine PMN
45k
33k
1
2
3
4

Figure 2.8. Western blot analysis of canine bone marrow mononuclear cells (BMMCs). A. mELA269 was used as primary antibody. B. mELA269 was incubated with peptide #269-282, and then used as primary antibody. Horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG was used as secondary antibody. The incubation completely inhibited the binding of mELA269 to target proteins. Lane 1 to 4: canine BMMCs lysates.

Figure 2.8

A


Canine BMMCs
Canine BMMCs
Canine BMMCs
Canine BMMCs
33k
26k
1
2
3
4

B

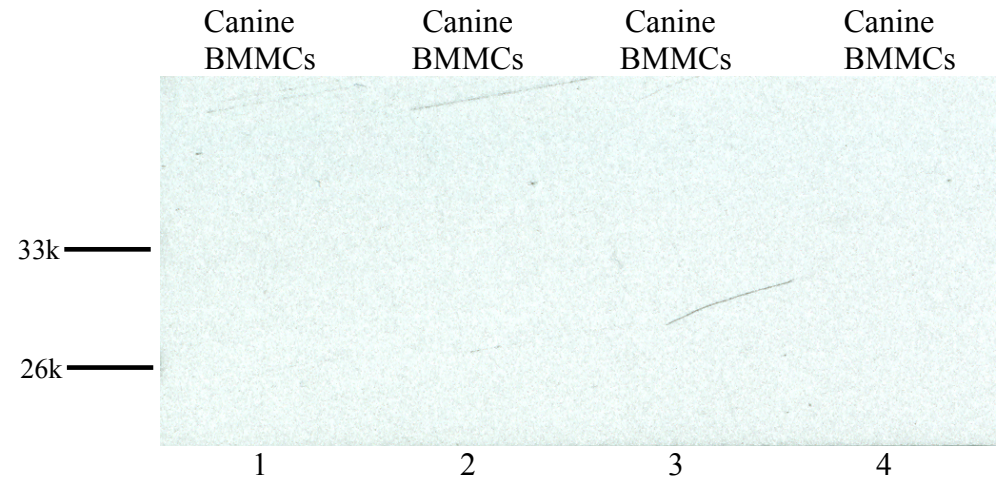

Canine BMMCs
Canine BMMCs
Canine BMMCs
Canine BMMCs
33k
26k
1
2
3
4

Figure 2.9. Western blot analysis of bone marrow mononuclear cells (BMMCs). BMMCs were cultured with stem cell factor (SCF) and granulocyte-colony stimulating factor (G-CSF), and then collected at 0, 6, 18, 30, 42, 54 and 66 hour time points as described in Materials and Methods. Cell lysates were made from the collected cells and subjected to western immunoblotting. mELA269 was used as primary antibody. Secondary antibody was horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG. Lane 1 to 7: BMMCs collected at 0, 6, 18, 30, 42, 54 and 66 hour time points. Protein expression profiles of NE preprotein (~33kD) and modified NE preprotein (~35kD) were determined. NE preprotein (~33kD) was induced to express in BMMCs after 6 hours in culture. The modified preprotein (~35kD) expressed after 18 hours in culture and gradually increased its expression in the subsequent time points.

Figure 2.9

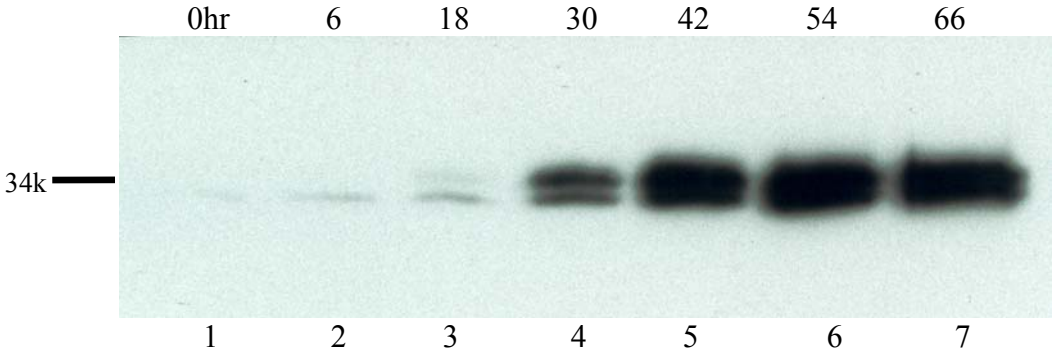
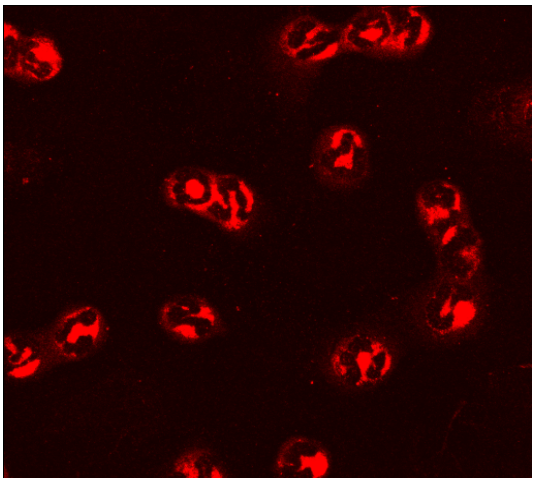


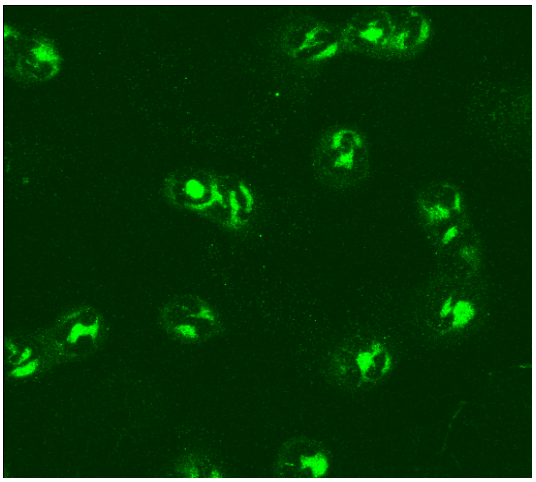
Figure 2.10. Double immunocytochemistry of canine PMNs. Either mELA85 or monoclonal antibody to canine myeloperoxidase (MPO) was used as primary antibody. Secondary antibodies were Alexa Fluor®488 or Alexa Fluor®574-conjugated goat anti-mouse IgG. A. Staining of neutrophil elastase (NE) with mELA85 in PMNs was indicated by green fluorescence. B. Staining of MPO in PMNs was detected by red fluorescence. C. Merged image of A and B illustrating that NE was co-localized with MPO in primary granules. D. PMNs were incubated only with secondary antibody and then mounted with Vectashield® with propidium iodide (PI). There was no non-specific staining of secondary antibody in the cells. E. Mouse IgG1 negative control was used as primary antibody. Secondary antibody was the same as previous. The cells were mounted with Vectashield® with PI. There was no non-specific staining of mouse IgG1 negative control antibody in the cells.

Figure 2.10

A



B



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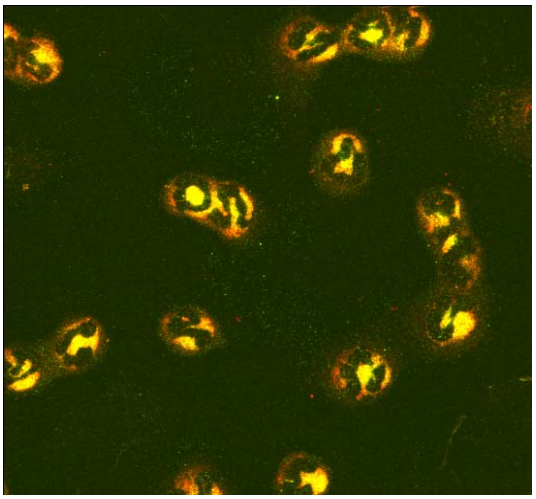
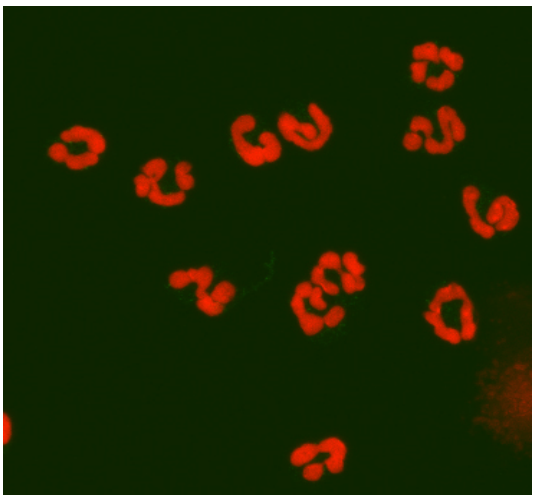


D



E

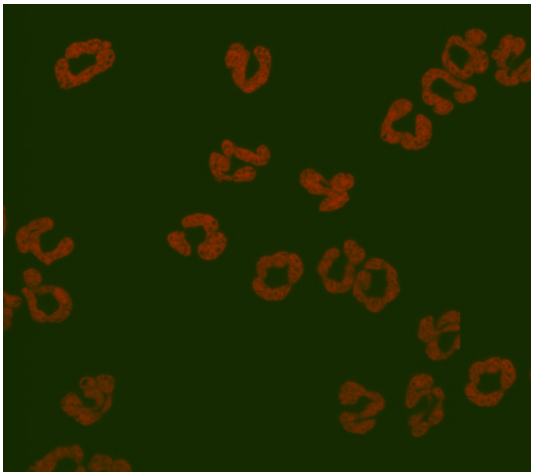


Figure 2.11. PMN ultrathin sectioning. An ultrathin section of normal PMNs was labeled with mELA85 as primary antibody. The 15nm gold-conjugated goat anti-mouse IgG/IgM was used as secondary antibody. The micrograph illustrating that gold particles (arrows) were found in granule-like subcellular compartments.

× 67,500.

Figure 2.11

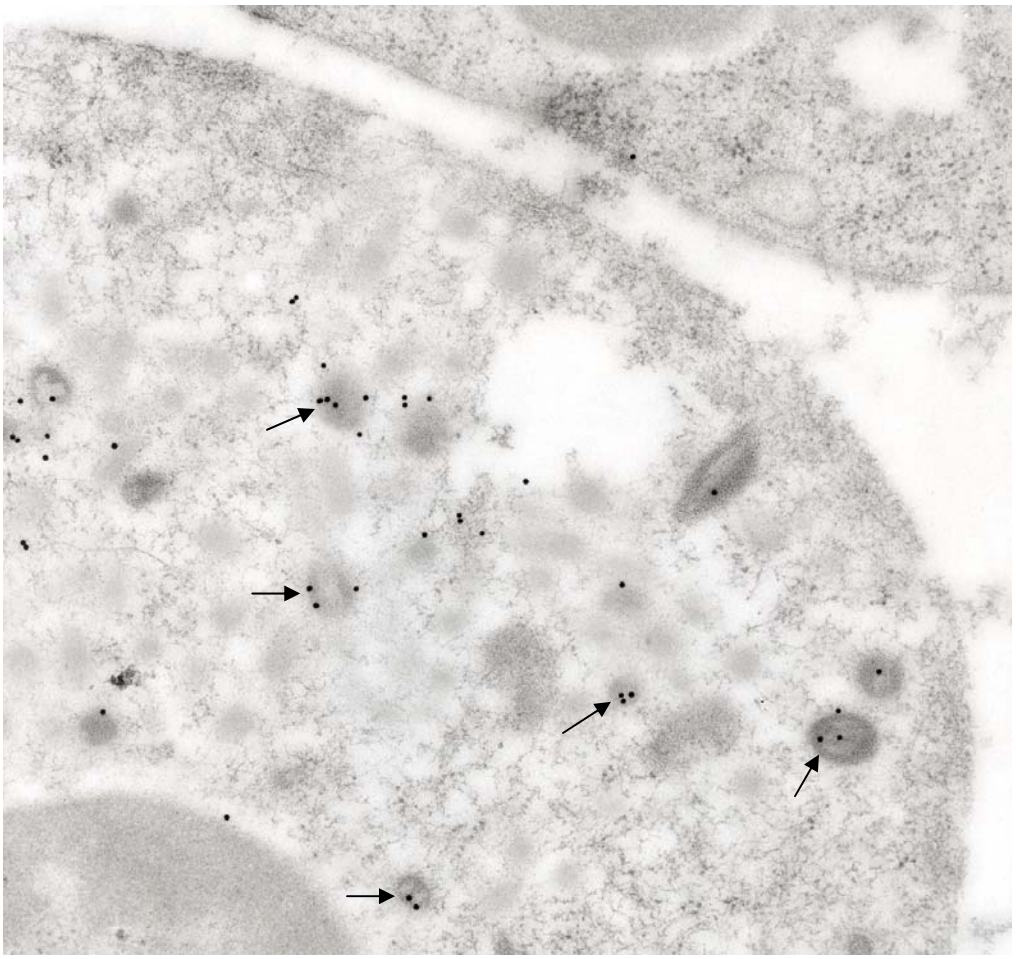
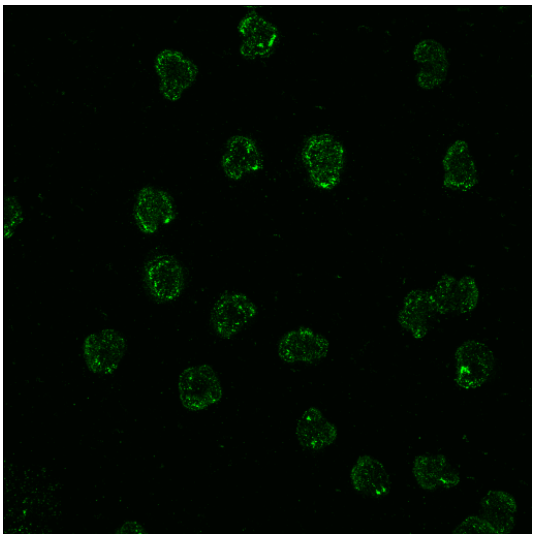


Figure 2.12. Double immunocytochemistry of canine PMNs. Either mELA269 or monoclonal antibody to canine myeloperoxidase (MPO) were used as primary antibody. Either Alexa Fluor®488 or Alexa Fluor®574-conjugated goat anti-mouse IgG was used as secondary antibody. A. Staining of canine neutrophils with mELA269 was detected by green fluorescence. B. Staining of MPO with antibody to canine MPO was detected by red fluorescence. C. Merged image of A and B illustrating that the protein recognized by mELA269 was not co-localized with MPO in neutrophil primary granules.

Figure 2.12

A



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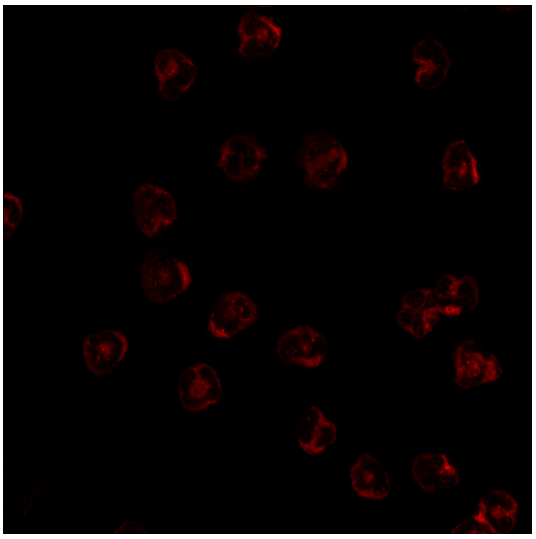

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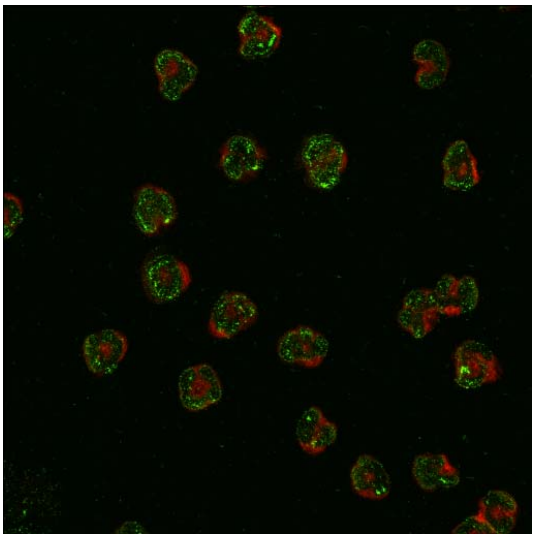
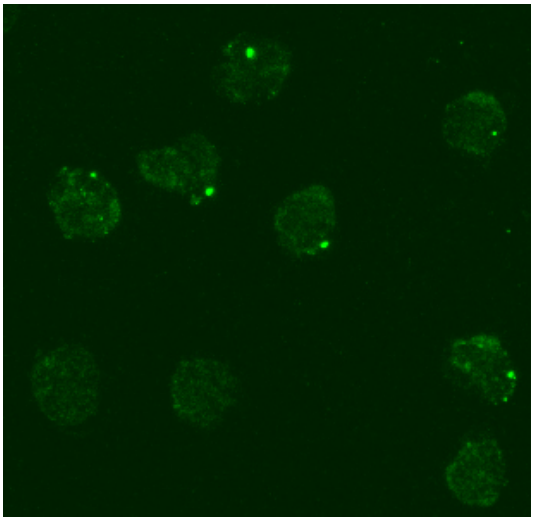


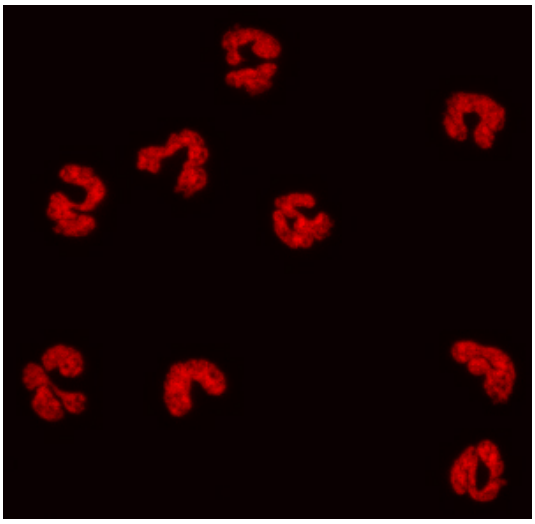
Figure 2.13. Immunocytochemistry of canine PMNs. mELA269 was used as primary antibody and Alexa Fluor®488-conjugated goat anti-mouse IgG was used as secondary antibody. The cells were mounted with VECTASHIELD® mounting medium with propidium iodide (PI). A. Staining of canine neutrophils with mELA269 was detected by green fluorescence. B. Staining of neutrophil nuclei was detected by red fluorescence. C. Merged image of A and B illustrating that proteins recognized by mELA269 were not found in PMN nuclei.

