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EFFECTS OF CELLULAR HETEROGENEITY AND IMMUNE CELLS IN
ANGIOTENSIN II-INFUSED HEMORRHAGED ASCENDING AORTAS

THESIS

A thesis submitted in partial fulfillment of the
requirements for the degree of Master of Science in the
Graduate Center for Toxicology, College of Medicine
at the University of Kentucky

By
Kyung Sik Jung
Lexington, Kentucky

Director: Dr. Alan Daugherty, Professor of Internal Medicine

Lexington, Kentucky

2013

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ABSTRACT OF THESIS

EFFECTS OF CELLULAR HETEROGENEITY AND IMMUNE CELLS IN ANGIOTENSIN II-INFUSED HEMORRHAGED ASCENDING AORTAS

A previous thoracic aortic aneurysm time course study from our laboratory determined that ascending aortic dilation was significantly increased by day 5, and reached a plateau by day 28 of angiotensin II (AngII) infusion. We also found that mice had hemorrhage localized to the ascending aortas by day 5 of AngII infusion. The purpose of these studies was to provide mechanistic insight into the development of AngII-induced ascending aortic hemorrhage.

Male C57BL/6 mice fed normal diet were subcutaneously infused with either AngII (1000 ng/kg/min) or saline for 5 days. To examine cellular heterogeneity, hemorrhaged ascending aortas were collected and sectioned serially for histological staining and immunostaining. I was unable to identify an entry point for blood into the media of the aortic root and ascending aorta. However, I found intimal, medial dissection near the hemorrhaged regions that may potentially be contiguous with the blood. To investigate infiltration of immune cells during AngII infusion, immunohistochemistry of hemorrhaged ascending

aortas was performed. The numbers of macrophages and neutrophils in AngII-infused aortas were increased in both medial and adventitial areas when compared with saline-infused aortas.

Therefore, infiltration of immune cells at the point of dissection is associated with aortic hemorrhage during AngII infusion.

KEYWORDS: Ascending Aortic Aneurysms, Hemorrhage, Angiotensin II, Neutrophil, Cellular Heterogeneity

Kyung Sik Jung
Student's Signature

August 21, 2013
Date

EFFECTS OF CELLULAR HETEROGENEITY AND IMMUNE CELLS IN
ANGIOTENSIN II-INFUSED HEMORRHAGED ASCENDING AORTAS

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TABLE OF CONTENTS

Acknowledgement.....	iii
List of Figures.....	v
Chapter I: Background	
1. Introduction.....	1
Chapter II: Cellular Heterogeneity	
1. Introduction.....	8
2. Materials and Methods.....	9
3. Results.....	11
4. Conclusion.....	18
Chapter III: Macrophages and Neutrophils in Ascending Aortic Dissection	
1. Introduction.....	19
2. Materials and Methods.....	20
3. Results.....	21
4. Discussion.....	32
5. Future.....	33
References.....	37
Vita.....	44

LIST OF FIGURES

Figure 2.1: Proximal edge of the AngII-induced hemorrhage does not co-localize with the transition to smooth muscle phenotype in the ascending aorta.....	13
Figure 2.2: AngII infusion develops not only hemorrhage but also dissection in the ascending region.....	15
Figure 2.3: Hemorrhaged ascending aorta compromises medial integrity in the anterior wall.....	17
Figure 3.1: Macrophages are abundant and surround the medial hemorrhage of the ascending aorta.....	23
Figure 3.2: Macrophages accumulate at sites of elastin breaks.....	25
Figure 3.3: Neutrophils are detected in murine spleen using an anti-Ly-6G antibody.....	28
Figure 3.4: Neutrophils are increased, not only in hemorrhaged regions, but also non-hemorrhaged ascending aortas during AngII infusion.....	30
Figure 3.5: A potential pathway: Neutrophils promote the infiltration of macrophages during AngII-infused TAA formation.....	34

Chapter One

I. Thoracic Aortic Aneurysms

Thoracic aortic aneurysm (TAA) is defined as a localized dilatation of at least a 50% increase compared to the normal aorta. Incidence of TAA is 5.6 to 10.4 cases per 100,000 patient-years. The prevalence of TAA in the general population without hypertension is up to 4.2 %.¹⁻³ TAAs are classified into four anatomic categories: ascending aortic, aortic arch, descending aortic, and thoracoabdominal aortic regions.⁴ Sixty percent of TAAs commonly develop in the ascending aorta,⁵ while forty percent of TAAs occur in descending aortic region. The increased prevalence of TAAs in the population is thought to be due to improve screening techniques.

TAA is an asymptomatic pathology until rupture occurs, thereby causing devastating health consequences. In general, growth rate of isolated idiopathic or degenerative ascending aortic aneurysm will be about 0.1 cm/year.⁶ If the aorta is >5 cm at the time of diagnosis, the growth rate may be accelerated up to 0.1 to 0.15 cm/year.⁷ Other than diagnosis and monitoring, the only treatment is surgery to repair advanced TAAs.

Etiology

TAA etiologies include degenerative processes, genetic mutations, infection, inflammation, trauma, and dissection.^{3, 4} The most common cause of TAA is a degenerative process associated with age. Genetic disorders involving

TAA include Marfan's syndrome (fibrillin-1 mutation), type IV Ehlers-Danlos syndrome (type III collagen deficiency), Loeys-Dietz syndrome (TGF- β receptors 1/2 mutations), bicuspid aortic valve disease, and familial non-syndromic TAA.

Marfan's syndrome is an inheritable, autosomal dominant disease caused by over 800 mutations in the fibrillin-1 (FBN-1) gene.⁴ FBN-1, a connective protein, is the major component of elastin microfibrils. Marfan's syndrome affects about 1 in 5000 humans.⁴ Cystic medial degeneration in young patients commonly occurs in Marfan's syndrome and less-commonly in Ehlers-Danlos syndrome. The mutations of FBN-1 result in a reduction and loss in structure of elastin in the aortic wall. In addition, defects in FBN-1 lead to increased transforming growth factor- β (TGF- β) signaling, and subsequently increased tissue degradation and weakening of the aortic wall^{8, 9} leading to aortic dilation.