Figure 2.13

A



B



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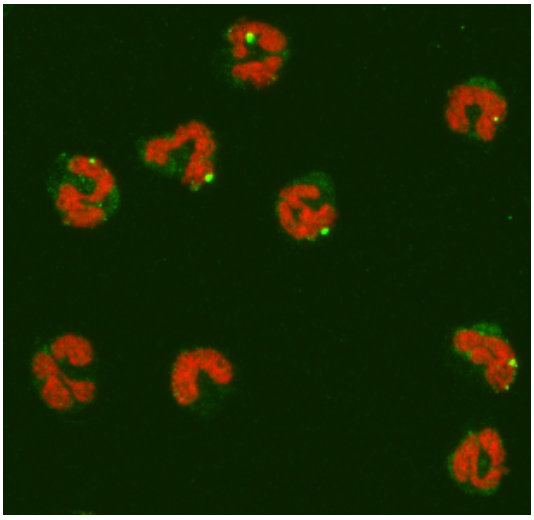


CHAPTER 3

EXPRESSION AND INTRACELLULAR LOCALIZATION OF NEUTROPHIL ELASTASE IN CANINE CYCLIC HEMATOPOIESIS

ABSTRACT

Canine cyclic hematopoiesis (CH) is characterized by cyclic fluctuation of polymorphonuclear neutrophils (PMN) and the other blood cells with a 14-day periodicity. Canine CH, an autosomal recessive disease, is caused by an insertional mutation in *AP3B1*, which encodes the β3A subunit of AP-3. Canine CH resembles human cyclic neutropenia (CN), which results from heterogeneous point mutations in *ELA2*, encoding neutrophil elastase (NE). It was proposed that in CH dogs, the mutation of β3A perturbs the function of AP-3, and impairs the shuttling of NE to lysosome-like organelles, the primary granules. Therefore, NE might be translocated to its default location the plasma membrane. To address this question, an elastase enzyme activity assay and western immunoblotting were performed following subcellular fractionation of neutrophils using discontinuous Percoll gradients in normal and CH dogs. Reduced amounts of NE protein and decreased enzymatic activity were observed in neutrophil subcellular fractions and whole cell lysates from the affected dogs. Interestingly, in CH dogs, mature NE was found exclusively present in the subcellular fractions which mainly contain primary granules. This was confirmed by the co-localization with the primary

granule markers, myeloperoxidase (MPO), by both western blot and immunocytochemistry. These data indicated that mature NE might not be accumulated on neutrophil plasma membrane as it was originally proposed. Using antibodies specifically to canine NE precursor proteins, CH dog neutrophils were found to contain increased NE proteins with intact C-terminus, while normal dog PMNs only contained a trace amount of the same precursor protein. This indicated that the processing of NE preproteins during neutrophil maturation is defective in CH dogs. Western immunoblotting of canine PMN and cultured bone marrow mononuclear cells (BMMCs) indicated that the expression of μ3A subunit of AP-3 is normal in canine CH. Furthermore, DAB staining of primary granules in CH dogs demonstrated less dense, smaller and abnormal shaped primary granules compared to normal dogs. These observations of PMN, along with the decreased storage of the mature elastase protein in primary granules and increased accumulation of NE precursor proteins suggested that not only the processing and modification of elastase is affected due to the mutation of *AP3B1*, but also the biogenesis of granules is altered in CH dogs.

3.1 Introduction

Cyclic neutropenia in grey collie dogs was first reported in 1967 (Lund *et al.*, 1967). The affected dogs can be identified by a diluted coat color and small body size at birth. This disorder in the dog is also known as canine cyclic hematopoiesis (CH) and is inherited in an autosomal recessive pattern. CH dogs are susceptible to bacterial infections during the nadir of the 13-14 day neutrophil cycle. In addition to cyclic fluctuation of neutrophils, other circulating blood cells including monocytes,

lymphocytes, eosinophils, reticulocytes and platelets also oscillate periodically but out of phase with neutrophils (Dale *et al.*, 1972a). Allogenic bone marrow transplantation demonstrated that affected dogs can be completely cured through transplantation and the same procedure can induce the disease in normal dogs (Weiden *et al.*, 1974; Dale & Graw, 1974). Therefore, this disease is caused by abnormalities in hematopoietic stem cells, which result in periodically reduced production of neutrophils and other blood cells.

Because of the clinical and hematologic similarities, CH dogs have been used as an animal model for human cyclic neutropenia (CN), an autosomal dominant disorder with neutrophil cycles in a 21-day interval (Guerry *et al.*, 1973). Hereditary human CN is exclusively caused by heterozygous point mutations in *ELA2*, encoding a neutrophil granule serine protease – neutrophil elastase (NE) (Horwitz *et al.*, 1999). The expression of *ELA2* is tightly regulated during myelopoiesis, which results in rigidly controlled, lineage-specific production of neutrophil elastase (Fouret *et al.*, 1989; Yoshimura & Crystal, 1992). Neutrophil elastase is initially synthesized as a preprotein that is subsequently processed and modified through intracellular translocation, and either constitutively secreted extracellularly as an inactive enzyme or packaged into primary granules of segmented neutrophils as an active enzyme (Gullberg *et al.*, 1995; Garwicz *et al.*, 2005). Upon neutrophil activation, intracellular neutrophil elastase is released along with other granular contents into phagosomal vacuoles or extracellular milieu through degranulation (Pham, 2006). Neutrophil elastase substrates include a broad range of extracellular matrix proteins, such as cytokines, adhesion molecules, receptors, and complement proteins (El Ouriaghli *et al.*, 2003; Lane & Ley, 2003; Chua & Laurent, 2006).

In contrast to human CN, canine CH is caused by an insertional mutation in exon 20 of *AP3B1*, encoding adaptin β3A, which is a large subunit of the adaptor-related protein complex -3 (AP-3) (Benson *et al.*, 2003). The other three subunits in AP-3 are a small σ3A, a medium size μ3A, and a large δ3A (Simpson *et al.*, 1996; Robinson & Bonifacino, 2001). There are at least four heterotetrameric adaptor-related protein complexes (AP-1, AP-2, AP-3 and AP-4) which have been identified in eukaryotes. The AP complexes are primarily responsible for intracellular protein transport at different stages in the secretion pathway (Nakatsu & Ohno, 2003). The four subunits of the AP-3 function differently but coordinately to transfer nascent integral membrane proteins from the trans-Golgi network (TGN) to lysosome or lysosome-like organelles, such as primary granules in neutrophils (Robinson, 2004). Yeast two hybrid assay experiments indicated that mature form of human neutrophil elastase interacts with μ3A through a tyrosine-based sorting signal. It was proposed that the mutation in *AP3B1* might disrupt the assembly of AP-3 and perturb the intracellular trafficking of neutrophil elastase and misdirect neutrophil elastase to the plasma membrane (Benson *et al.*, 2003). To determine whether the trafficking and storage of neutrophil elastase is altered in CH dogs, canine specific elastase antibodies which react with the elastase preprotein and the active enzyme were generated. Neutrophil elastase enzymatic assay was also used to determine the subcellular localization of active elastase in neutrophils. The results provided direct evidence that mature active elastase is dramatically reduced in neutrophil primary granules from CH dog, while there was an excessive accumulation of elastase precursor proteins. The processing of elastase preproteins during neutrophil maturation is defective in CH dog, which results in decreased deposit of mature elastase in the primary granules. This defect is apparently

closely related to ineffective sorting and translocation of elastase among intracellular compartments, presumably due to decreased binding of NE with AP-3 because of the mutation of β3A subunit. It is the first time using canine specific antibodies to study mature and precursor protein forms of NE in canine CH.

3.2 Materials and Methods

3.2.1 Experimental animals and determination of neutrophil cycle

CH dogs, CH heterozygotes and normal dogs were from a dog colony that has been well described (Yang *et al.*, 1974; Jones *et al.*, 1975a; Jones *et al.*, 1975b; Jones *et al.*, 1975c). All animals were housed in AAALAC, Intl. accredited facilities and the experimental protocols were approved by the institutional IACUC (OLAW assurance #: A-3152-01). Dogs were administered routine vaccinations and used only when free of concurrent infections. The CBC was determined on alternating days to establish the CH cycle days.

3.2.2 Blood and bone marrow sample collection

Peripheral blood was collected from normal, CH and CH carrier dogs into EDTA-containing BD Vacutainer® tubes (BD, Franklin Lakes, NJ). Polymorphonuclear (PMN) cells were isolated by Ficoll-Hypaque (Amersham Biosciences, Piscataway, NJ) density gradient sedimentation. Erythrocytes were removed by Dextran T-500 (Sigma) sedimentation. Finally, cells were suspended in 0.3% NaCl for hypotonic lysis of residual erythrocytes. The purity of neutrophils from each sample was greater than 95% based on evaluation of cytocentrifuge cell preparations (Shandon Cytospin III).

Bone marrow from dogs was collected in a 12ml syringe containing 3ml Iscove's modified Dulbecco's medium (IMDM) (Invitrogen Corporation, Carlsbad, CA) and 150 units of Heparin (Sigma). Following centrifugation on Ficoll-Hypaque gradients, bone marrow mononuclear cells were collected, and washed three times in phosphate buffered saline (PBS, Invitrogen) and resuspended in IMDM containing 10% heat-inactivated fetal bovine serum (FBS; Hyclone, Logan, UT) and 100U/ml penicillin and 100 μg/μl streptomycin (Invitrogen).

3.2.3 Culture of bone marrow mononuclear cells

Freshly isolated bone marrow mononuclear cells (BMMCs) were cultured in IMDM containing 10% FBS and 100U/ml penicillin, 100μg/μl streptomycin (Invitrogen), 25ng/ml canine stem cell factor (SCF; R&D Systems, Inc. Minneapolis, MN), and 10ng/ml canine granulocyte colony stimulating factor (G-CSF; Stem Cell Technologies, Inc.) at 37°C in a CO_2 water-jacketed incubator (Thermo Electron Corporation, Waltham, MA). Cells were collected at seven successive time points: 0, 6, 18, 30, 42, 54 and 66 hour. The cells were washed in cold PBS to remove culture medium, and then cell lysate were prepared for immunoblot analysis.

3.2.4 Antibodies of immunoblot analysis

Four clones of monoclonal antibodies to canine myeloperoxidase (MPO) were generous gifts from Dr. William Vernau (University of California, Davis, CA). Rabbit polyclonal antibody (pELA85) to antigenic peptide of canine neutrophil elastase was generated by Zymed Laboratories Inc. (Invitrogen Corporation, San Francisco, CA).

Monoclonal antibodies (mELA85 and mELA269) to canine neutrophil elastase antigenic peptides were generated by Auburn University Hybridoma Facility. Monoclonal antibody to P47A (μ3A) was purchased from BD Biosciences Pharmingen (San Diego, CA). Monoclonal antibody to GAPDH (Abcam Inc., Cambridge, MA) was used as protein loading control. HRP-conjugated antibodies (Santa Cruz Biotechnology, Inc. Santa Cruz, CA) to the relevant species of generating primary antibodies were used as secondary antibodies.

3.2.5 Cell lysate preparation and western immunoblot analysis

For immunoblotting, cells were washed in cold PBS and resuspended in 1×RIPA lysis buffer (Upstate, Lake Placid, NY) containing 1× protease inhibitor (Roche Applied Science, Mannheim, Germany). The cell suspension was further homogenized by passage through a 21 gauge needle and centrifuged at 15,000×g to obtain cell lysates. Samples were denatured at 95°C for 5 minutes for immuno-blot analysis. Proteins were separated in a 4-20% linear gradient gel (Bio-Rad Labs, Hercules, CA) using a Ready Gel Precast Gel System (Bio-Rad, CA), and transferred in Towbin transfer buffer to a nitrocellulose membrane under high intensity transfer conditions. After initial blocking with StartingBlock™ Tris-buffered saline (TBS) blocking buffer (Pierce Biotechnology, Rockford, IL), blots were incubated with primary antibodies overnight at 4°C, and then washed in TBS washing buffer containing 0.05% Tween (Bio-Rad). Blots were incubated with HRP-conjugated secondary antibodies for 1 hour at room temperature and then washed again in the same washing buffer. Blots were incubated with SuperSignal® West Dura Extended Duration Substrate (Pierce Biotechnology, Rockford, IL) for 5 minutes

and visualized by exposure to a Kodak BioMax XAR film (Fisher Scientific, Pittsburgh, PA).

The specificity of the polyclonal and monoclonal antibodies to canine neutrophil elastase was determined by incubating the primary antibody with the corresponding peptides prior to blotting. The mixture was centrifuged for 15 minutes at 4°C (15,000rpm) to pellet immune complexes, and then diluted in blocking buffer before immunoblotting.

3.2.6 Subcellular fractionation

Subcellular fractionation was according to a protocol previously described with minor modifications (Kjeldsen *et al.*, 1999) . Freshly isolated neutrophils were washed in PBS and centrifuged at 400×g for 10 minutes. The cell pellet was resuspended at 0.5-1.0×10^8 cell/ml in disruption buffer (100mM KCl, 3mM NaCl, 1mM $ATPNa_2$, 3.5mM $MgCl_2$, 10mM Piperazine, N, N'-bis-2 ethanesulfonic acid, pH7.2) and then pressurized on ice under nitrogen for 5 minutes at 380psi in a nitrogen bomb (Parr Instrument, Moline, IL). Cavitates were collected in disruption buffer containing 1.5mM EGTA, and then centrifuged at 400×g for 15 minutes to pellet nuclei and intact cells. The postnuclear supernatant was applied to a three-layer discontinuous Percoll (Amersham Biosciences, Piscataway, NJ) gradient (1.050/1.090/1.120g/ml), then centrifuged at 37,000×g for 30 minutes at 4°C in a SM-24, fixed angle rotor in a Sorvall RC-5C centrifuge (Sorvall Instruments, Duluth, GA). The fractions (1 ml) were collected by pipetting from top to bottom. Percoll was removed by centrifugation at 100,000×g for 90 minutes at 4°C in a 70.1Ti, fixed angle rotor in a Beckman L8-M ultracentrifuge (Beckman Instrument, Inc. Palo Alto, CA).

3.2.7 Enzymatic activity assay

Neutrophil cell lysates or neutrophil subcellular fractions were gently solubilized with M-PER® mammalian protein extraction reagent (Pierce Biotechnology, Rockford, IL). A *DC* protein assay kit (Bio-Rad, Hercules, CA) was used to determine protein concentration. MPO enzymatic activity from neutrophils and cell fractions was measured using EnzChek® myeloperoxidase (MPO) activity assay kit (Molecular Probes, Eugene, OR). Neutrophil and cell fraction elastase activity was determined by EnzChek® elastase assay kit (Molecular Probes, OR). All samples were standardized to 60µg protein per MPO assay, and 40µg protein per elastase assay. The fluorescence was read on a Synergy™ HT multi-detection microplate reader (BioTek Instruments, Inc. Winooski, Vermont).

3.2.8 Electron microscopy / primary granule labeling with diaminobenzidine

3.2.8.1 Primary granule labeling in neutrophils

The MPO activity of the primary (azurophil) granules in the neutrophils was determined using 3,3'-diaminobenzidine (DAB) as a substrate as described by Graham and Karnovsky (1966). Peripheral blood samples were fixed in 3% glutaraldehyde in 0.1M Millonig's phosphate buffer (pH 7.3) (Fittschen *et al.*, 1983). Briefly, fresh, EDTA-treated blood was centrifuged for 3 minutes at 3000 rpm at 4°C in Kimax borosilicate glass tubes (6 × 50 mm O.D.xL, Fisher Scientific, Suwanee, GA). The overlying plasma layer was carefully removed and cold fixative (4% paraformaldehyde + 0.05% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4) was gently added to the top of the test tube so as not to disrupt the buffy coat cells. After fixation (10 minutes), the buffy

coats were gently released from the test tubes and minced with a razor blade in the same fixative solution at 4°C. Subsequently, the buffy coat pieces were rinsed in the cacodylate buffer. At this point, the samples were divided in two aliquots, the first being processed for routine transmission electron microscopy (TEM) for ultrastructure, and the second for MPO cytochemistry. For the demonstration of MPO activity, the samples were incubated in 0.05% DAB-Tris solution (0.05 M Tris buffer, pH 7.6) at RT for 10 minutes. Hydrogen peroxide (final conc. 0.01%) was added to the DAB-Tris solution and the preparation was incubated for an additional 60 minuntes at RT. The buffy coat pieces were washed with the same Tris buffer containing 5% sucrose (Bainton, 1999), and post-fixed in 1% osmium tetroxide in Millonig's phosphate buffer (0.1 M, pH 7.3) for 1 hour. The samples were then dehydrated in an ascending series of ethanol to propylene oxide, and embedded in Durcupan ACM plastic (Electron Microscopy Sciences, Ft. Washington, PA). The ultrathin sections for routine TEM were stained with uranyl acetate and lead citrate. The sections for MPO cytochemistry were not counterstained to permit easier observation of the electron-dense DAB-staining product. All specimens were examined using Philips 301 TEM (FEI Co., Hillsboro, OR) operating at 60 kV.

3.2.8.2 Labeling of subcellular fractions

Subcellular fractions of neutrophils were processed as described above. Percoll was removed from the subcellular fractions by ultracentrifugation (100,000x g, 1 hour). The biological material was carefully aspirated and resuspended in fixative (1:1 vol/vol, 3% glutaraldehyde in 0.1 M Millonig's phosphate buffer, 0.1 M, pH 7.3) and kept at 4°C until further processing. The fractions were sedimented (16,000 × g, 5 minutes) in an

Eppendorf Microcentrifuge (Eppendorf North American, Westbury, NY) to remove the supernatant. The pellets were then processed as above, for routine TEM and MPO cytochemistry.