Ehlers-Danlos syndrome is prevalent in approximately 1 in 5000 humans.⁴ Type IV Ehlers-Danlos results from a defect of type III collagen due to mutations in the COL3A1 gene. This disease is manifested by severe aortic aneurysm and dissection.⁴ These patients have remarkably impaired vascular and connective tissues with an extremely high risk of aortic rupture, and are therefore difficult to repair surgically.¹⁰

Loeys-Dietz syndrome has mutations in TGF- β receptors 1 and 2. This disorder is phenotypically similar to Marfan's syndrome. Patients with this disorder develop TAA, rupture, or dissection at a young age.¹¹

Bicuspid aortic valve (BAV) disease is an autosomal dominant inherited

disease with reduced penetrance and affects 1-2% of the general population. It is the most common congenital abnormality in adulthood.¹² The relationship between BAV and aortic aneurysms is well established. The aortic valve and ascending aorta share the same embryonic origin; the neural crest.¹³ BAV patients also have aortic pathology similar to patients with Marfan's syndrome. Aortic aneurysms associated with BAV may occur in up to 50% of cases and involve the aortic root through the arch.¹⁴ These aneurysms have medial degeneration, increases in matrix metalloproteinase activity and reduction of FBN-1 in the aortic wall.¹⁵

Familial non-syndromic TAA is a term used for patients who have a family or personal history of aortic aneurysm with unknown etiology. Twenty percent of TAA patients will have a family member with a similar history.⁷ This disorder has mutations in ACTA2 (Alpha-actin-2), MYH11 (Myosin-11), or TGFBR2 (transforming growth factor, beta receptor II) genes.^{16, 17} The defect of ACTA2 reduces smooth muscle α 2 actin, decreases numbers of smooth muscle cells (SMCs), and increases elastin fiber degradation.

Syphilis was the most common cause of TAA before development of antibiotics. Spirochetes of syphilis infect the aortic media, causing obliterative arteritis of the vasa vasorum in the proximal ascending aorta. Degeneration of collagen and elastic tissues leads to dilatation of the aorta.⁵

There are two common non-infective and inflammatory arteritises: Takayasu's and giant cell arteritis. Takayasu's arteritis is a chronic inflammatory

disease of unknown etiology. This disease occurs more often in women.¹⁸ The cause of this disease is obliterative luminal changes in aorta and other involved arteries, which arise from acute inflammation at an early stage. Giant-cell arteritis may affect the aorta even though this disease typically affects temporal or cranial arteries.¹⁹

Treatment and Management

Most patients with TAA are asymptomatic. Diagnosis of TAA is usually found incidentally when imaging (chest X-ray, CT scan, MRI, or echocardiogram) is ordered for other indications. The only current therapy for patients with TAAs is surgery.

Recently, Dr. Dietz's group reported that angiotensin receptor blockers attenuated the growth of TAA in Marfan's patients.²⁰ Even though blood pressure of these patients was not changed, the dilation of TAA was significantly reduced. In addition, renal functions were also normal in all patients after 3 months of ARB treatment.

Other studies have reported benefits of β -blockers^{21, 22} and statins²³ in chronic aortic aneurysms, although several large population studies have also shown no effects of these inhibitors.^{24, 25}

Pathology

All of the TAA etiologies cause medial degeneration through inflammation, degradation of extracellular matrix, and apoptosis of vascular SMCs.⁴ One possible mechanism of medial degeneration is accumulation of reactive oxygen

species (ROS) that activate matrix metalloproteinases (MMPs), thereby, degrading extracellular matrix in aorta.^{26, 27} TGF- β , increased in Marfan's syndrome and other inherited diseases, has been associated with thickening of the aortic wall and fragmentation of elastin in the media.²⁸ Pathways of medial degeneration, including ROS production and TGF- β signaling in the cells, are part of common inflammatory cascades. Thus, the complexity of signal transduction pathways including mitogen-activated protein kinase cascade has been implicated in formation of aortic aneurysms.

Animal Models

AngII is the main effective molecule in the renin-angiotensin system. AngII infusion leads to a variety of vascular diseases in different animal models including atherosclerosis and aneurysms.^{29, 30} Recent studies demonstrate that AngII infusion induces TAAs in C57BL/6³¹ and hypercholesterolemic mice.^{32, 33} These TAAs have similar pathological characteristics, including ascending aortic dilatation, medial thickening, and elastin destruction, when compared to the fibrillin-1 mutated mice, a Marfan's syndrome model.³⁴

There are three main animal models of Marfan's syndrome: FBN-1^{C1039G/+}, FBN-1^{mg Δ /mg Δ} , and mgR/mgR.³⁴⁻³⁶ A complete deficiency of fibrillin-1 in mice, FBN-1^{C1039G/C1039G} has embryonic lethality. FBN-1^{mg Δ /mg Δ} mice are born with 10-fold-less expression of FBN-1 and live for 9-18 weeks.³⁵ Mice homozygous for the mgR mutation have a 5-fold reduction of FBN-1 expression and live about 27 weeks.³⁶ Thus, a commonly used animal model for Marfan's syndrome is

heterozygous FBN-1^{C1039G/+}.³⁴ This heterozygous mouse is born with a normal phenotype and life span but may develop ascending aortic aneurysm after 2 months.³⁴

Another genetically modified TAA animal model is fibulin-4 mutant mice. Fibulin-4^{-/-} mice die perinatal due to aortic rupture,³⁷ and fibulin-4^{R/R} mice also start to die after birth due to reduction of fibulin-4 leading to aortic dissection.³⁸

Lastly, abluminal calcium chloride application on the descending aorta of 129/SvE mice creates TAA formation. Mice have ascending aortic dilation but reduced medial thickness.³⁹

Renin Angiotensin System (RAS) in Vascular Pathology

AngII is a major effector in the RAS. This octapeptide is formed from enzymatic cleavage of angiotensinogen to angiotensin I (AngI) by renin which is secreted from the kidney. Next, angiotensin converting enzyme (ACE) cleaves AngI between amino acid 8 (Phe) and 9 (His), converting it to AngII.

Increased concentrations of AngII lead to binding to the angiotensin type 1 receptor (AT1R), resulting in activation of several signaling cascades, that regulate various physiological and pathological effects. AngII affects all vascular cells, including endothelial cells, SMCs, fibroblasts, and monocyte/macrophages. Thus, AngII is important to the development of vascular diseases. Changes in phenotype and morphology of these cells, as well variations in gene expression lead to vascular pathogenesis.

Pathologic AngII-induced signaling in various cells, including immune cells, SMCs, and endothelial cells, promotes reactive oxygen species (ROS) generation, inflammation, migration, growth, platelet activation, and fibrosis. All of these effects generate and contribute to various vascular diseases, including atherosclerosis and abdominal aortic aneurysms. These vascular diseases may be attenuated by antagonism of AngII actions. Recent studies partially explained the benefits of angiotensin converting enzyme inhibitors and angiotensin II receptor blocker therapy.^{4, 28, 40, 41}

Chapter Two

1. Introduction

Many studies have reported that AngII infusion leads to formation of abdominal aortic aneurysms. Recently, it has been observed that AngII infusion into mice also leads to aneurysms of the ascending aorta.^{32, 33} Ascending aortic aneurysms have distinctly different pathological characteristics to aneurysms formed in the abdominal aorta. For instance, abdominal aortic aneurysm is highly prevalent in aged males with smoking being a major risk factor;^{42, 43} whereas ascending aortic aneurysms frequently occur in young people and are more commonly associated with genetic disorders in connective tissues.⁴⁴ During a study to determine sequential pathological changes in AngII-induced ascending aortic aneurysms, we noted that medial disruption including intralamellar hemorrhage (blood accumulation within lamellae), was a common occurrence within days of initiating AngII infusion.

The ascending aorta is composed of smooth muscle cells (SMCs) from two different developmental origins: second heart field and cardiac neural crest.⁴⁵ SMCs mainly play a role in contraction, and maintain considerable plasticity based on their response to changes in local environmental information.^{46, 47} Thus, neural crest derived SMCs play a key instructive role in the complex morphogenesis of brachial arch-derived vessels.

Moreover, myofibroblasts were increased by consequences of tissue injury and promoted expression of several SMC differentiation markers. For instance, SM α -actin suppressed by SMCs under vascular injury was increased by adventitial myofibroblasts.^{48, 49}

Based on the above background, different origins of SMCs in the ascending aorta may be crucial in morphogenesis of TAAs. In addition, two different cell types in the ascending aorta, myofibroblasts and SMCs, may have opposite effects in vascular injury. These differences may play an important role in development of vascular diseases including aortic aneurysms. The purpose of this study was to examine the origin of intralamellar hemorrhage present in the ascending aorta during AngII infusion.

2. Materials and Methods

2.1. Mice and Diet

C57BL/6J mice were purchased from The Jackson Laboratory (Cat# 0664; Bar Harbor, ME), and fed a normal laboratory diet (Harlan Teklad; Indianapolis, IN). All studies were performed with approval of the University of Kentucky Institutional Animal Care and Use Committee (IACUC).

2.2. Drug Administration

Saline or AngII (Bachem; Cat# H-1706; Torrance, CA) was infused for 5 days via subcutaneously implanted mini-osmotic pumps (Alzet Model#2001;

Durect Corp; Cupertino, CA) into 8-week-old male mice. Two groups of mice were studied: 1. vehicle (saline) , or 2. AngII (1,000 ng/kg/min) infusion.

2.3. *Pathology of ascending aortic dissection*

Blood was collected via cardiac puncture. Saline was perfused through the left ventricle of the heart until the aorta was completely cleared of blood. The aorta with attached heart was harvested.

For cross sections: The aorta was cleaned and the heart was removed from the aorta. The lower three fourths of heart was discarded and the upper portion of the heart was set apex down in a mold filled with Optimal Cutting Temperature (OCT) compound for frozen sectioning. Serial sections (10 μ m) were acquired through the entire aortic root in sets of 10 slides with 9 sections per slide.

For longitudinal sections: Vertical serial sections of the ascending aorta were collected on 10 consecutive slides with 6 sections on each slide.

Immunostaining was performed on serial sections of the aortic root. Alpha-actin (Abcam; Cat# ab5694) and reticular fibroblast (ER-TR7; Abcam; Cat# ab51824) antibodies were used for detecting SMCs and fibroblasts, respectively. Biotinylated goat anti-rabbit IgG (Vector; Cat# BA1000) and rabbit anti-rat IgG (Vector; Cat# BA4001) were used as secondary antibodies, respectively. Peroxidase-based ABC kits (Vector; Cat# PK6001) and the red chromogen, AEC

(Vector; Cat# SK4205), were used to detect the antigen-antibody reaction. Non-immune IgGs (rabbit for α actin; rat for fibroblast) were used as negative controls.

Histology: Serial sections were stained with Movat's pentachrome (Cat# S2087; PolyScientific; Bay Shore, NY) and Hematoxylin & Eosin (H&E; Eosin; Sigma; Cat# E8761; St. Louis, MO).

3. RESULTS

3.1 The proximal edge of AngII-induced hemorrhage did not co-localize with the transition of smooth muscle phenotype in the ascending aorta

To locate the source of blood in intralamellar hemorrhage of the ascending aorta, 8-week-old male C57BL/6J (N=20) were fed normal laboratory diet during 5 days of AngII infusion. Hemorrhage was visible from the ascending aorta to the carotid branches of the aortic arch at day 5 of AngII infusion. The incidence of hemorrhage was 30% (6 out of 20).

Ascending aortas were collected and sectioned serially. Sections were immunostained for detection of SMCs by an anti- α -actin antibody (Figure 2.1 A, D, G), and fibroblast cells by an anti-ERTR7 antibody (Figure 2.1 B, E, H). Elastin breaks were detected using Movat's pentachrome staining (Figure 2.1 C, F, I). The sections in Figure 2.1 were located from the ascending aorta to aortic root and intervals between A, B, C and D,E,F, or from D, E, F to G, H, I were 400 μ m or 300 μ m, respectively.

The major medial cell type was fibroblasts not SMCs in aortic root sinuses. (Figure 2.1 G, H) Fibroblast cells gradually diminished from the aortic root to the ascending aorta and located predominately in the perivascular area, whereas SMCs gradually increased in the media. (Figure 2.1 A, B, D, E, G, H) Elastin layers were intact in the ascending aorta proximal to the aortic root. (Figure 2.1 C, F, I)

The proximal edge of the ascending aorta with hemorrhage was not present in these sections. Hemorrhage in the ascending aortas of mice infused with AngII did not reach the aortic root region.