3.2.9 Double immunofluorescence labeling and confocal microscopy

Cytospin preparations of freshly isolated neutrophils were fixed with 4% paraformaldehyde. Following fixation, the cytospins were permeabilized with 1% Triton X-100 in PBS with 1% bovine serum albumin (BSA; Invitrogen). Nonspecific binding was blocked with 5% goat serum (Jackson ImmunoResearch Laboratories, West Grove, PA) and 2.5% BSA in PBS. The cytospin was incubated with mELA85 or mouse IgG1 negative control (AbD Serotec, Raleigh, NC) overnight at 4°C. Incubation with Alexa Fluor®488-conjugated goat anti-mouse IgG (Invitrogen) was performed at room temperature for 1 hour. The cytospin was blocked with mouse serum (1:20 in PBS) and then with 20 µl/ml Fab-fragment goat anti-mouse IgG (H+L) (Jackson ImmunoResearch Laboratories) for 1 hour, respectively. Cytospins were then incubated with a second monoclonal antibody to myeloperoxidase (MPO) overnight at 4°C, and finally incubated with Alexa Fluor®574-conjugated goat anti-mouse IgG (Invitrogen) for 1 hour at room temperature. The cytospin preparations were washed with PBS three times between each incubation step.

Finally, the cytospin preparations were mounted with Vectashield® Mounting Medium (Vector Laboratories Inc., Burlingame, CA). Immunofluorescence was observed under BioRad MRC-1024 laser scanning confocal microscope (Bio-Rad Laboratories).

3.3 Results

3.3.1 Neutrophil primary granules of CH dog are round shaped, and they are smaller and less dense than those of normal dog

Neutrophil cytoplasmic granules are important compartment for storage of enzymes involved in the host defense against invading pathogens. Previous studies indicated that CH dog neutrophils had decreased bactericidal capacity (Chusid *et al.*, 1975). To identify whether CH neutrophil granules were morphologically different from normal neutrophil, DAB staining was performed on paraformaldehyde fixed blood buffy coat cells, which are mainly neutrophils. Under electron microscopy, CH neutrophils had less DAB-stained granules and the granules were smaller than normal neutrophils. In addition, the shape of the stained granules was not similar between normal and CH dogs (Figure 3.1A, B). To further characterize the differences between normal and CH dog granules, neutrophils were disrupted in a nitrogen bomb and the cell cavitates were subjected to a 3-layer Percoll gradient centrifugation. To determine which fraction had maximum amount of primary granule, western immunobloting was performed to identify the location of a primary granule marker protein – MPO. The result indicated that MPO was primarily found from fraction 5 to 7 and peaked in fraction 6 in CH dogs, whereas normal dogs had MPO distributed mainly from fraction 6 to 8 and peaked in fraction 7 (Figure 3.2A, B). Based on the density of the marker beads, the density of fractions containing MPO was 1.14 in normal dog and 1.12 in CH dog (data not shown). This indicated that neutrophil primary granules from CH dogs were less dense than those from normal dogs.

Based on the distribution of the primary granule marker protein MPO in neutrophil subcellular fractionation, fraction 6 in CH dog and fraction 7 in normal dog were

identified as the major primary granule fraction (Table 1). These two fractions were stained with DAB again. Consistent with previous observations of whole neutrophil, DAB-staining of these two fractions indicated that the shape and size of the primary granules in CH dogs were dissimilar to those from normal dogs. CH dog primary granules were mostly round shaped, whereas normal dog granules were mainly oval shaped (Figure 3.3A, B). Generally, the size of primary granules was 389 ± 17.5 × 210 ± 27nm in normal dogs, and 249 ± 35 × 193 ± 21nm in CH dog, which indicated an apparent size difference between normal and CH dogs.

3.3.2 CH dog mature NE is localized in neutrophil primary granules

Previously studies suggested that NE is a cargo protein of AP-3, and the mutation of *AP3B1* in CH dog causes elastase mistrafficking to the default location the plasma membrane. To determine whether there is an accumulation of elastase on plasma membrane, antibody pELA85 was used to determine the distribution of elastase in neutrophil subcellular fractions using western immunoblotting (Figure 3.4A, B). Elastase was found primarily in fraction 6 of CH dog, and in fraction 7 of normal dog. The distribution of elastase was similar to the primary granule marker protein – MPO. The molecular weight of mature NE from CH dogs was the same as of normal dog. No elastase was detected in the plasma membrane fractions. NE enzymatic assay on each subcellular fraction confirmed the results of western immunoblotting (see below). In addition, immunocytochemistry was performed to determine whether there is an accumulation of mature elastase on plasma membrane. Using mELA85 and antibody to

canine MPO, the merged image illustrated that mature NE is exclusively co-localized with MPO in neutrophil primary granules from both normal and CH dogs (Figure 3.5).

3.3.3 CH dogs have decreased elastase enzymatic activity, reduced amount of mature elastase protein, and increased elastase preproteins in neutrophils compared to normal dog

Although neutrophil elastase was localized in the primary granules as in normal dog, CH dogs were found to have decreased elastase enzymatic activity in both the whole cell assay and subcellular fraction assay (Figure 3.6). To determine whether the decease of enzymatic activity is caused by the storage of inactive elastase protein in primary granules, western immunobloting was performed using mELA85 and mELA269 on neutrophil whole cell lysate (Figure 3.7). In CH dog, the majority elastase that segmented neutrophils store was the precursor protein form, while normal dog neutrophils contained large amounts of processed mature elastase. Correspondingly, normal dogs had only a trace amount of elastase precursors in segmented neutrophils, while CH dogs had a small amount of mature elastase. Western immunoblotting using pELA85 on neutrophil subcellular fractions confirmed that mature elastase in the CH dog is dramatically decreased, and present in the fraction where primary granules are located (Figure 3.4). Immunocytochemistry using mELA85 indicated that the fluorescence in CH neutrophils is apparently weaker than normal dog, confirmed the lack of mature elastase in CH neutrophils (Figure 3.5). The decreased enzymatic activity of CH dog elastase is due to reduced accumulation of mature proteins in neutrophil primary granules.

Immunocytochemistry using mELA269 demonstrated that NE precursor proteins were greatly accumulated in the neutrophils from CH dogs than from normal dogs (Figure 3.8). In both CH and normal dogs, NE precursor molecules were not co-localized with MPO, suggesting they were localized outside of the primary granules in the cell, which was probably in the ER or Golgi complex.

3.3.4 The AP-3 subunit μ3A is normally expressed in CH dog neutrophils and during the culture of bone marrow mononuclear cells stimulated with SCF and G-CSF

CH dog has a mutation in *AP3B1*, encoding adaptin β3A, which is one of the four subunits of the AP-3 complex (Simpson *et al.*, 1996). As such, was the reduced accumulation of elastase in mature neutrophils due to the mutation of *AP3B1*? Due to the limitation of antibodies, only canine μ3A was successfully determined on western immunoblotting. None of the commercially available antibodies to β3A cross reacted with canine β3A (data not shown). The result indicated that μ3A was normally expressed in CH neutrophils (Figure 3.9). There is no degradation of this adaptin in the affected dog. Culture of bone marrow mononuclear cells stimulated with SCF and G-CSF showed CH dog BMMCs constitutively expressed μ3A during the induction, and the expression level of μ3A was the same as in the normal dog (Figure 3.10A, B). Immunocytochemistry using antibodies to μ3A indicated that μ3A was not co-localized with elastase preproteins in CH dog neutrophils. While in normal dog PMNs, μ3A subunit was co-localized with elastase precursor proteins (Figure 3.11).

3.4 Conclusions and discussion

Neutrophils are professional phagocytes, and they play a key role in the innate and induced immune response through phagocytosis against invasive pathogens (Smolen & Boxer, 1996). The function of neutrophils is largely dependent on the content of their cytoplasmic granules, especially primary granules, containing a variety of proteases which work coordinately to destroy molecular components of invading pathogens (Borregaard & Cowland, 1997). Early studies indicated that neutrophils of CH dogs have functional defects against various bacteria (Chusid *et al.*, 1975). Correspondingly from a morphological assessment, CH neutrophil primary granules were misshapen, smaller, and less dense than primary granules from normal dog neutrophils. This leads to a hypothesis that CH dog neutrophil primary granules might have less protein content than normal neutrophils. The decreased storage of bactericidal proteins in the primary granules might cause the altered size and shape of their primary granules, and therefore contribute to the bactericidal defects of CH dog neutrophils.

Further studies on NE, a neutral protease primarily stored in neutrophil primary granules, confirmed that CH neutrophils contained fewer NE mature proteins. The enzymatic activity of NE was also significantly decreased in CH dog neutrophils. This decrease was not due to accumulation of inactive proteins in primary granules, or the misallocation of mature proteins into other subcellular compartments, such as the plasma membrane. It is solely caused by the decreased storage of active, mature elastase proteins in the primary granules.

Using antibodies which specifically react with canine NE preprotein and mature protein, segmented neutrophils from CH dogs were shown to accumulate excessive

amount of NE precursor proteins (with intact C-terminus), and a small amount of mature NE in the primary granules. In contrast, neutrophils from normal dogs only contained a small amount of NE precursor proteins, but a large amount of mature NE enzyme in primary granules. The NE precursor proteins from both CH and normal dogs were not co-localized with MPO, suggested that NE precursor proteins might be localized in the ER, Golgi, cytoplasm or the plasma membrane portion of the cell.

In normal myelopoiesis, NE is secreted constitutively as an inactive preprotein or sorted to the primary granules as an active enzyme (Lindmark *et al.*, 1990; Gullberg *et al.*, 1995; Gullberg *et al.*, 1997). There are at least two possible reasons of the reduced storage of mature elastase enzyme in neutrophil primary granules from CH dogs. The first might be that CH dog has increased constitutive secretion of elastase zymogens during the differentiation of myeloid progenitor cells. The other one might be that NE is not properly folded, and the sorting and packaging into primary granules is not as efficient as in normal myelopoiesis. These results supported that in CH dog elastase is abnormally processed and sorted, resulted in decreased storage of mature elastase enzymes in neutrophil primary granules. Therefore, segmented neutrophils from CH dogs contained excessive amount of unprocessed, immature elastase preproteins.

Removing the C-terminal peptide of NE preprotein is critical for NE trafficking through plasma membrane route then back to granules (Tapper *et al.*, 2006). However, NE is able to target to granules when C-terminal coding sequence is deleted in the genome (Gullberg *et al.*, 1995). This suggested that NE sorting into granules at least through two pathways. One is direct translocation from the TGN to granule, and the other is an indirect route through internalization of NE from the plasma membrane. The

mutation of *AP3B1* in CH dogs might interrupt the direct translocation of NE from the TGN to granules, resulted in accumulation of NE preproteins. However, the plasma membrane routing might still facilitate the trafficking of NE to granules. This is supported by the observation of small amount of NE in neutrophil granules in HPS-2 patients, although their β3A subunit is missing and AP-3 is not stable due to the mutation of *AP3B1* (Fontana *et al.*, 2006).

HPS-2 patients and *pearl* mice which have β3A mutations are both characterized with oculocutaneous albinism due to abnormal melanosomes in melanocytes, and prolonged bleeding due to platelet storage pool deficiency (SPD) (Feng *et al.*, 1999; Di Pietro & Dell'Angelica, 2005). Human HPS-2 patients generally have mild neutropenia while mice neutrophil counts are normal. In HPS-2, NE protein is severely reduced and it is co-localized with the cell surface marker CD43 (Fontana *et al.*, 2006). Human HPS-2 and *pearl* mice have mutations in *AP3B1* and the deficiency of AP-3 causes abnormal cell surface accumulation of lysosomal membrane proteins. The paradox is that NE is a well-defined soluble protein, while AP-3 mainly is responsible for intracellular transport of transmembrane proteins. In HPS-2 and *pearl* mice, deficiency of β3A results in the instability of other three subunits, hence the disruption of AP-3 (Zhen *et al.*, 1999; Feng *et al.*, 1999; Dell' Angelica *et al.*, 1999b; Fontana *et al.*, 2006). However, small amount of NE was still sorted into neutrophil granules in HPS-2 patients (Fontana *et al.*, 2006). This suggested that NE might be capable of being sorted into granules independent of AP-3. It was proposed by Benson et al. that NE might have a transmembrane conformation which allows the interaction between NE and μ3A subunit of AP-3 (Benson *et al.*, 2003; Horwitz *et al.*, 2004). But there is lack of direct evidence to prove this assumption.

Similar to HPS-2 patients, CH dogs have a grey coat color, SPD and neutropenia. But the mutation in *AP3B1* in the dog seems not affecting the expression of μ3A. The limitation of antibodies against canine species does not allow us to quantify the other three AP-3 subunits. The constitutive expression of μ3A implicated that a small amount of a functional AP-3 complex might be assembled in CH dogs, since the transcriptional slippage of mutated *AP3B1* can eventually generate partial normal β3A transcripts (Benson *et al.*, 2004). The results obtained in this study indicated that there is no surface accumulation of mature NE in neutrophils from CH dogs, albeit CH dogs contained a reduced amount of mature elastase in the granules. Collectively, it appears that elastase might not be simply the cargo protein of AP-3. A protein modification and trafficking system which involves a third or even more proteins might be associated with pathogenesis of this disorder in the dog.

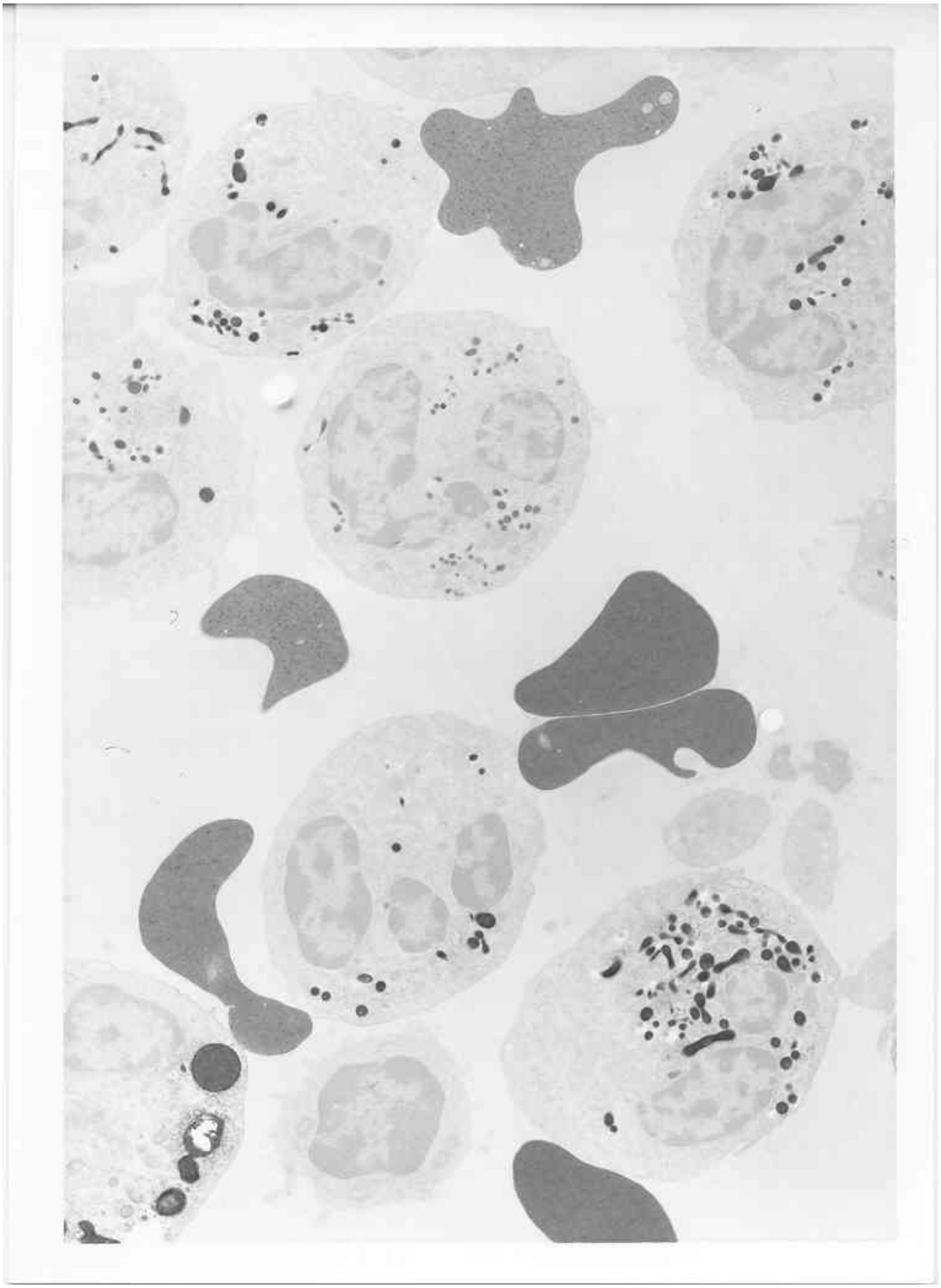
Fraction 1	Fraction 2	Fraction 3	Fraction 4	Fraction 5	Fraction 6	Fraction 7	Fraction 8
cytoplasm	cytoplasm	plasma membrane	tertiary granule	tertiary granule	secondary granule	primary granule	primary granule
	plasma membrane	secretory vesicle		secondary granule	primary granule		nuclei
	secretory vesicle						

Table 3.1. Distribution of PMN subcellular contents in each fraction. Normal dog PMNs were disrupted by nitrogen cavitation. PMN subcellular compartments were separated using a 3-layer Percoll gradient. Distribution of the contents in each fraction was confirmed by electron microscopy and western immunoblotting using antibody to marker proteins. Note: some intracellular components are present in overlapping fractions.

Figure 3.1. DAB staining of PMNs. Micrographs showing DAB staining of PMNs from A. a CH dog and B. a normal dog. MPO positive primary granules in PMNs stained darkly (opaque). Neutrophils from the CH dog have fewer dark-stained granules compared to the normal dog. × 6,000.