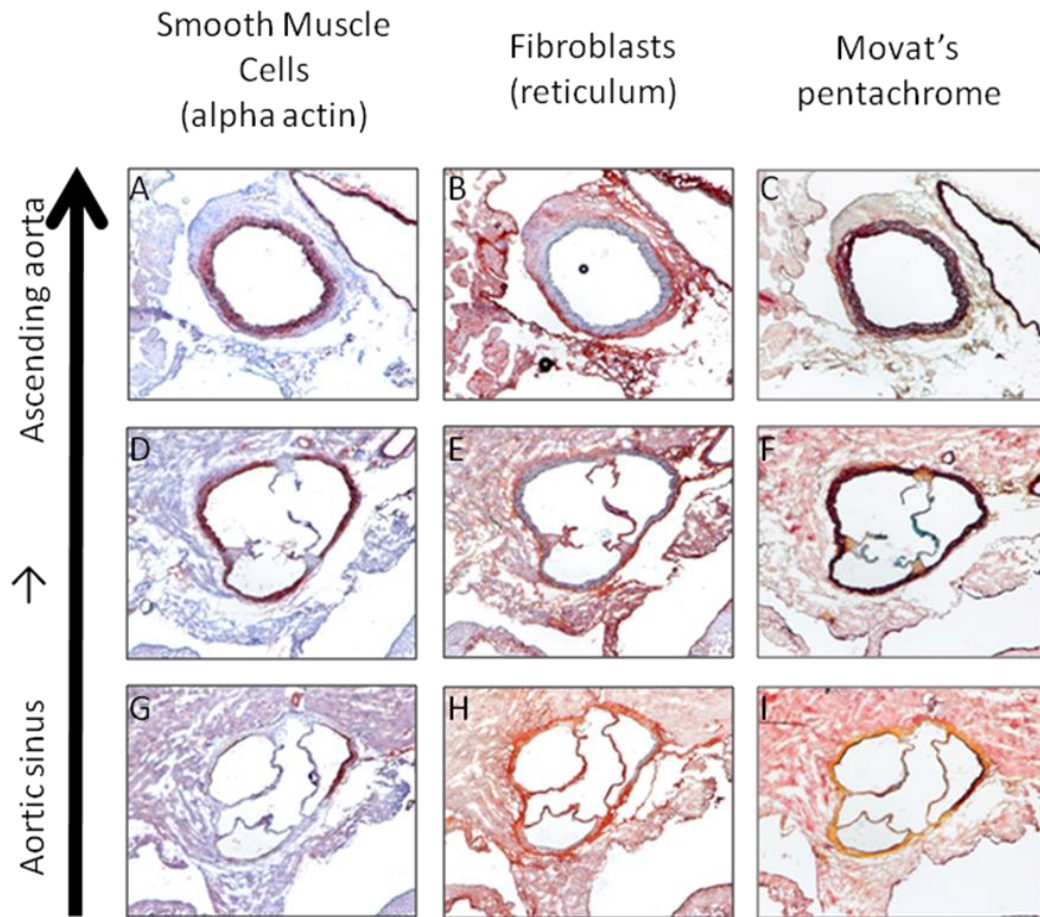


Figure 2.1 The proximal edge of AngII-induced hemorrhage did not co-localize with the transition of smooth muscle phenotype in the ascending aorta. Immunostaining for SMCs and fibroblasts, and histological staining using Movat's pentachrome of cross-sections from the aortic root through the ascending aorta of an AngII-infused mouse. Serial sections A, B, C are the ascending aorta distal to the aortic root; serial sections D, E, F are 400 μm proximal from upper panels, and G, H, I are 300 μm proximal from the middle panels. Panels A, D, G are immunostained by anti- α -actin antibody for detecting SMCs, panel B, E, H by anti-ERTR7 antibody for fibroblast cells. Red color indicates positive cells. The right panels (C, F, I) were stained with Movat's pentachrome to detect elastic fibers in media indicated by black color.

3.2 AngII infusion developed not only hemorrhage, but also medial breaks in the ascending region

Aortic dissection was found in AngII-infused hemorrhaged aortas at 5 days of AngII infusion. Two out of 6 hemorrhaged aortas had medial breaks. Serial sections were cut through ascending aortas with hemorrhage. These sections were stained and examined for pathological characteristics. I detected a dissection in serial sections of the ascending aorta. The distal section of the aortic dissections nearest the aortic arch had two regions of intralamellar hemorrhage in the media. (Figure 2.2 A) The section of the ascending aorta in Figure 2.2 B was located in 400 μm distal to Figure 2.2 A. This aortic dissection was extensive and maximally dilated. The section in Figure 2.2 C was 300 μm distal to Figure 2.2 B.

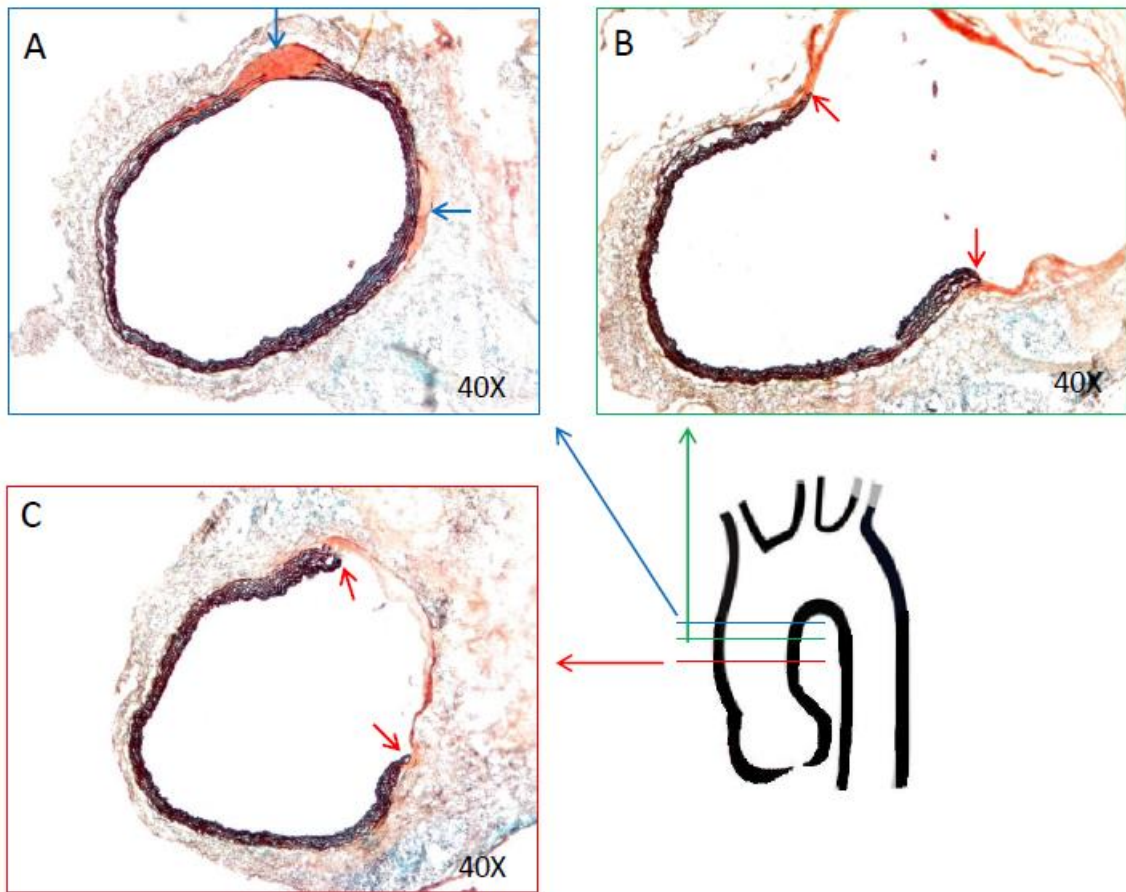


Figure 2.2 AngII infusion developed both hemorrhage and dissection in the ascending region. Movat's pentachrome staining of the hemorrhaged ascending aortas shows aortic dissection. (A) The ascending aorta near the aortic arch showed two hemorrhaged regions indicated by blue arrows. (B) This section was 400 μm proximal to (A) and had large luminal dilation. (C) was 300 μm proximal to (B) and the dilation was attenuated. Red arrows indicate dissection in the aorta.

3.3 Hemorrhaged ascending aorta compromised medial dissection in the anterior wall

Longitudinal serial sections of hemorrhaged aortas infused for 5 days with AngII may identify the origin of blood. For this study, we infused mice (N=20) with AngII and three hemorrhaged aortas were observed. These aortas were serially sectioned longitudinally and H&E staining was performed. In Figure 2.3, panel A shows vertical sections of a hemorrhaged aorta. Panels B, C, and D are magnified 200X to focus on specific parts of the hemorrhaged aorta. In panel B, the dissected region on the anterior aortic wall can be observed. The area of blood accumulation was detected using H&E staining. Several medial layers of disrupted elastin fibers can be seen in panel C. However, there were no medial elastin breaks visible in posterior regions of the aorta. (Figure 2.3 D)

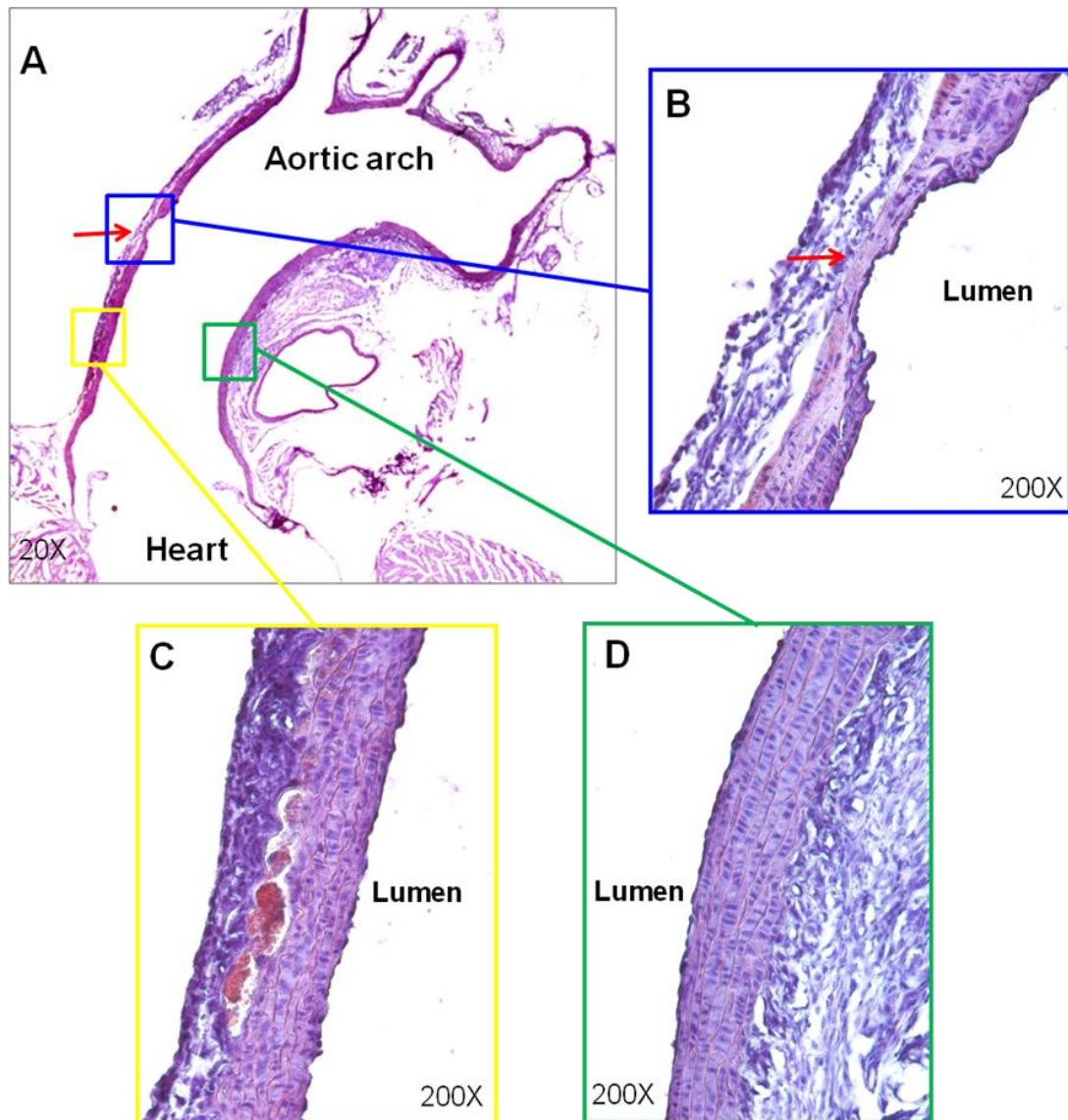


Figure 2.3 Hemorrhage of the ascending aorta compromised medial dissection in the anterior wall H&E staining of a longitudinal section of the hemorrhaged ascending aorta detected accumulated blood (A). A magnified view of the compromised medial integrity on the anterior wall is shown in the blue inset (B). Elastin fragmentation was frequent in the anterior wall as shown in the yellow inset (C). Intact elastin layers in the posterior ascending aorta are shown in the green inset (D). Red arrows indicate areas of decreased medial integrity.