Figure 3.1

A



B

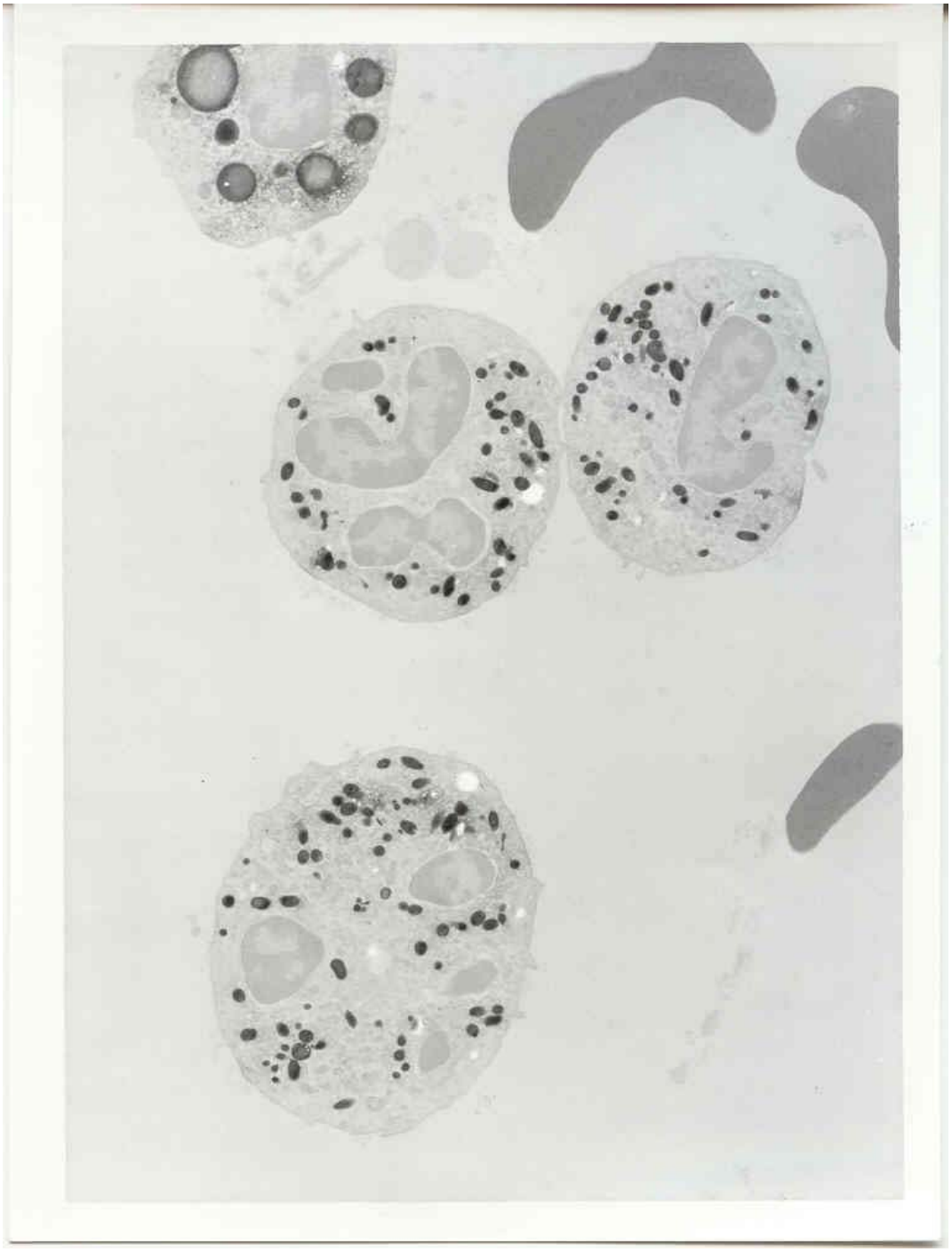
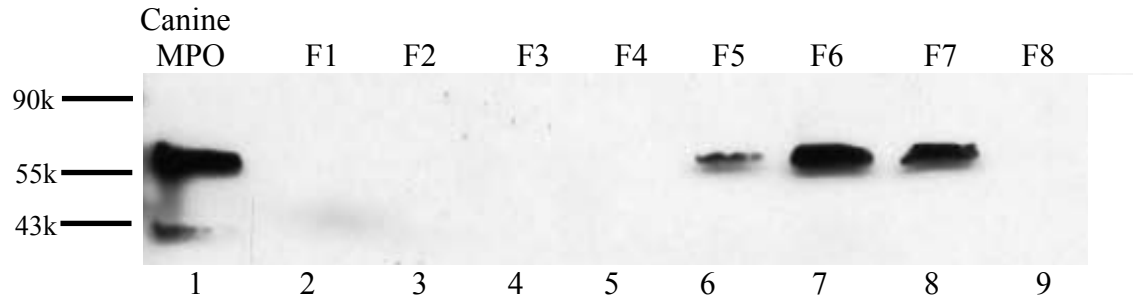


Figure 3.2. Western blot analysis of PMN subcellular fractions. Figures were showing western immunoblots of PMN subcellular fractions from A. a CH and B. a normal dog. Monoclonal antibody to canine MPO was used as primary antibody. Horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG was secondary antibody. Lane 1: canine MPO was served as positive control. Lane 2 to 9: PMN subcellular fraction 1 to fraction 8. MPO was maximally distributed in neutrophil fraction 7 in normal dog, and in fraction 6 in CH dog.

Figure 3.2

A


Canine MPO
F1
F2
F3
F4
F5
F6
F7
F8
90k
55k
43k
1
2
3
4
5
6
7
8
9

B

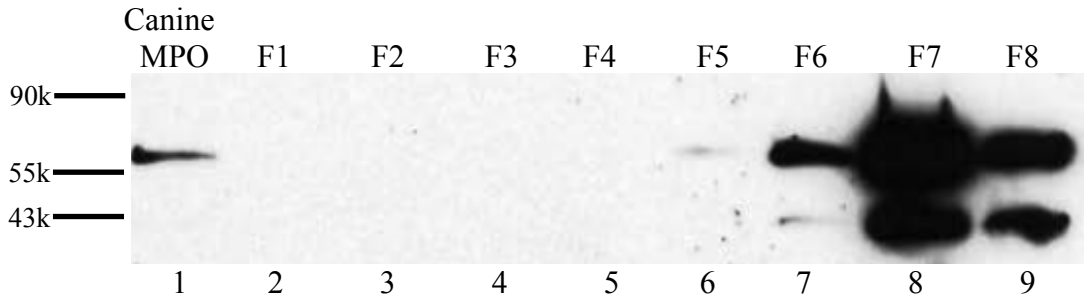
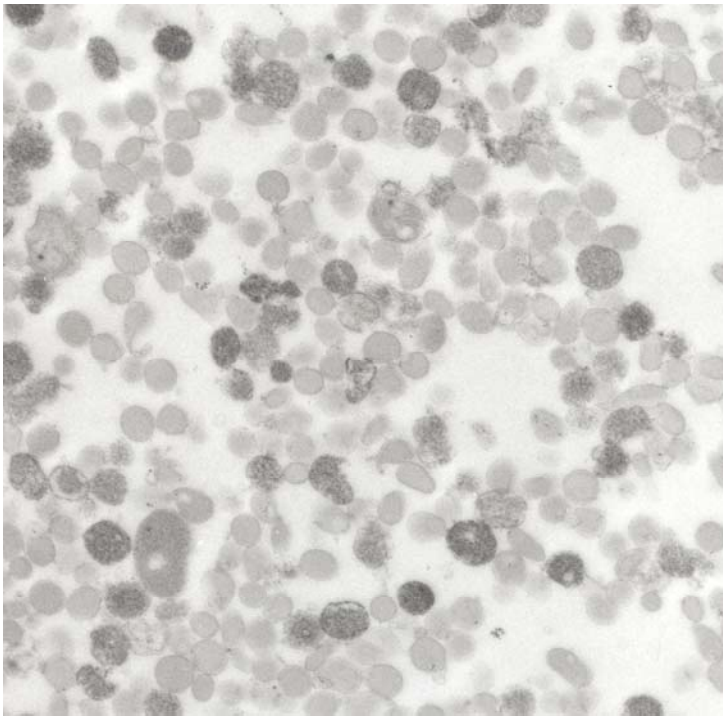

Canine MPO
F1
F2
F3
F4
F5
F6
F7
F8
90k
55k
43k
1
2
3
4
5
6
7
8
9

Figure 3.3. DAB staining of PMN subcellular fractions. Micrographs were showing DAB staining of A. PMN subcellular fraction 6 from a CH dog and B. PMN subcellular fraction 7 from a normal dog. The average size of primary granules from the CH dog was 249 ± 35 × 193 ± 21nm, and most of them were round-shaped. PMNs from normal dogs contained primary granules with an average size 389 ± 17.5 × 210 ± 27nm, and mostly were oval-shaped. × 29,700.

Figure 3.3

A



B

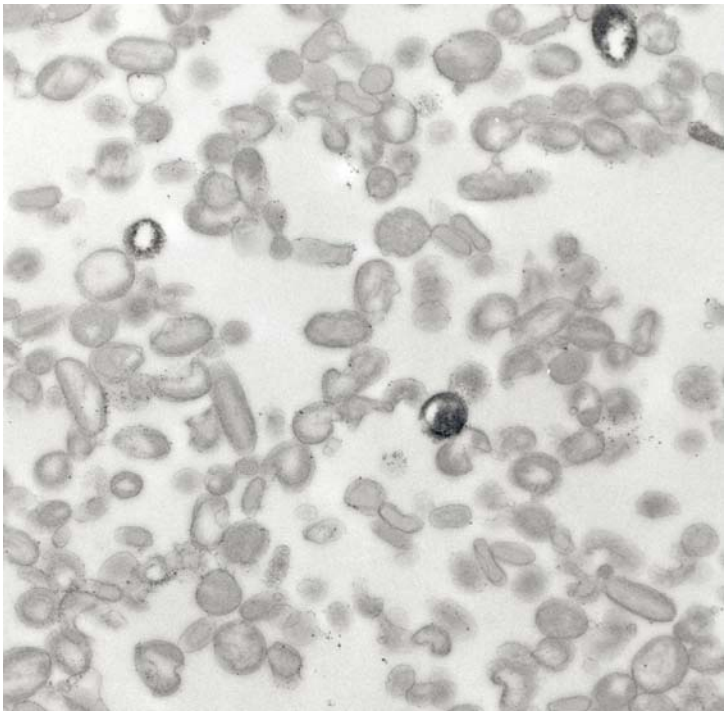
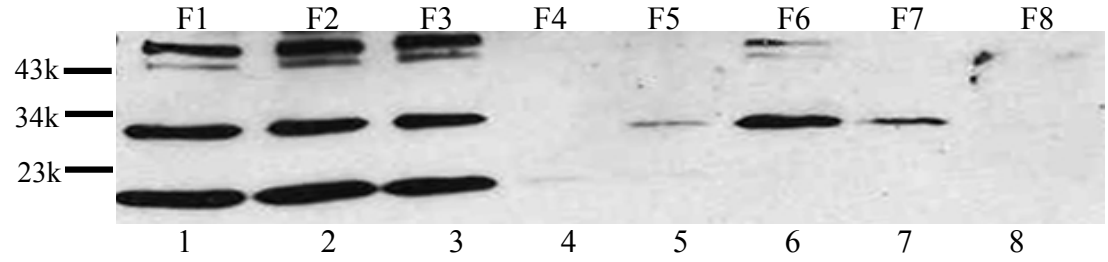


Figure 3.4. Western blot analysis of PMN subcellular fractions. Neutrophils were isolated, homogenized by nitrogen cavitation, and fractionated into 8 subcellular fractions using discontinuous Percoll gradients. pELA85 was used as primary antibody. Horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG was secondary antibody. A. PMN subcellular fractions from a CH dog. B. PMN subcellular fractions from a normal dog. For A and B, lane 1 to 8 corresponded to PMN fraction 1-8. Mature neutrophil elastase (NE) recognized by pELA85 was found primarily in PMN fraction 6 from the CH dog, while in fraction 7 from the normal dog. The protein expression level of mature NE was significantly decreased in the CH dog compared to the normal dog.

Figure 3.4

A


F1
F2
F3
F4
F5
F6
F7
F8
43k
34k
23k
1
2
3
4
5
6
7
8

B

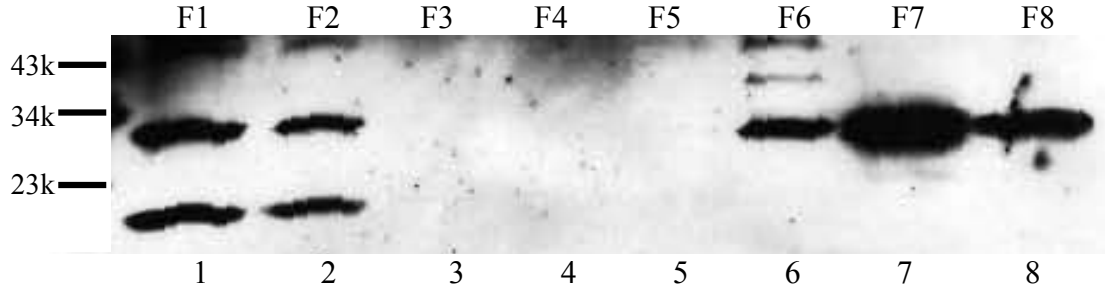

F1
F2
F3
F4
F5
F6
F7
F8
43k
34k
23k
1
2
3
4
5
6
7
8

Figure 3.5. Double immunocytochemistry of canine PMNs. mELA85 and monoclonal antibody to canine myeloperoxidase (MPO) were used as primary antibody. Either Alexa Fluor®488 or Alexa Fluor®574-conjugated goat anti-mouse IgG was used as secondary antibody. A. Staining of neutrophil elastase (NE) in CH dog neutrophils was indicated by red fluorescence. B. Staining of MPO in CH dog neutrophils was detected by green fluorescence. C. Merged image of A and B illustrating that NE was co-localized with MPO in primary granules. D. Staining of NE in neutrophils from a normal dog was demonstrated by red fluorescence. E. MPO in normal dog neutrophils was identified by green fluorescence. F. Merged image of D and E illustrating that NE was co-localized with MPO in primary granules. Compared to the staining of NE in normal dog PMN, the staining of NE in CH dog was weaker, indicating that CH dogs contained decreased NE abundance.

Figure 3.5

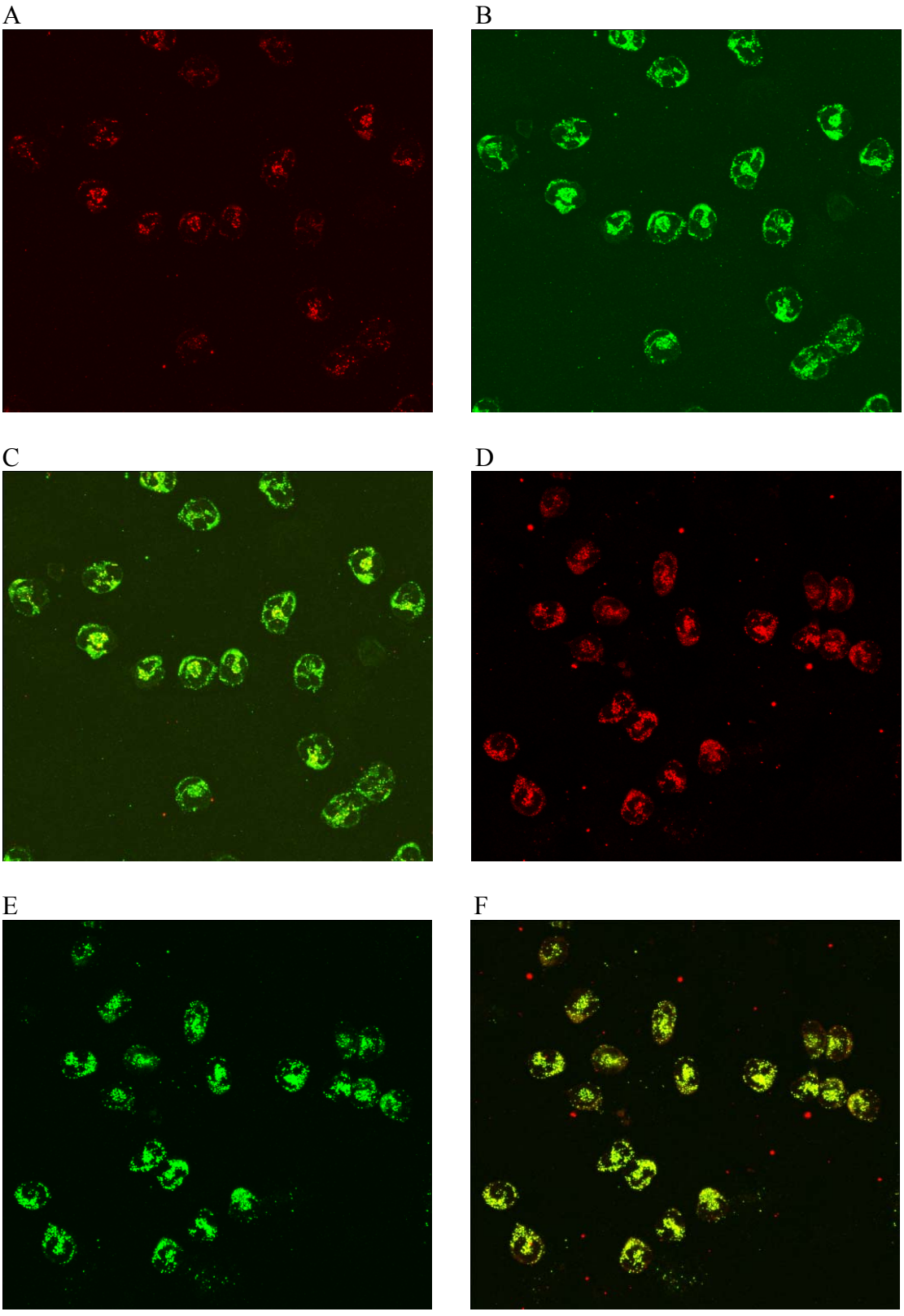
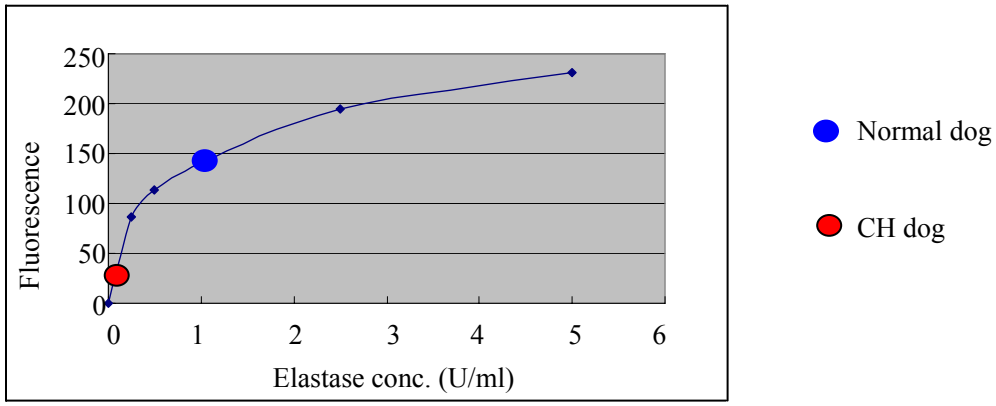
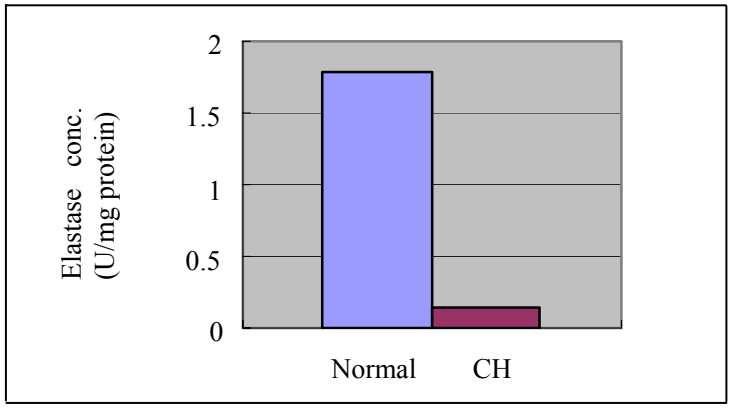


Figure 3.6. Elastase enzymatic activities in dog PMNs. A. PMN cell lysates from a normal and CH dog. B. PMN subcellular fraction lysates from a normal and CH dog. The results indicated that the CH dog retained approximately 5-10% elastase enzymatic activity of normal dogs.