4. Conclusions

As illustrated in Figure 2.1, the source of blood in AngII-infused hemorrhaged aortas was not located in the proximal region near the aortic sinus, which is the transition point between two origins, the second heart field and the cardiac neural crest. Although the source of thrombus is still unknown, I found that myofibroblasts are the major cell type in the aortic sinus, and different origins of SMCs, located in the media of the ascending aorta, were not directly implicated with the source of blood in intralamellar hemorrhage.

Aortic dissection in cross-sections was found in AngII-infused hemorrhaged ascending aortas. Dissection may be the entry point of blood into the media of ascending aorta, but all of hemorrhaged aortas for 5-day-AngII infusion did not show dissection and the number of aortic dissections were few. In longitudinal aortic sections, hemorrhage in ascending aortas was located on the anterior region and had pathological characteristics of medial breaks and elastin degeneration. However, elastin degradation was not observed in the posterior region of ascending aortas without hemorrhage.

In summary, the origin of blood in AngII-infused hemorrhaged aortas is yet unknown but dissection may be one possible candidate of blood source for forming the intralamellar hemorrhage.

Chapter Three

1. Introduction

Leukocytes and TAA: Numerous studies have demonstrated that AngII infusion increases the presence of immune cells including macrophages, lymphocytes, and neutrophils in the aorta.^{50, 51} Medial macrophage accumulation and MMPs secretion are related to fragmentation of elastin fibers. Published papers from our laboratory have demonstrated that the deficiency of monocyte-colony stimulating factor, a growth factor to influence the proliferation and differentiation of macrophages, reduces monocyte numbers yet hematomas were present in thoracic aortas.⁵² C-C chemokine receptor type 2 (CCR2) deficiency attenuated TAA formation in apolipoprotein E^{-/-} mice and also decreased macrophage accumulation in the ascending aorta.³² Therefore, these studies demonstrated increase in macrophage numbers in TAAs during AngII infusion. Recently, Kurihara and colleagues demonstrated that neutrophils initiate acute aortic dissection.⁵¹ However, they used a neutralizing anti-granulocyte receptor-1 (Gr-1) antibody that recognizes not only neutrophils (Ly-6G) but also monocytes (Ly-6C) for neutrophil depletion.

Monocytes/Macrophages: Monocytes are heterogeneous, circulating blood cells that rapidly extravasate into inflamed tissues. Many studies have determined that AngII infusion leads to macrophage accumulation in TAAs.^{31, 32} Many published papers have determined that patients with TAAs have increased immune cells including macrophages in aortic tissue.^{51, 53} One possible role of

AngII could involve inflammatory responses, triggering the infiltration of immune cells into aortic tissue. Ruiz-Ortega and colleagues stated that AngII can induce infiltration of immune cells, especially monocytes during inflammation.⁵⁴

Neutrophils: Neutrophils are abundant immune cells in blood and are the first leukocyte recruited into sites of acute inflammatory injury. Their survival in inflammatory sites is increased as 3-4 times compared with their basal half life in circulation.^{55, 56}

Many studies have demonstrated that an anti-Ly-6G antibody is specific for neutrophils,^{57, 58} and neutrophils were increased in patients with acute aortic dissection and rupture.^{51, 59}

2. Material and Methods

2.1. Mice and Diet

Mice and diet were used from the same source in the previous chapter.

2.2. Drug Administration

Saline or AngII (Bachem; Cat# H-1706; Torrance, CA) was infused for 5 days via subcutaneously implanted mini-osmotic pumps (Alzet Model#2001; Durect Corp; Cupertino, CA) into male mice at the age of 8 weeks. Two groups of mice were studied: 1. vehicle (saline) infusion (N=5), and 2. AngII 1,000 ng/kg/min infusion (N=15).

2.3. Pathology of ascending aortas

Pathological procedures were the same as discussed in the previous chapter.

Immunostaining was performed on serial sections of the aortic root. Anti-CD68 (AbDSerotec; Cat# MCA1957) and anti-Ly-6G (BD Pharmingen; Cat# 551459) antibodies were used for detection of macrophages and neutrophils, respectively. Biotinylated goat anti-rabbit IgG (Vector; Cat# BA1000) and rabbit anti-Rat IgG (Vector; Cat# BA4001) were used as secondary antibodies, respectively. Peroxidase-based ABC kits (Vector; Cat# PK6001) and the red chromogen, AEC (Vector; Cat# SK4205), were used to detect antigen-antibody reactions. Non-immune IgG (rat for macrophages or neutrophils) were used as negative controls.

Histology: Serial sections were stained with Movat's pentachrome (PolyScientific; Cat# S2087; Bay Shore, NY) and H & E (Eosin; Sigma; Cat# E8761; St. Louis, MO).

3. RESULTS

3.1 Macrophages were abundant and surrounded the intralamellar hemorrhage of the ascending aorta

Based on a laboratory defined measurement process of TAA, Jessica Moorlegghen and Debra Rateri (staff in Dr. Alan Daugherty's laboratory) measured lumen dilation of ascending aorta by ultrasonography. AngII infusion

into C57BL/6 mice rapidly increased luminal diameter on day 5, and lumen dilatation reached a plateau after day 5 through day 28. (Data not shown)

Aortas were harvested on day 5 of AngII infusion. Collected aortas were serially sectioned as mentioned in the previous chapter. Serial sections of AngII-infused hemorrhaged aortas were stained with H&E (Figure 3.1 A, B) or immunostained with an anti-CD68 antibody (Figure 3.1 C, D). Figure 3.1 shows a section of AngII-infused aorta showing dissection (red arrows). There is CD68 positive staining around the dissection of the media and adventitia. (Figure 3.1 C, D)

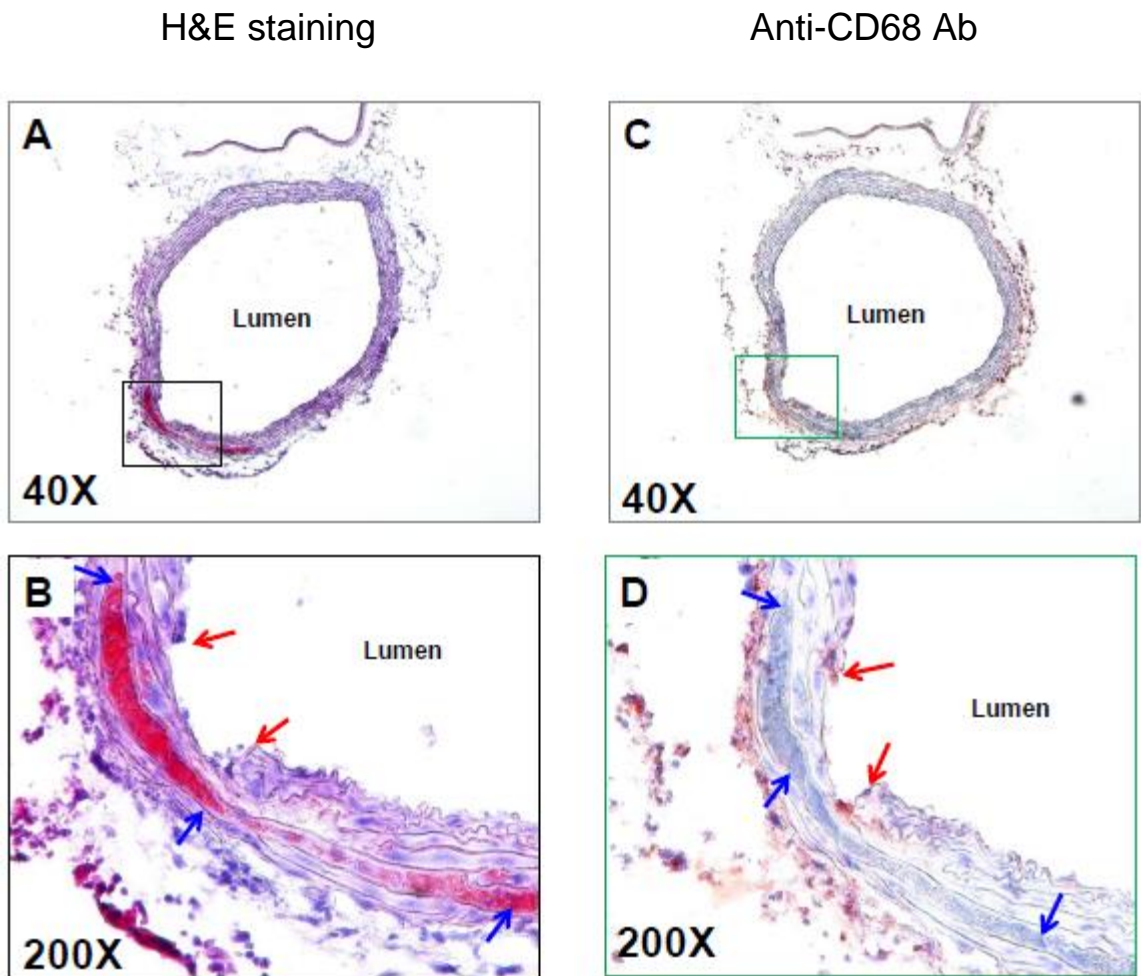


Figure 3.1 Macrophages were abundant and surrounded the intralamellar hemorrhage of the ascending aorta. H&E staining of the ascending aorta detected medial hemorrhage (blue arrows) and elastin breaks (red arrows) (A, B). CD68 positive cells stained red (C, D).

3.2 Macrophages were increased in ascending aortic aneurysm

Regions of aortic dissections without hemorrhage (Figure 3.2 A, B, C) and no dissection (Figure 3.2 D) in AngII-infused ascending aorta were stained by Movat's pentachrome. From this staining, medial breaks were detected in Figure 3.2 A, B, C, but not D. In addition, an abundance of macrophages were detected in the media and adventitia of the dissected region (Figure 3.2 E, F, G), but only in the adventitia of the non-dissected aortas (Figure 3.2 H).