Figure 3.6

A


250
200
150
100
50
0
Fluorescence
0
1
2
3
4
5
6
Elastase conc. (U/ml)
Normal dog
CH dog


2
1.5
1
0.5
0
Elastase conc. (U/mg protein)
Normal
CH

B

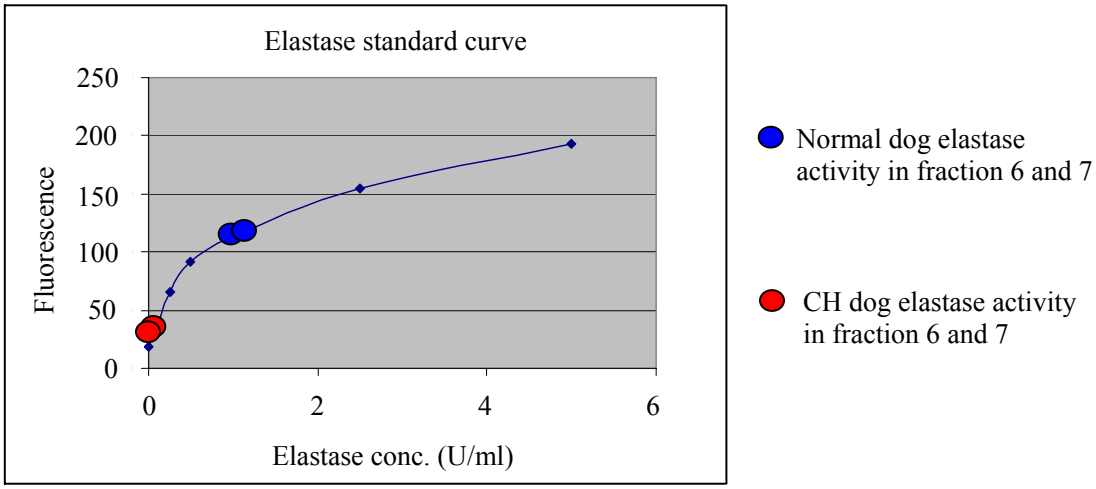
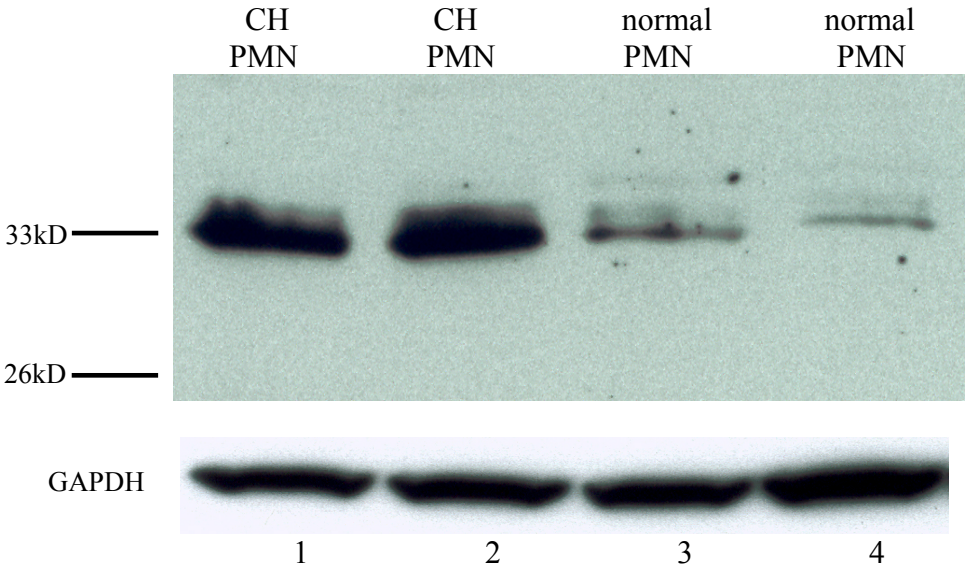

Elastase standard curve
250
200
150
100
50
0
Fluorescence
0
2
4
6
Elastase conc. (U/ml)
Normal dog elastase activity in fraction 6 and 7
CH dog elastase activity in fraction 6 and 7

Figure 3.7. Western blot analysis of canine PMNs. A. mELA269 was used as primary antibody. B. mELA85 was used as primary antibody. Secondary antibody was horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG. Lane1 and 2: CH dog neutrophil lysates. Lane 3 and 4: normal dog neutrophil lysates. CH dog accumulated large amount of neutrophil elastase preprotein with MW ~33kD. Mature neutrophil elastase was significantly decreased in CH dog PMNs compared to normal dog.

Figure 3.7

A


CH PMN
CH PMN
normal PMN
normal PMN
33kD
26kD
GAPDH
1
2
3
4

B

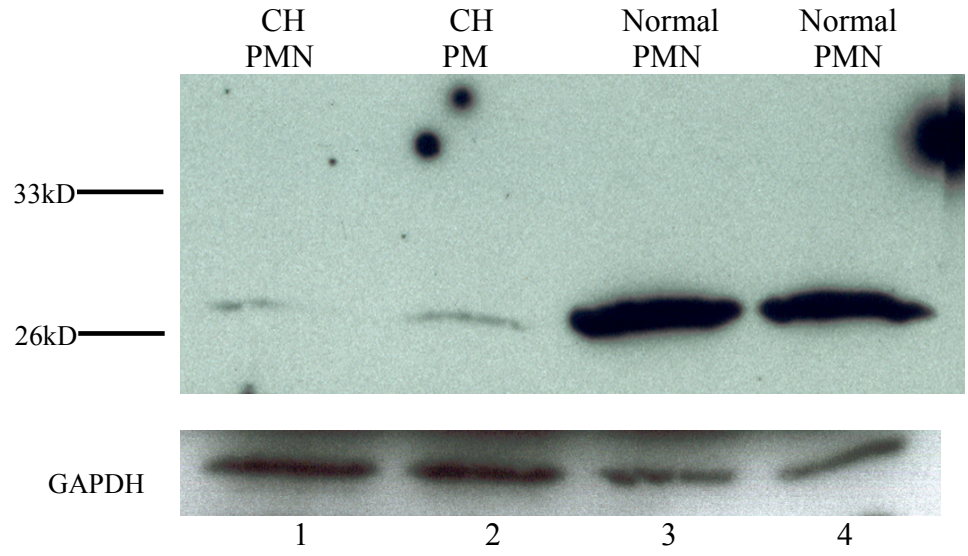

CH PMN
CH PM
Normal PMN
Normal PMN
33kD
26kD
GAPDH
1
2
3
4

Figure 3.8. Double immunocytochemistry of canine PMNs. mELA269 and monoclonal antibody to canine MPO were used as primary antibody. Either Alexa Fluor®488 or Alexa Fluor®574-conjugated goat anti-mouse IgG was used as secondary antibody. A. Staining of neutrophil elastase (NE) preprotein with mELA269 in PMNs from a CH dog was indicated by green fluorescence. B. Staining of MPO in PMNs from the CH dog was detected by red fluorescence. C. Merged image of A and B illustrating that NE preproteins were not co-localized with MPO in primary granules from the CH dog. D. Staining of NE preprotein in PMNs from a normal dog was indicated by green fluorescence. E. Staining of MPO in PMNs from a normal dog was detected by red fluorescence. F. Merged image of D and E illustrating that NE preproteins were not co-localized with MPO in primary granules in the normal dog. The staining of NE preprotein with mELA269 indicated that PMNs from CH dog accumulated large amount of NE preproteins compared to normal dog.

Figure 3.8

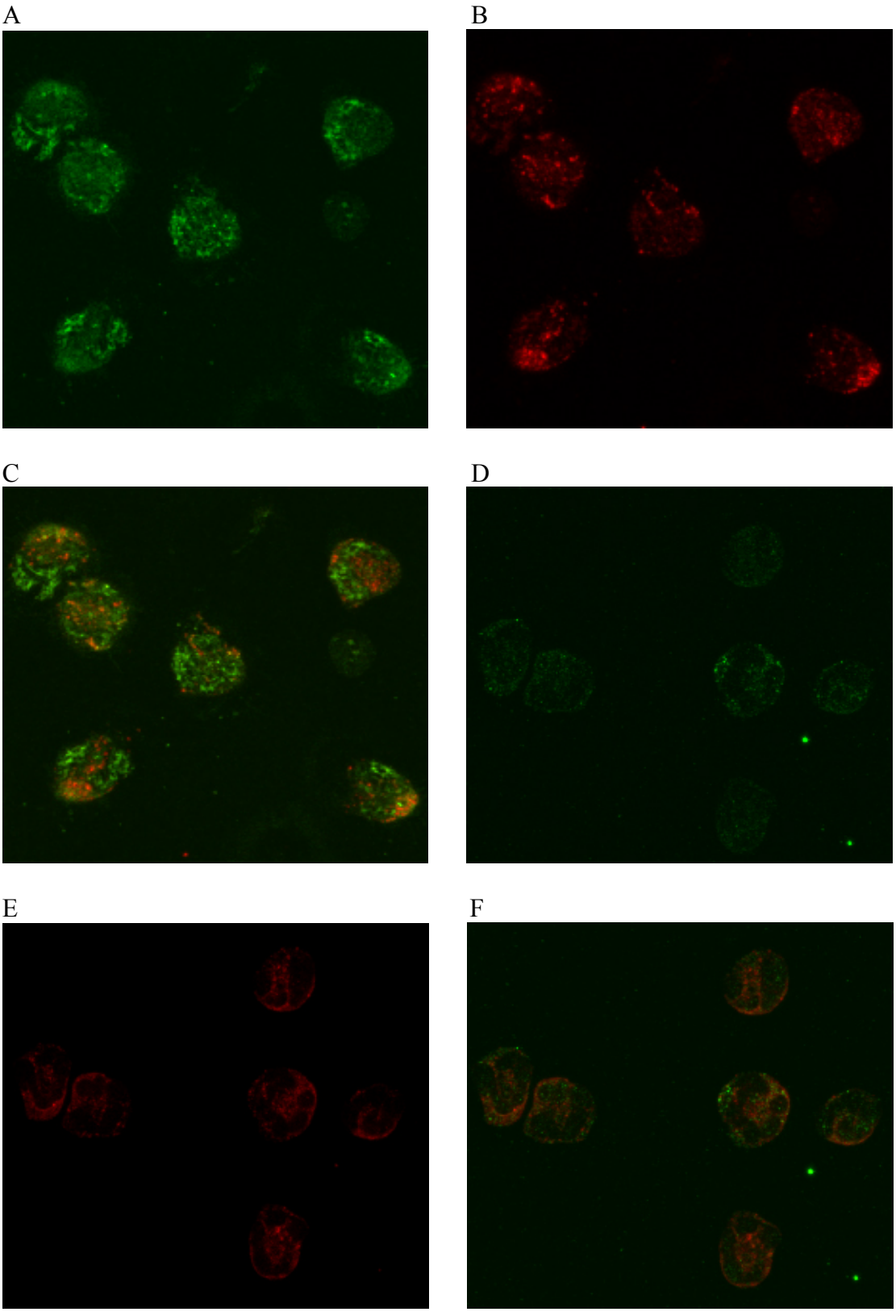


Figure 3.9. Protein expression of μ3A in PMNs. Monoclonal antibody to human P47A (μ3A) was used as primary antibody. Secondary antibody was horseradish peroxidase (HRP) -conjugated goat anti-mouse IgG. Lane 1: Madin-Darby canine kidney (MDCK) cell lysate; Lane 2 and 3: CH dog neutrophils; Lane 4-6: CH carrier dog neutrophils; Lane 7-9: normal dog neutrophils. The result demonstrated that μ3A subunit of AP-3 was normally expressed in PMNs from CH dog. The blot was stripped and reprobed with monoclonal antibody to GAPDH.

Figure 3.9

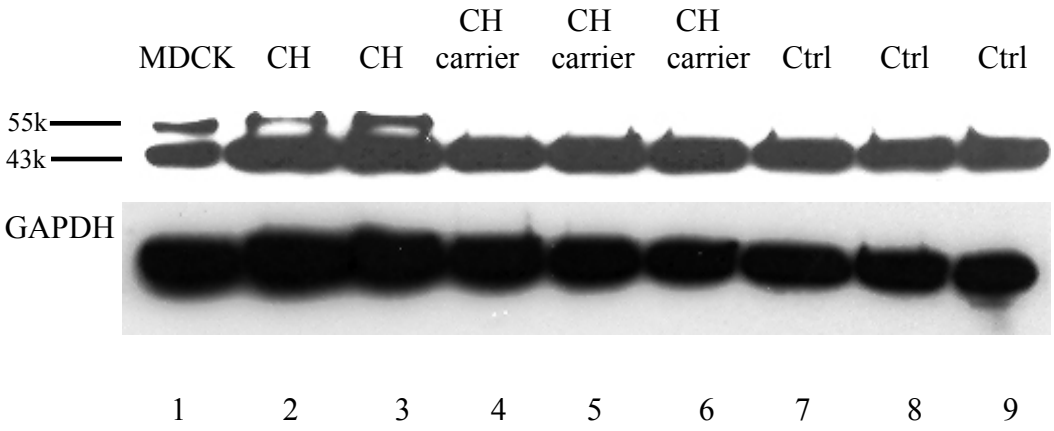
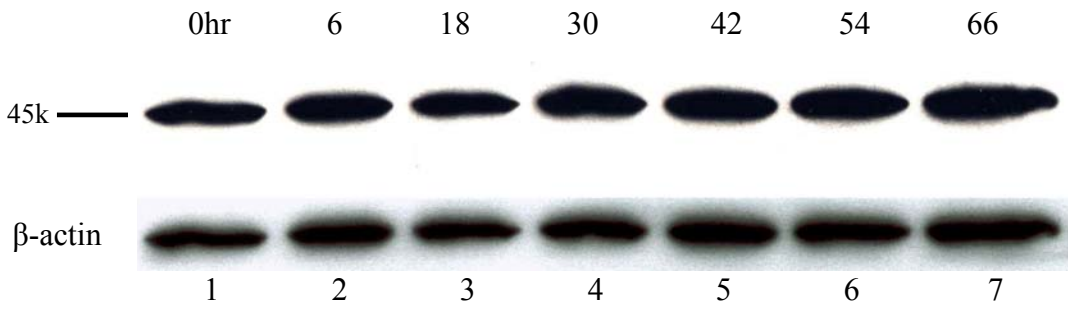

MDCK
CH
CH
CH carrier
CH carrier
CH carrier
Ctrl
Ctrl
Ctrl
55k
43k
GAPDH
1
2
3
4
5
6
7
8
9

Figure 3.10. Protein expression of μ3A in bone marrow mononuclear cells (BMMCs). BMMCs were cultured with the stimulation of stem cell factor (SCF) and granulocyte-colony stimulating factor (G-CSF) and collected at 0, 6, 18, 30, 42, 54, and 66 hour time points. A. BMMCs from a normal dog. Lane 1-7: BMMCs were collected at different time point as indicated on the figure. B. BMMCs from a CH dog. Lane 1-7: BMMCs were collected at different time point as indicated on the figure. Primary antibody was monoclonal antibody to human P47A (μ3A). Secondary antibody was horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG. The blots were stripped and reprobed with monoclonal antibody to β-actin. The results demonstrated that μ3A was normally expressed at the protein level in BMMCs when BMMCs were stimulated towards myelopoiesis in CH dog.

Figure 3.10

A



B

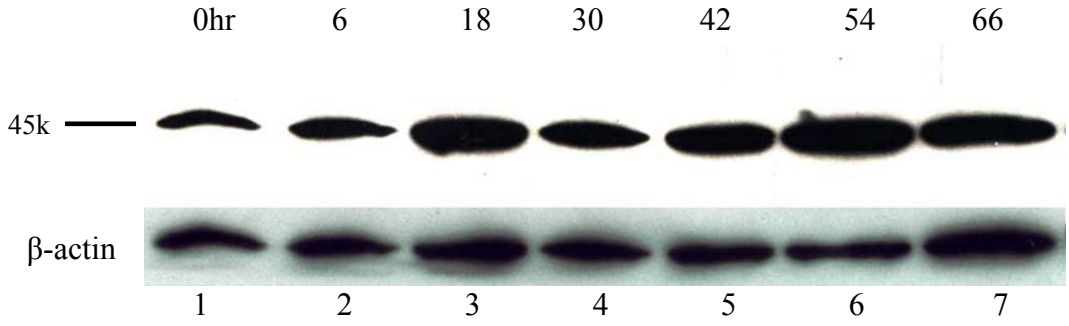
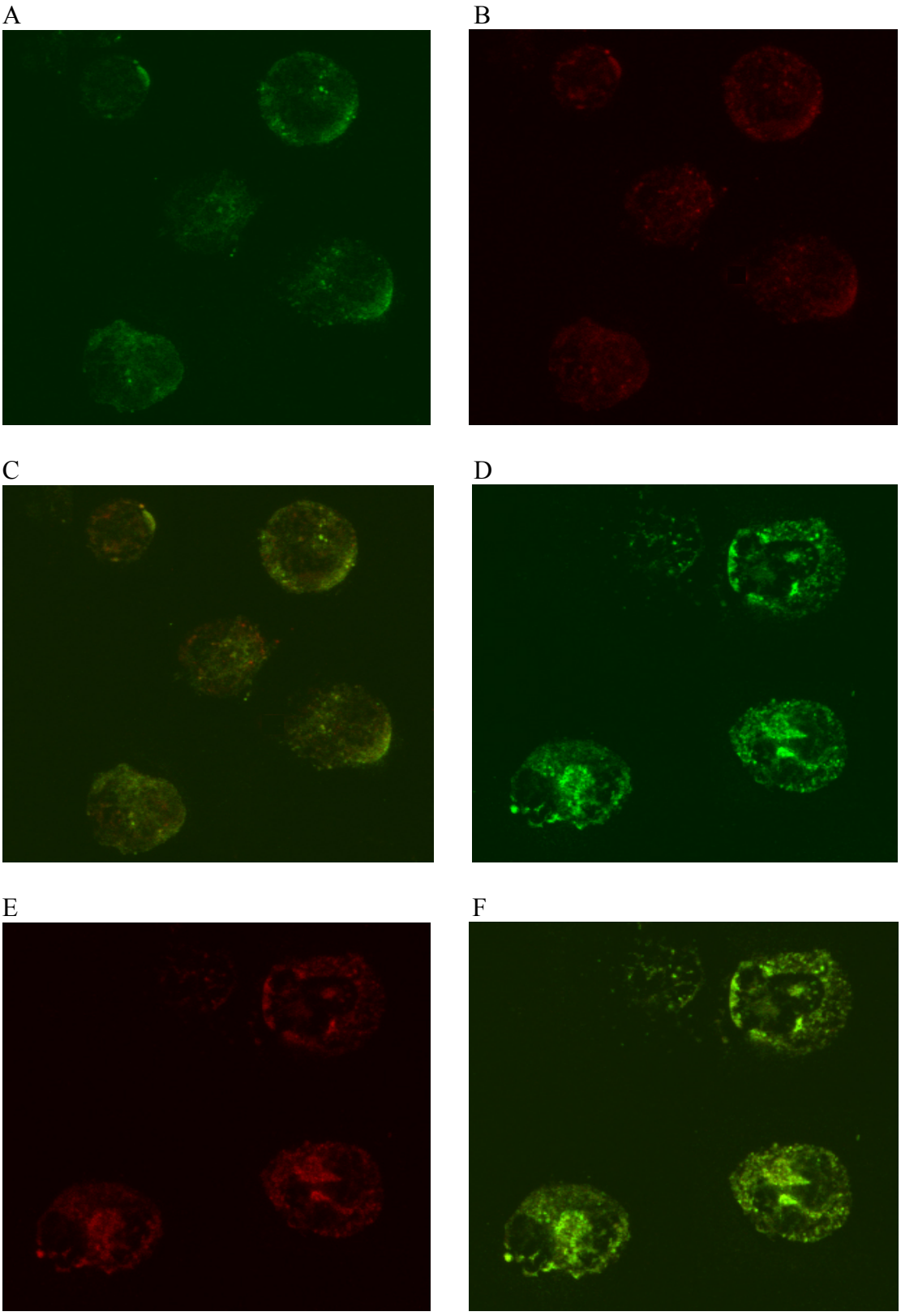


Figure 3.11. Double immunocytochemistry of PMN cells. Monoclonal antibodies to P47A (μ3A) and to mELA269 were used as primary antibodies. Secondary antibodies were either Alexa Fluor®488 or Alexa Fluor®574 -conjugated goat anti-mouse IgG. A. Staining of μ3A subunit in PMNs from a CH dog was indicated by green fluorescence. B. Staining of neutrophil elastase (NE) preproteins with mELA269 in PMNs from the CH dog was detected by red fluorescence. C. Merged image of A and B illustrating that NE preproteins were not co-localized with μ3A in PMNs from the CH dog. D. Staining of μ3A in PMNs from a normal dog was indicated by green fluorescence. E. Staining of NE preproteins in PMNs from the normal dog was demonstrated by red fluorescence. F. Merged image of D and E illustrating that NE preproteins were co-localized with μ3A in PMNs from the normal dog.

Figure 3.11



CHAPTER 4

ABNORMAL TRAFFICKING OF NEUTROPHIL ELASTASE INDUCES PROTEIN EXPRESSION OF BIP/GRP78 AND APOPTOSIS OF MYELOID PROGENITOR CELLS IN CANINE CYCLIC HEMATOPOIESIS

ABSTRACT

Cyclic hematopoiesis (CH) in collie dogs is caused by a mutation in the β3A subunit of adaptor-related protein complex – 3 (AP-3). However, the mechanism by which the mutation causes periodic fluctuation of hematopoietic blood cells remains unknown. Phenotypically, the CH dogs are analogous to human cyclic neutropenia (CN) and severe congenital neutropenia (SCN) patients. However these human disorders are caused by point mutations in neutrophil elastase (NE). Previous data indicated that CH dog neutrophils contain a significantly reduced amount of active NE in primary granules, and there was no detectable elastase on the plasma membrane. Using canine specific antibodies to NE preproteins, differentiating bone marrow mononuclear cells (BMMCs) from CH dogs generated large amounts of elastase precursor proteins comparing to normal dogs. This resulted in accumulation of elastase preproteins in segmented neutrophils from CH dogs and reduced storage of mature elastase in primary granules. During the culture of BMMCs, increased apoptotic cell death was observed in CH dogs. The expression of BiP/GRP78 was also up-regulated in segmented neutrophils from CH

dogs. These results suggested that abnormal processing and trafficking of elastase preproteins during myelopoiesis leads to induction of the unfolded protein response (UPR) and apoptosis of the progenitor cells from CH dogs. Collectively, the accumulation of NE preproteins, the UPR, and increased apoptosis of myeloid progenitor cells results in reduced production of neutrophils in CH dogs, but the mechanism of the cyclic phenomenon remains undetermined.

4.1 Introduction

Canine cyclic hematopoiesis (CH), an autosomal recessive disorder of collie dogs, is characterized by periodic reduced production of multiple lineages of blood cells, predominantly neutrophils (Lund *et al.*, 1967; Dale *et al.*, 1972a; Dale *et al.*, 1972b). Functional analysis indicated that neutrophils of the CH dog have bactericidal defects (Chusid *et al.*, 1975). Characterization of neutrophils showed that their primary granules are smaller and less dense compared to normal dogs. Further studies on neutrophil elastase (NE), a neutral serine protease stored mainly in neutrophil primary granules, indicated that CH dog has significantly decreased granular deposit of active elastase enzyme.

Genetic analysis revealed that canine CH is caused by an insertional mutation of *AP3B1*, encoding the β3A subunit of adaptor-related protein complex – 3 (AP-3), which shuttles nascent membrane proteins from the trans-Golgi network (TGN) to lysosomes and lysosome-like granules (Dell' Angelica *et al.*, 1999b; Benson *et al.*, 2003). AP-3 is a heterotetrameric protein complex containing four subunits: β3A, μ3A, σ3A, and δ3A (Simpson *et al.*, 1996; Robinson & Bonifacino, 2001). The interaction between mature

NE and the μ3A subunit has been demonstrated in a yeast two-hybrid assay (Benson *et al.*, 2003). It was hypothesized that the transmembrane conformation of NE interacts with AP-3 to be transported to the primary granules. Hence in CH dogs, the mutation of *AP3B1* causes mis-trafficking of NE to the default location the plasma membrane instead of primary granules. However, in CH dog neutrophils, elastase was not detectable on the cell surface using subcellular fractionation and immunocytochemistry experiments. The decreased storage of elastase enzyme in CH dog neutrophils is unlikely associated with distribution of mature NE proteins on the cell surface.

NE is synthesized as a preprotein and the fate of the preprotein is diverged into two pathways (Gullberg *et al.*, 1995; Gullberg *et al.*, 1997; Gullberg *et al.*, 1999; Garwicz *et al.*, 2005). One is the constitutively secretion and the other one is the regulated secretion by first storage in neutrophil granules and subsequent exocytosis upon stimulation. Previous studies indicated that mutations in *ELA2*, which causes all cases of human cyclic neutropenia (CN) and some cases of severe congenital neutropenia (SCN), might induce premature apoptosis of neutrophil precursor cells (Aprikyan *et al.*, 2001; Mackey *et al.*, 2003; Carlsson *et al.*, 2004; Massullo *et al.*, 2005; Grenda *et al.*, 2007). Canine CH has long been used as animal model to study human CN and SCN. To study the biosynthesis of canine NE, two newly generated antibodies were used with specificity to either processed mature elastase protein or elastase preprotein containing the C-terminus. The results demonstrated that in CH dogs, elastase preprotein not only accumulated in segmented neutrophils, but was also greatly induced during culture of bone marrow mononuclear cells (BMMCs) from CH dogs comparing to normal dog BMMCs in the culture. BiP/GRP78, a sensor of ER stress and the unfolded protein response (UPR), was

up-regulated in CH dog PMNs. During the culture of BMMCs, the increased apoptosis of BMMCs indicated that CH dog myeloid progenitor cells undergo continuous ER stress due to accumulation of NE preproteins. The induction of the UPR and apoptosis of myeloid progenitor cells is a plausible novel mechanism to explain the pathogenesis of cyclic hematopoiesis in the dog.

4.2 Materials and Methods

4.2.1 Experimental animals and the determination of neutrophil cycle

CH dogs, CH heterozygotes and normal dogs were from a dog colony that has been well described (Yang *et al.*, 1974; Jones *et al.*, 1975a; Jones *et al.*, 1975b; Jones *et al.*, 1975c). All animals were housed in AAALAC, Intl. accredited facilities and the experimental protocols were approved by the institutional IACUC (OLAW assurance number: A3152-01). Dogs were administered routine vaccinations and used only when free of concurrent infections. The CBC was determined on alternating days to establish the CH cycle days.

4.2.2 Blood and bone marrow sample collection

Peripheral blood was collected from normal, CH, and CH carrier dogs into EDTA-containing BD Vacutainer® (BD, Franklin Lakes, NJ). Polymorphonuclear (PMN) cells were isolated by Ficoll-Hypaque (Amersham Biosciences, Piscataway, NJ) density gradient sedimentation. Erythrocytes were removed by Dextran T-500 (Sigma) sedimentation. Finally, cells were suspended in 0.3% NaCl for hypotonic lysis of residual

erythrocytes. The purity of neutrophils from each sample was greater than 95% based on evaluation of cytocentrifuge cell preparations (Shandon Cytospin III).

Bone marrow from dogs was collected in a 12ml syringe containing 3ml Iscove's modified Dulbecco's medium (IMDM) (Invitrogen Corporation, Carlsbad, CA) and 150 units of Heparin (Sigma). The bone marrow mixture (5 ml) was applied slowly on Ficoll-Hypaque gradients and then centrifuged at 400 ×g for 30 minutes to separate bone marrow mononuclear cells (BMMCs). Following centrifugation on Ficoll-Hypaque, BMMCs were collected, and washed three times in phosphate buffered saline (PBS, Invitrogen) and resuspended in IMDM containing 10% heat-inactivated fetal bovine serum (FBS; Hyclone, Logan, UT) and 100U/ml penicillin and 100 μg/μl streptomycin (Invitrogen).

4.2.3 Culture of bone marrow mononuclear cells

Freshly isolated BMMCs (5×10^6/ml) were cultured in IMDM containing 10% FBS and 100U/ml penicillin, 100μg/μl streptomycin (Invitrogen), 25ng/ml canine stem cell factor (SCF; R&D Systems, Inc. Minneapolis, MN), and 10ng/ml canine granulocyte colony stimulating factor (G-CSF; Stem Cell Technologies, Inc.) at 37°C in a CO_2 water-jacketed incubator (Thermo Electron Corporation, Waltham, MA). Samples (3 ml each) were collected at seven successive time points: 0, 6, 18, 30, 42, 54 and 66 hour. The cells were washed in cold PBS to remove culture medium, and then cell lysate were prepared for immunoblot analysis.

4.2.4 Antibodies

Four clones of monoclonal antibody to canine myeloperoxidase were gifts from Dr. William Vernau (University of California, Davis, CA). Monoclonal antibodies to canine neutrophil elastase antigenic peptide were generated by the Auburn University Hybridoma Facility. Rabbit polyclonal to BiP/GRP78 (ab32618) was purchased from Abcam Inc. (Cambridge, MA). Monoclonal antibody to GAPDH (Abcam Inc., Cambridge, MA) was used as protein loading control. Secondary antibodies used on western immunoblotting were goat anti-mouse IgG or goat anti-rabbit IgG HRP-conjugated antibodies (Santa Cruz Biotechnology, Inc. Santa Cruz, CA).

4.2.5 Cell lysate preparation and immunoblot analysis

For immunoblot analysis, cells were washed in cold PBS and resuspended in 1×RIPA lysis buffer (Upstate, Lake Placid, NY) containing 1× protease inhibitor (Roche Applied Science, Mannheim, Germany). The cell suspension was further homogenized by passage through a 21 gauge needle and centrifuged at 15,000×g to obtain cell lysates. Samples were denatured at 95°C for 5 minutes for immuno-blot analysis. Proteins were separated in a 4-20% linear gradient gel (Bio-Rad Labs, Hercules, CA) using a Ready Gel Precast Gel System (Bio-Rad, CA) and transferred in Towbin transfer buffer to a nitrocellulose membrane under high intensity transfer conditions. After initial blocking with StartingBlock^{TM} Tris-buffered saline (TBS) blocking buffer (Pierce Biotechnology, Rockford, IL), blots were incubated with primary antibodies overnight at 4°C with gentle shaking, and then washed in TBS washing buffer containing 0.05% Tween (Bio-Rad). Blots were incubated with HRP-conjugated secondary antibodies for 1 hour at room

temperature with gentle shaking and then washed again in the same washing buffer. Blots were incubated with substrate (Pierce Biotechnology, Rockford, IL) for 5 minutes and then visualized by exposure to a Kodak BioMax XAR film (Fisher Scientific, Pittsburgh, PA).

4.2.6 Apoptosis Assay

BMMCs from CH and normal dogs were cultured as described. Bone marrow from CH dogs was obtained on the same cycle day (day 6-8) as in bone marrow immunoblot assays. The cell culture was stimulated with 25ng/ml canine SCF (R&D Systems), and 10ng/ml canine G-CSF (Stem Cell Technologies). Samples were collected at 0, 6, 18, 30, 42, 54 and 66 hour. The apoptotic cells were measured by a Cytomation MoFlo flow cytometer (Daka, Carpinteria, CA) using an Annexin-V FITC apoptosis detection kit from BD Pharmingen (San Diego, CA) and evaluated by Summit 4.3 from Dako Colorado, Inc. (Fort Collins, CO).

4.3 Results

4.3.1 Neutrophil elastase preproteins are accumulated in myeloid progenitors and strongly induced by growth factors during myelopoiesis in CH dogs

Previous data indicated that the mature form of NE is significant reduced in CH dog neutrophils. Since elastase is synthesized primarily during the promyelocyte stage, BMMCs were cultured in IMDM media stimulated with growth factors which induce myelopoiesis. In normal dog BMMCs, expression of elastase preproteins were detected after 6 hours in culture. In the subsequent time point, expression and glycosylation of the

preprotein was gradually increased up to 66 hours in culture (Figure 4.1A). In contrast, CH dog BMMCs already contained a large amount of elastase preproteins at 0 hour time point (Figure 4.1B). The expression and glycosylation of preprotein in CH dogs was strongly induced after 18 hours, and continues up to 66 hours in culture. These results indicated that CH dog myeloid progenitors accumulated large amount of elastase preproteins during the differentiation. This accumulation is commonly not found in normal dog BMMCs. The expression of elastase preproteins in CH dog was higher than normal dog, indicating that myeloid progenitor cells in CH dogs were strongly responded to the stimulation of growth factors – SCF and G-CSF.

4.3.2 Increased expression of BiP/GRP78 in CH neutrophils but not in cultured bone marrow mononuclear cells

Accumulation of unfolded or misfolded proteins in the ER will generate stress in the cell and hence induce the unfolded protein response (UPR) (Zhang & Kaufman, 2006a). BiP/GRP78, a sensor of the UPR, was up-regulated ~5 fold in CH dog neutrophils (Figure 4.2). The increased expression of BiP/GRP78 indicated that myeloid progenitor cells might be undergoing increased intracellular stress due to the accumulation of NE precursor proteins. In contrast, the expression of BiP/GRP78 was not so dramatically changed when BMMCs of CH and normal dogs were cultured with SCF and G-CSF (Figure 4.3). Only after 66 hours in culture, BMMCs from CH dogs has up-regulated ~2 fold expression of BiP/GRP78 compared to normal dogs. These results indicated that there was an up-regulation of BiP/GRP78 expression during neutrophil

maturation in response to increased accumulation of NE precursor proteins in CH neutrophils.

4.3.3 Increased apoptosis of bone marrow progenitor cells cultured with growth factors – SCF and G-CSF

Intracellular overload of improperly folded proteins induces the UPR and eventually causes programmed cell death – apoptosis (Zhang & Kaufman, 2004; Zhang & Kaufman, 2006a; Zhang & Kaufman, 2006b). Previous studies have shown that the UPR and increased apoptosis of bone marrow progenitor cells are molecular mechanisms associated with SCN. To determine whether the same mechanism is applied to canine CH, BMMCs isolated from CH and normal dogs were cultured with SCF and G-CSF. Samples were collected after 24 and 48 hours in culture and then stained with Annexin-V and propidium iodide (PI). The results showed that fresh isolated BMMCs from CH dog had a cellular apoptosis rate 15%, which is in contrast to 9% of normal dog (Figure 4.4). After cultured overnight, the apoptosis rate of BMMCs from CH dog increased to 33%. The apoptosis of BMMCs was relatively unchanged (10%) in normal dog myeloid progenitor cells, which was similar to the freshly isolated BMMCs (Figure 4.5). After two days in the culture, BMMCs of normal dog had a constant apoptosis rate, and CH dog progenitor cells still had a 32% apoptotic death rate (Figure 4.6).

4.4 Discussion

Myeloid lineage differentiation is coupled with transcription of genes which protein products are packaged into neutrophil cytoplasmic granules for future use in the innate

immune response (Gullberg *et al.*, 1997; Gullberg *et al.*, 1999). *ELA2*, encoding neutrophil elastase (NE), is a central member of a gene family which is turned on during the early stages of myelopoiesis (Fouret *et al.*, 1989; Garwicz *et al.*, 2005). Besides storage as an active enzyme in neutrophil granules, elastase is constitutively secreted from the cell as an inactive protein during the differentiation of progenitor cells (Garwicz *et al.*, 2005). The exact time point and compartment where the two pathways diverge is not known. Concurrently, the translocation of nascent integral membrane proteins into the primary granules is dependent on a specific interaction between the cargo protein and its carrier proteins.

Canine CH is caused by an insertional mutation of *AP3B1*, encoding the β3A subunit of AP-3 (Benson *et al.*, 2003). AP-3 is localized on the trans-Golgi network (TGN) membrane as well as peripheral membranes in which endocytosed materials are contained, and mainly functions as a carrier protein responsible for translocation of membrane-bound cargo proteins from the TGN to lysosomes and lysosome-like granules (Robinson & Bonifacino, 2001; Nakatsu & Ohno, 2003; Peden *et al.*, 2004). It was proposed that NE is a cargo protein of AP-3, and the canine β3A mutation might cause abnormal trafficking and cell surface accumulation of NE proteins (Benson *et al.*, 2003). The dissertation studies indicate that mature elastase is not detectable on the neutrophil cell surface, while a significantly reduced amount of elastase protein is present in CH dog primary granules. Culture of BMMCs towards myeloid lineage showed an up-regulation and subsequent accumulation of elastase preproteins in the progenitor cells and segmented neutrophils from CH dogs. Thus increased accumulation of elastase precursors suggested a processing defect that might induce the unfolded protein response (UPR).

Indeed, an up-regulation of BiP/GRP78 was observed in CH dog neutrophils and bone marrow myeloid progenitor cells after 66 hours in culture, indicating that the concentration of misfolded or unfolded proteins is increased to a level requiring more protein chaperones to promote folding and prevent aggregation of proteins. Corresponding to this cellular response, accelerated apoptotic death of bone marrow myeloid progenitors was observed in differentiating BMMCs stimulated with growth factors. These results suggest that there was increased apoptotic cell death due to the accumulation of improperly folded NE precursor proteins.

The extraordinary accumulation of elastase precursor proteins in CH dog neutrophils indicated that the normal processing of the preprotein is perturbed during the differentiation of myeloid progenitor cells. Since elastase C-terminus is not necessary for its granular targeting or enzymatic activity, the C-terminus might be removed after the translocation into primary granules. However, studies have shown that elastase C-terminus has to be removed before the interaction with the μ3A subunit (Benson *et al.*, 2003). Since CH dog has the β3A mutation, there might be a third or even more proteins involved in the translocation systems. The accumulation of elastase preproteins in CH dog neutrophils indicated that the constitutive and regulated secretion pathway might diverge before the protein departure from the TGN. Without the assistance of AP-3, elastase preprotein might not able to be normally processed and translocated into granules. The small amount of mature elastase protein in CH dog is presumably due to the limited assembly of functional AP-3 complex, which is demonstrated by the expression of intact μ3A subunit protein and trace amount of normal β3A gene transcripts in CH dogs

(Benson *et al.*, 2004). Alternatively, the cells might utilize plasma membrane route to transport elastase to the granules.

Human HPS-2 patients and *pearl* mice, both of which are caused by mutations in *AP3B1* and the mutation affects the stability of the other three subunits, especially the degradation of μ3A, which plays a central role in cargo selection. Phenotypically, HPS-2 patients are characterized with oculocutaneous albinism, platelet storage pool deficiency (SPD) and neutropenia. In contrast, *pearl* mice have hypopigmentation, prolonged bleeding due to SPD, and reduced sensitivity in the dark-adapted state. However, *pearl* mice do not have detectable neutropenia, which is a major hallmark in human HPS-2 patients and CH dogs. In HPS-2 patients, neutrophils still contain a small amount of elastase protein in the granule, which suggested that disassembly of AP-3 does not completely disrupt the trafficking of elastase into granules. Cells might utilize other mechanisms, such as the plasma membrane routing, which requires the presence of intact NE C-terminus (Tapper *et al.*, 2006). Further analysis on the differentiating myeloid progenitor cells in long term culture might be helpful in elucidating the mechanism whereby NE translocates to the primary granules in the absence of AP-3.

The cell employs either residual AP-3 or other undefined alternative route to shuttle elastase and other granular-localized proteins at greatly has a reduced efficiency. This results in the accumulation of elastase precursor protein in the segmented neutrophils and differentiating progenitor cells. Correspondingly, an intracellular stress condition is triggered and the cells undergo apoptotic death. This is consistent with previous studies in which increased apoptosis of myeloid progenitor cells were found in human CN and SCN, due to cytoplasmic accumulation of elastase mutants (Aprikyan *et al.*, 2001; Carlsson *et*

al., 2004; Massullo *et al.*, 2005). PMNs isolated from HPS-2 patients were also found to contain significantly reduced amount of NE. Therefore, the phenomenon of intracellular stress, UPR and apoptosis of myeloid progenitor cells, might be a common mechanism which triggers neutropenia in these human disorders and cyclic hematopoiesis in the dog.

The severity of neutropenia is varied in human congenital neutropenia depending on the mutation of *ELA2* gene. Generally, elastase mutants of human CN and SCN retain most of the enzymatic activity. However, the structure of the protein is dependent on amino acid sequences, while point mutations might perturb the protein structure and the processing and modification, then the interaction with the carrier proteins. As a model of human congenital neutropenia (CN and SCN), these studies provide a link between the genetic basis of CH dogs and human disorders. The mutation of *AP3B1* in the dog or *ELA2* in human both affect the communication between AP-3 and elastase, resulting in abnormal protein trafficking and accumulation of NE precursor proteins. To cope with this stress, cells either go to apoptotic death, or utilize other mechanisms to help shuttle elastase, which ultimately results in the generation of neutrophils with functional defects and periodic neutropenia.

Figure 4.1. Protein expression of neutrophil elastase (NE) preprotein in bone marrow mononuclear cells (BMMCs). Stem cell factor (SCF) and granulocyte-stimulating factor (G-CSF) stimulated BMMCs were cultured and collected at 7 different time points: 0, 6, 18, 30, 42, 54 and 66 hour. Monoclonal antibody mELA269 was used as primary antibody. Secondary antibody was horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG. A. BMMCs from a normal dog. B. BMMCs from a CH dog. For A and B, lane 1 – 7: BMMCs collected after 0, 6, 18, 30, 42, 54 and 66 hours in culture. BMMCs cell lysates were loaded again in the same normalized protein amount and probed with monoclonal antibody to GAPDH. The results showed that NE preproteins recognized by mELA269 was highly induced expression during the induction of myelopoiesis in BMMCs from CH dog compared to normal dog BMMCs in the culture.

Figure 4.1

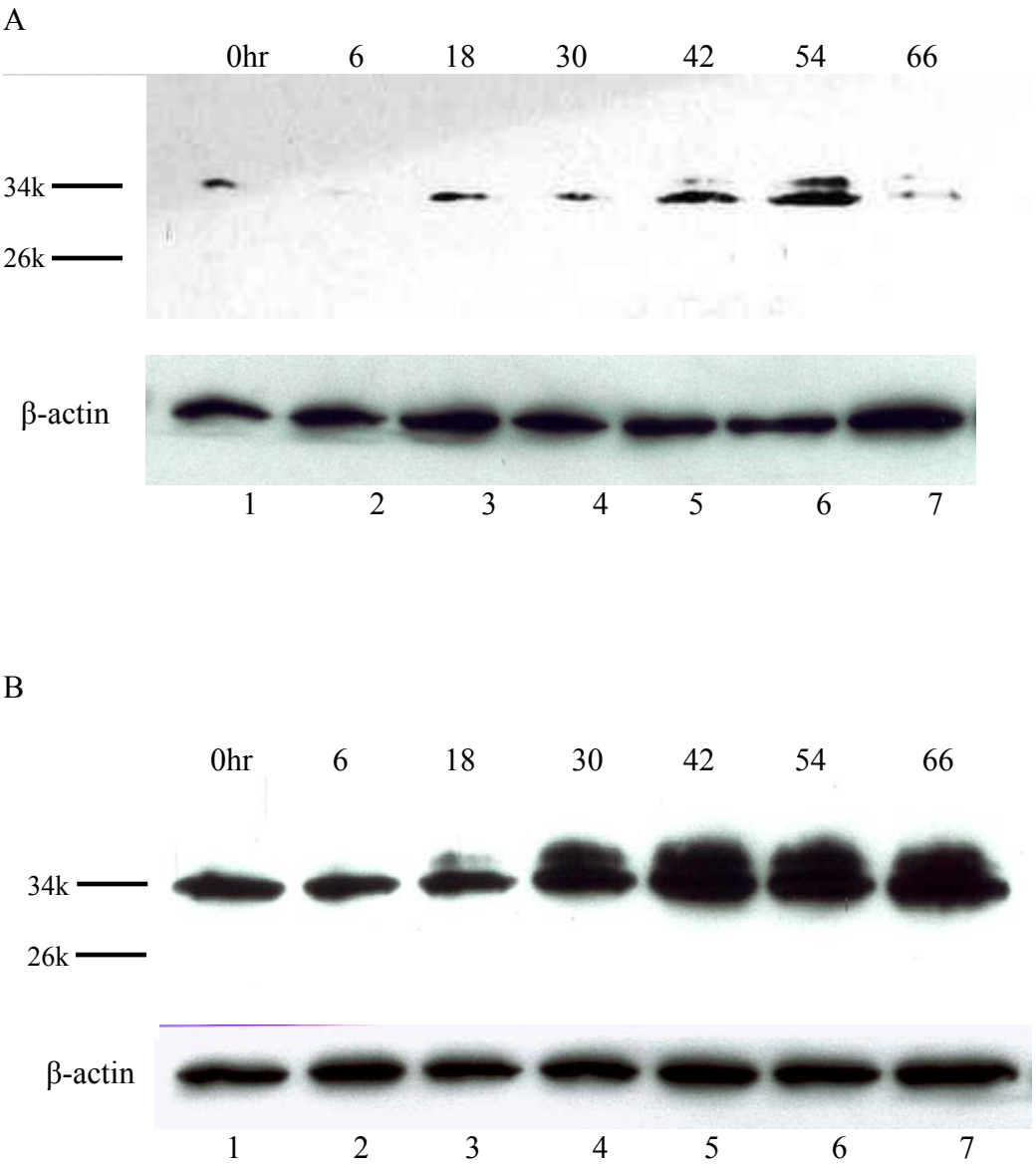

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Figure 4.2. Protein expression of BiP/GRP78 in canine PMNs. Polyclonal antibody rabbit to BiP/GRP78 was used as primary antibody. Horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG was secondary antibody. Lane 1 and 2: CH dog neutrophils; Lane 3 and 4: normal dog neutrophils. The blot was stripped and reprobed with monoclonal antibody to GAPDH. Protein expression of BiP/GRP78 was up-regulated ~5 fold in neutrophils from the CH dog compared to normal dog.

Figure 4.2

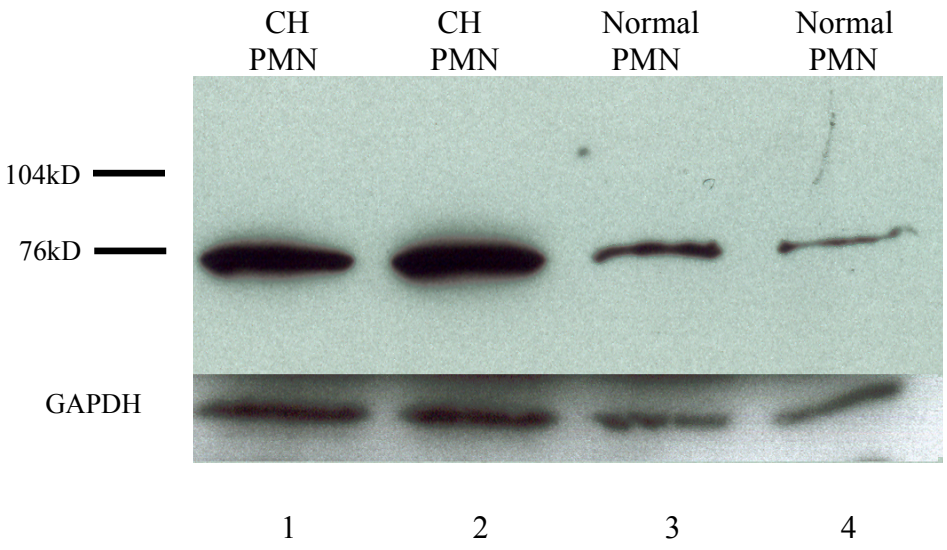
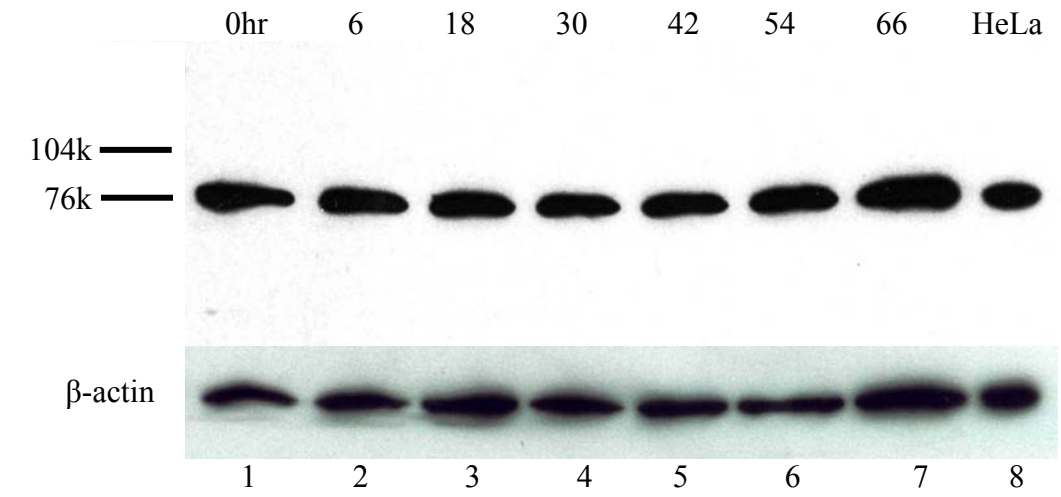


Figure 4.3. Protein expression of BiP/GRP78 in bone marrow mononuclear cells (BMMCs). BMMCs were cultured with stem cell factor (SCF) and granulocyte-colony stimulating factor (G-CSF). The cells were collected at 0, 6, 18, 30, 42, 54 and 66 hour time points and the cell lysates were subjected to western blot. Antibodies used were the same as in Figure 4.2. A. BMMCs from a normal dog. B. BMMCs from a CH dog. For A and B, lane 1 to 7: BMMCs collected at different time point as indicated on the top of the figure. Lane 8: HeLa cell lysate. BMMCs cell lysates were loaded again in the same normalized protein amount and probed with monoclonal antibody to GAPDH. Protein expression of BiP/GRP78 was slightly up-regulated at 54 hour time point in cultured BMMCs from the CH dog compared to normal dogs. At 66 hours in culture, protein expression of BiP/GRP78 was ~2 fold upregulated in BMMCs from a CH dog compared to normal dogs. HeLa cells served as a positive control.

Figure 4.3

A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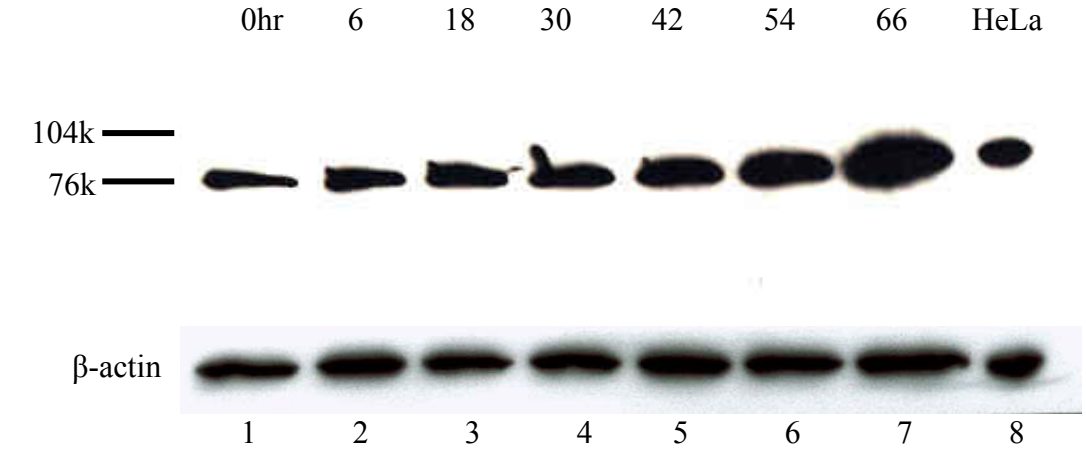
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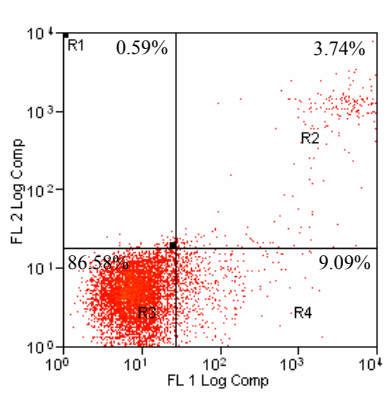
Figure 4.4. Apoptosis in freshly isolated bone marrow mononuclear cells (BMMCs). The cells were stained with Annexin V-FITC and propidium iodide (PI). A total of 10,000 cells were counted in each sample. A. Apoptosis of BMMCs from a normal dog. 9.09% BMMCs were Annexin V-FITC positive and PI negative. B. Apoptosis of BMMCs from a CH dog. 14.56% BMMCs were Annexin V-FITC positive and PI negative. Freshly isolated BMMCs from the CH dog had an increased apoptotic death rate compared to the normal dog.

Figure 4.4

A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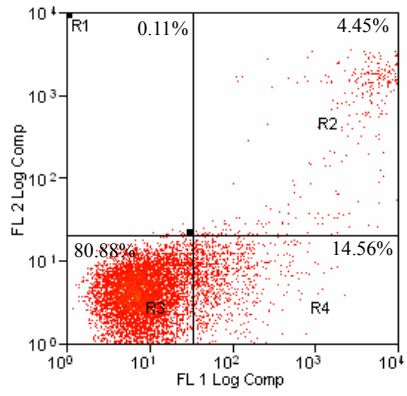
B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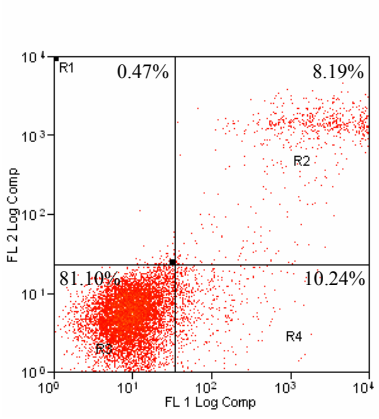
FL 1 Log Comp

Figure 4.5. Apoposis in cultured bone marrow mononuclear cells (BMMCs). Stem cell factor (SCF) and granulocyte-colony stimulating factor (G-CSF) were used to stimulate BMMCs in culture. BMMCs were then collected after overnight culture (22 hours) and stained with Annexin V-FITC and propidium iodide (PI). A total of 10,000 cells were counted in each sample. A. Apoptotic death rate of BMMCs from a normal dog. 10.24% BMMCs were Annexin V-FITC positive and PI negative. B. Apoptotic death rate of BMMCs from a CH dog. 32.92% BMMCs were Annexin V-FITC positive and PI negative. During the induction of myelopoiesis with SCF and G-CSF, *in vitro* cultured BMMCs from the CH dog had an increased rate of apoptosis compared to the normal dog.

Figure 4.5

A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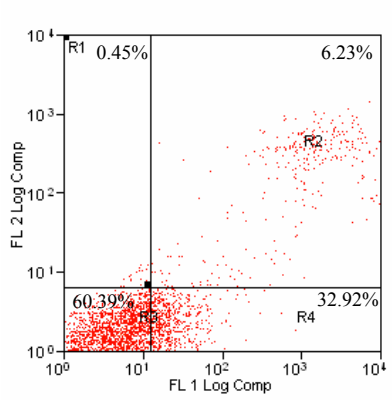
B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R2
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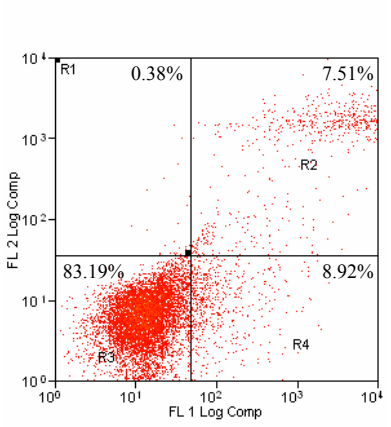
R4
R3
FL 2 Log Comp
FL 1 Log Comp

Figure 4.6. Apoptosis in cultured bone marrow mononuclear cells (BMMCs). Stem cell factor (SCF) and granulocyte-colony stimulating factor (G-CSF) were used to stimulate BMMCs in culture. BMMCs were then collected after 2 days in culture (48 hours) and stained with Annexin V-FITC and propidium iodide (PI). A total of 10,000 cells were counted in each sample. A. Apoptotic death rate in BMMCs from a normal dog. 8.92% cells were Annexin V-FITC positive and PI negative. B. Apoptotic death rate in BMMCs from a CH dog. 32.42% cells were Annexin V-FITC positive and PI negative. During the induction of myelopoiesis with SCF and G-CSF, *in vitro* cultured BMMCs from CH dog had increased apoptosis compared to normal dog.

Figure 4.6

A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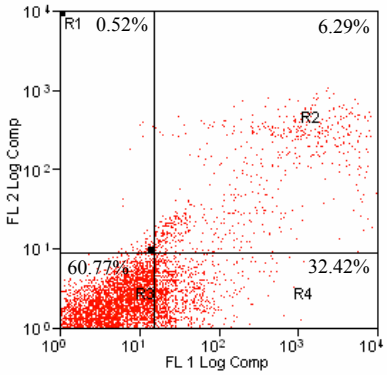
B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CHAPTER 5

CONCLUSION AND FUTURE STUDIES

Hematopoiesis is a continuous process which gives rise to myeloid and lymphoid progenitor cells and all types of blood cells. Cyclic fluctuation of blood cells in human beings and dogs has long been considered as an intriguing but enigmatic phenomenon, since hematopoiesis is not periodic in normal subjects. Finding the mechanism of these disorders will further our understanding on some complicated issues in hematology, such as how hematopoiesis is regulated under normal or stressful circumstances requiring increased WBC production. A better understanding of the molecular mechanisms for cyclic neutropenia (CN) and cyclic hematopoiesis (CH) will facilitate the discovery of new treatment regimens for these inherited hematologic disorders.

In this study, the generation of canine specific antibodies to neutrophil elastase (NE) was critical for understanding of pathogenesis of CH in the dog. Newly generated antibodies mELA85 and pELA85 recognize the mature form of canine elastase with a MW of 28kD, while antibodies mELA269 and pELA269 react with canine elastase precursor protein with a MW of 33kD. These antibodies are useful reagents for studying the intracellular trafficking and localization of NE in normal subjects, as well as diseases which are associated with NE. Using mELA85 and pELA85, canine neutrophils were

shown to contain large amount of mature elastase enzymes in primary granules. These cells also retain a small amount of elastase precursor proteins which are not localized in primary granules. Using these new canine specific antibodies, the decreased enzymatic activity of NE in CH dogs was caused by reduced storage of elastase mature forms in primary granules. In contrast to normal neutrophils, PMNs from CH dogs contain large amount of elastase precursor proteins and these proteins were not localized in primary granules, as in normal cells. These data suggest that neutrophils from CH dogs have a defect which results in abnormal sorting and trafficking of granule-localized protein – NE.

During myelopoiesis, elastase is constitutively secreted or stored in primary granules for regulated secretion. Sorting and trafficking of elastase into primary granules possibly includes two pathways. One is direct granule targeting independent of the presence of elastase C-terminal peptide. The second pathway is trafficking to plasma membrane and then to the granules through endocytosis. Absence of the C-terminal peptide bypasses the plasma membrane trafficking of the second route (Tapper *et al.*, 2006). In CH dogs, neutrophils accumulated excessive amount of elastase precursor proteins with intact C-terminus. Only a small amount of the mature elastase is stored in primary granules. The mutation in the CH dog and the function of AP-3 suggest that the direct granule targeting of elastase is possibly interrupted, resulting in the accumulation of elastase precursors in neutrophils. Results reported herein provide direct evidence that the sorting and trafficking of NE is mediated by AP-3, although how AP-3 interacts with NE has not been determined. The minimal amount of elastase in primary granules from CH dogs is possibly due to elastase trafficking through the plasma membrane and

endocytosis. These results also suggest that a second route for NE trafficking via the plasma membrane is inefficient in delivering elastase to primary granules. Hermansky-Pudlak syndrome type – 2 (HPS-2) is caused by the same gene mutation as in CH dogs. Neutrophils from HPS-2 patients contain reduced amount of elastase (Fontana *et al.*, 2006). Whether these cells accumulate elastase precursor proteins is unknown. The assembly of AP-3 in HPS-2 patients is disrupted due to the absence of β3A and μ3A subunits and reduced expression of the other two AP-3 subunits. Therefore, elastase trafficking to primary granules from HPS-2 patients is likely conducted through the route of plasma membrane and then endocytosis as may occur in the CH dog. Intriguingly, unlike HPS-2 and *pearl* mice, these studies show that the μ3A subunit is normally expressed in CH dog PMNs and cultured bone marrow mononuclear cells (BMMCs). CH dogs were found to produce a heterogeneous population of normal *AP3B1* transcripts due to a transcriptional slippage made by RNA polymerases (Benson *et al.*, 2004). Therefore, whether there is protein expression of normal β3A and the assembly of functional AP-3 in CH dogs remains to be investigated. Solving this issue will depend on the identification of canine specific or species cross-reactive antibodies to the AP-3 subunits.

NE is known as a soluble, secretory protein which is primarily synthesized in promyelocytes during neutrophil maturation. Although neutrophil primary granules are lysosome-like organelles, the sorting and trafficking of their soluble protein contents are different from other lysosomal proteins. The function of AP-3 has been designated as transfer of integral membrane proteins, since the structure of transport vesicles determines that AP-3 cargo proteins should exist in a transmembrane form in order to directly contact with adaptor protein complex which is localized on the outside of the

vesicles (Robinson & Bonifacino, 2001; Peden *et al.*, 2004). Benson and colleagues have predicted that NE has a transmembrane conformation, and the interaction between elastase and the μ3A requires the removal of elastase C-terminal peptide (Benson *et al.*, 2003; Horwitz *et al.*, 2004). However, there is lack of direct evidence for this transmembrane conformation. The C-terminal peptide has been shown not to be required for elastase translocation into primary granules and the maturation of the enzyme. Determining the compartment where elastase C-terminus is removed, the enzyme which cleaves off elastase C-terminus and the function of C-terminus for elastase maturation are critical for the understanding the abnormal accumulation of elastase precursor proteins and the pathogenesis of the cyclic hematopoiesis in both human beings and dogs.

The unfolded protein response (UPR) and increased apoptosis of hematopoietic progenitor cells represent a novel mechanism associated with human severe congenital neutropenia (SCN) patients with heterogeneous point mutations in NE (Kollner *et al.*, 2006; Grenda *et al.*, 2007). These point mutations do not change the NE protein stability or substrate specificity, but most of them reduce elastase proteolytic activities. Cells expressing the mutated NE proteins and myeloid progenitor cells from SCN patients accumulate NE in the cytoplasm which induces the UPR. These point mutations might affect protein tertiary structure, the proper folding of NE protein and its intracellular localization, causing the stress in the endothelium reticulum (ER). In CH dog, NE gene is integral and the mutation is different from SCN, but the phenotypes are similar: NE precursor proteins are abnormally accumulated in neutrophils. The accumulation of NE precursors up-regulates the expression of BiP/GRP78, the sensor of ER stress and the UPR, indicating CH dog myeloid progenitor cells are undergoing increased ER stress

during differentiation. Increased apoptosis of *in vitro* cultured BMMCs from CH dogs suggests a mechanism which might cause the reduced production of neutrophils in CH dogs. To further study the cellular response, culture of purified hematopoietic progenitor CD34+ cells and the study on the gene expression of proteins which are involved in apoptosis pathway, ER- or proteasome-associated protein degradation pathway are necessary to provide direct evidence for the UPR in CH dogs.

AP-3 transfers nascent proteins from the TGN to primary granules, while BiP/GRP78 is localized in the ER. In order to induce ER stress, these precursor proteins have to be accumulated in the ER. NE precursor proteins were not localized in the primary granules, but the localization of elastase precursors in the cell has not been determined. Immunocytochemistry using the ER, Golgi and cell surface markers to localize elastase precursor proteins will be needed to confirm the induction of ER stress in CH dogs.

The UPR and increased apoptosis of myeloid progenitor cells are presumably the molecular mechanism associated with CH in the dog. The increased apoptosis of progenitor cells has been demonstrated in human CN, although all cases of human CN are caused by point mutations in NE. It has not been determined whether the apoptosis of myeloid progenitor cells in human CN is caused by the accumulation of NE proteins and the UPR. Characterization of NE in neutrophils from human CN patients will be very useful for understanding the likely mechanisms associated with this hematologic disorder.

In this study, culture of BMMCs demonstrated that bone marrow progenitor cells from CH dogs have increased apoptotic rates when they are induced to differentiate towards the myeloid lineage. The expression of NE precursor proteins and the

glycosylation of these precursor proteins in CH dog BMMCs are strongly induced during myelopoiesis. However, BMMCs are a population of cells which are composed of hematopoietic cytokines, mature blood cells, and myeloid and lymphoid progenitor cells at different stages. Therefore, the results from BMMCs culture might be confounded when we need to study proteins expressed only in myeloid lineages. Culture of canine hematopoietic progenitor CD34+ cells with a longer culture period will be more powerful for characterizing the expression and maturation of NE proteins, and the corresponding cellular responses.

In the normal adult human, neutrophils spend their life in three compartments: bone marrow, blood and tissues. Bone marrow is where myelopoiesis occurs, and the mature neutrophils are stored in the marrow before release into the peripheral blood. In human beings, it generally takes about two weeks for segmented neutrophils to develop from myeloblasts (Babior & Golde, 1996). NE is primarily synthesized in promyelocyte stage, when the cells are still undergoing mitotic division. According to the time course and compartment that neutrophils develop, the possible scenario for the cycle of blood cells in CH dogs is proposed here. In CH dog bone marrow, when NE precursor proteins start to express in promyelocytes, abnormal sorting and trafficking results in accumulation of NE precursor proteins in the progenitor cells. Progenitor cells derived from the division stages when NE is highly expressed will accumulate a large amount of NE precursor proteins. When a small number of promyelocytes survive and enter myelocyte stage, NE transcription is down-regulated and these progenitor cells keep dividing and differentiating to the cells in the following stages. Apoptosis of early divided progenitor cells causes depletion of neutrophils in the marrow and the peripheral blood.

This creates the nadir of the cycle (neutropenia). It usually takes days for neutrophils to mature in the marrow, normal myeloid progenitor cells keep dividing and differentiation, and will be able to replenish peripheral PMNs in a timely manner. In CH dogs, most promyelocytes die at their early stage. Maturation of neutrophils from the small number of survived promyelocytes results in a delayed release of neutrophils into the blood. Once these neutrophils are matured and released into the blood, PMN counts go up to nearly or just beyond normal, creating a peak of the cycle. The cycle of neutrophils in human CN lasts longer than CH dogs. It is presumably because the maturation of neutrophils in the marrow takes more days in human beings than in dogs.

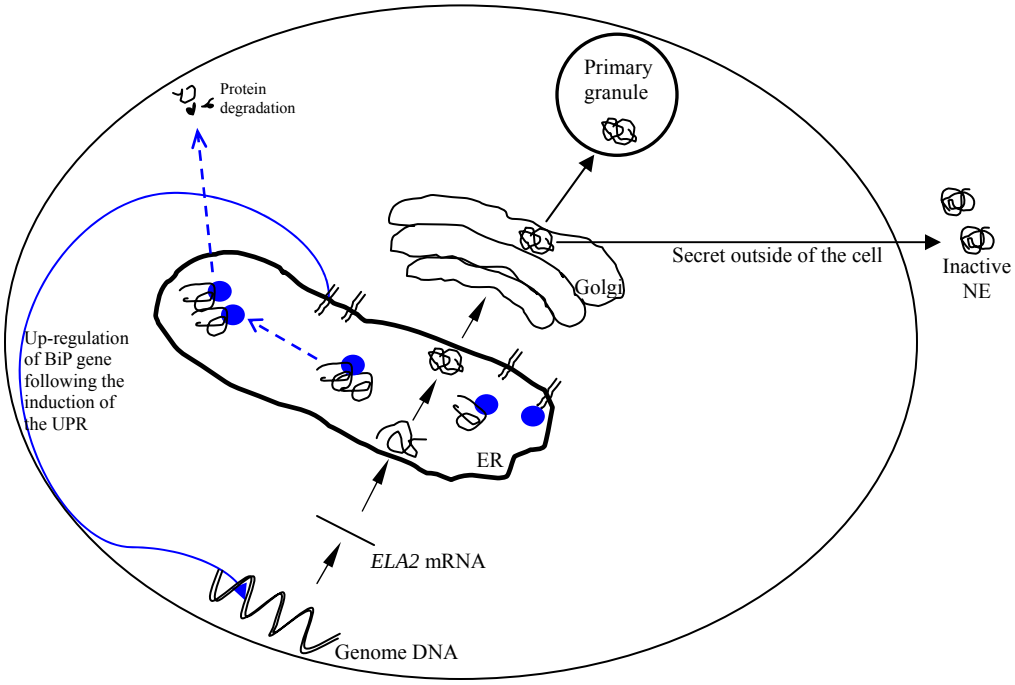
The cycle of other blood cells such as erythrocytes, platelets, monocytes in CH dogs indicate that all blood cells are originated from one common ancestor – multipotential hematopoietic stem cells. The mutation in *AP3B1* might not only affect the trafficking of NE, other proteins expressed during the differentiation of progenitor cells are possible target proteins of AP-3. To identify proteins which might be deficient in CH dogs, comparison of protein profiling from CH and normal dogs during the culture of hematopoietic progenitor CD34+ cells with growth factors inducing myelopoiesis, erythropoiesis, or thrombocytosis will be a very powerful approach. In addition, 2-D electrophoresis and subsequent mass spectrometry of neutrophil, monocyte, and platelet subcellular fractions from CH and normal dogs will be useful for identification of AP-3 associated cargo proteins.

In summary, these studies demonstrate for the first time that neutrophils from CH dogs accumulate large amount of elastase precursor proteins which results in decreased storage of mature elastase in primary granules. The accumulation is due to abnormal

processing and trafficking of NE and is likely caused by the β3A mutation. Therefore, NE intracellular trafficking is possibly mediated by AP-3. The decreased elastase enzymatic activity in CH dogs is caused by reduced storage of mature elastase proteins in neutrophil primary granules. In response to the accumulation of NE precursor protein, myeloid progenitor cells initiate the UPR, which is indicated by the up-regulation of BiP/GRP78 and increased apoptosis of these progenitor cells (Figure 5.1). The induction of the UPR and apoptosis of progenitor cells represent a common mechanism responsible for canine CH, as well as human SCN. Further characterization of the four subunits of AP-3 in CH dog, studies on the expression of NE in cultured bone marrow progenitor CD34+ cells from CH dogs, characterization of NE expression and localization in human CN, and identification of the defects of other proteins in neutrophil, platelet, monocyte from CH dogs will expand the understanding of the function of NE and AP-3, the general mechanism of hematopoiesis, as well as ultimately lead to development of new treatment regimens for these hematologic disorders.

Figure 5.1. Signaling the unfolded protein response (UPR) in cyclic hematopoietic dogs. During normal myelopoiesis, *ELA 2* gene is differentially expressed in the promyelocyte stage. The nascent NE preprotein is modified in the ER and then translocated into the Golgi complex. NE is either constitutively secreted outside of the cell or stored in primary granules for regulated secretion. In CH dogs, mutation in the β3A subunit results in abnormal trafficking of NE preprotein and accumulation of NE precursor proteins in the cell. As a feedback response, newly generated NE preproteins are accumulated in the ER and unable to be translocated into the Golgi complex. In the ER, protein chaperones BiP/GRP 78 are released from their receptors and bind to these NE preproteins to facilitate the degradation of these abnormally accumulated proteins. At the same time, BiP/GRP78 receptors (the UPR transducers) are activated and initiate the up-regulation of the UPR target genes, such as BiP/GRP78 gene, and genes which are involved in protein degradation and apoptosis pathway. As the ER stress continues, the cells eventually initiate programmed cell death (apoptosis) to release the intracellular stress.

Figure 5.1



BiP/GRP78

BiP/GRP78 receptor

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