Movat's pentachrome stain

Anti-CD68 antibody

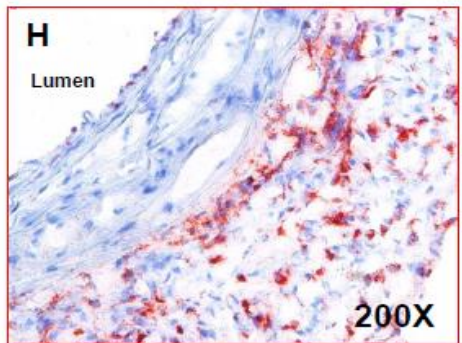
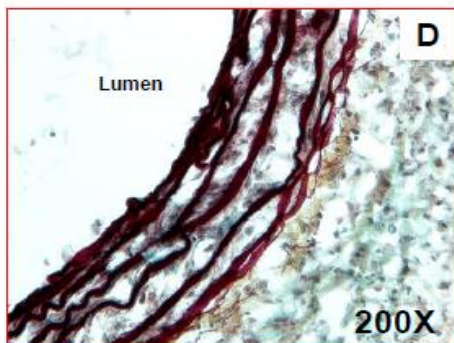
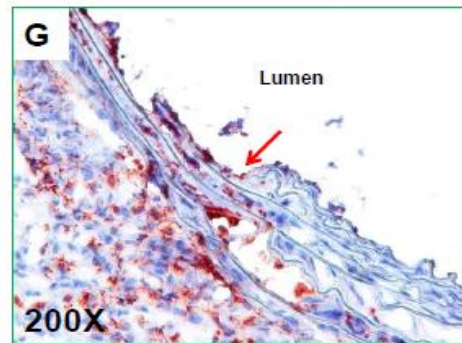
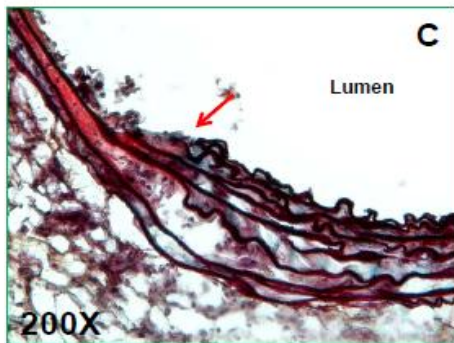
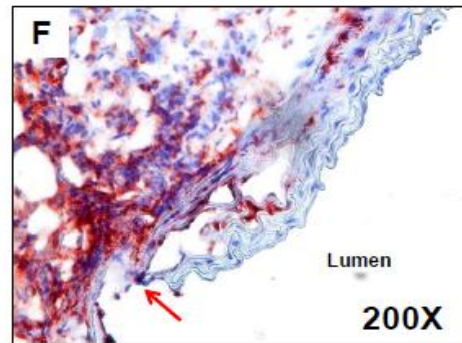
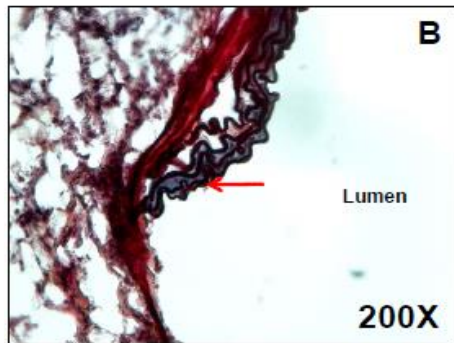
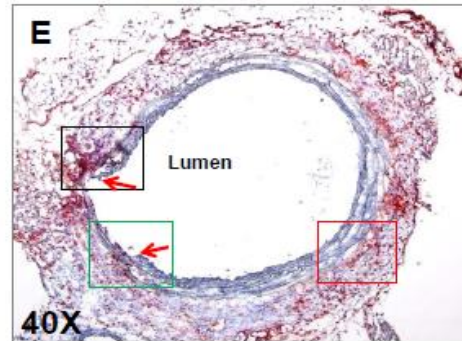
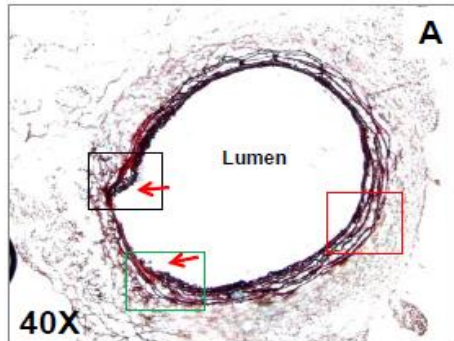


Figure 3.2 Macrophages accumulated at sites of elastin breaks. Cross sections of an ascending aorta infused with AngII. A-D represent histological staining using Movat's pentachrome. Macrophages (red color) were detected by an anti-CD68 antibody (E-H). B-D and F-H were magnified from different color boxes in A and E. Red arrows represent the sites of elastin breaks.

3.3 Neutrophils detected in murine spleen using an Ly-6G antibody

Dr. Van Leeuwen and colleagues demonstrated that neutrophils (anti-Ly-6G antibody) did not co-localize with macrophages (anti-CD68 antibody) in the merged immunofluorescent stain of murine spleen.⁶⁰ I repeated the immunohistochemistry for the anti-Ly-6G antibody in murine spleen. In this study, I observed that the anti-Ly-6G antibody only detected cells in red pulp, but not white pulp in the spleen. (Figure 3.3 B) However, the anti-CD68 antibody stained in both red and white pulp. (Figure 3.3 A) Therefore, we confirmed that the anti-Ly-6G antibody is specific for neutrophils because these antibodies have different staining patterns in spleen.

Anti-CD68 antibody

Anti-Ly-6G antibody

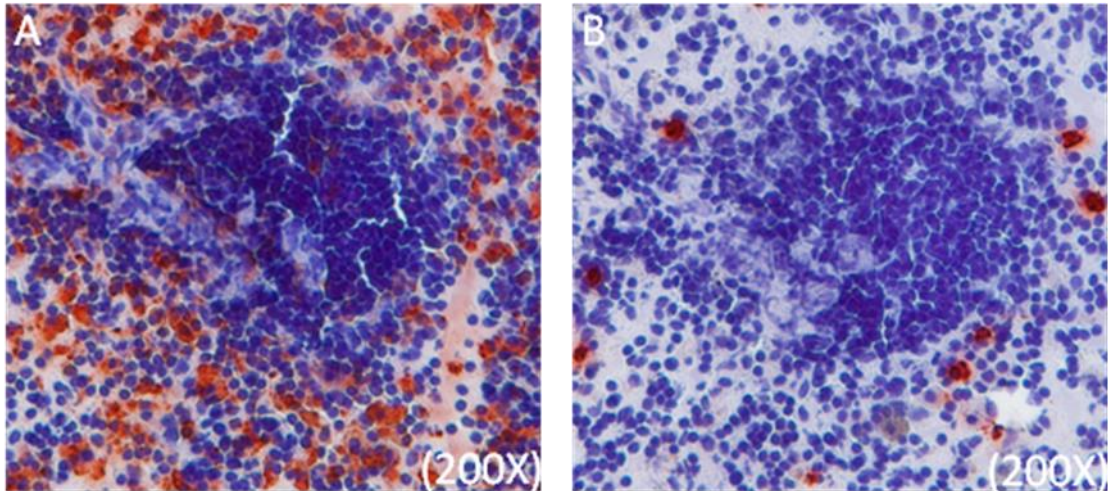


Figure 3.3 Neutrophils detected in murine spleen using an anti-Ly-6G antibody. Immunostaining of macrophages (A) or neutrophils (B) in murine spleen. Macrophages were detected by an anti-CD68 antibody (A), neutrophils were detected by an anti-Ly-6G antibody (B). Red color represents positive cells.

3.4 Neutrophils were increased in both hemorrhaged and non-hemorrhaged ascending aortas

After confirmation of the neutrophil antibody, I have immunostained aortas using anti-Ly-6G and anti-CD68 antibodies. (Figure 3.4) In A, C, E, macrophages were detected by anti-CD68 antibody and in B, D, F neutrophils were detected using the anti-Ly-6G antibody. Some macrophages were detected by anti-CD68 antibody in saline-infused ascending aortas. (Figure 3.4 A) In addition, macrophages increased in the media and adventitia of AngII-infused aortas (Figure 3.4 C, E) compared to saline controls. Neutrophils were present in the adventitia of AngII-infused aortas (Figure 3.4 F) and the media and adventitia of hemorrhaged aortas (Figure 3.4 D). There were no Ly-6G positive cells detected in saline-infused aortas (Figure 3.4 B).

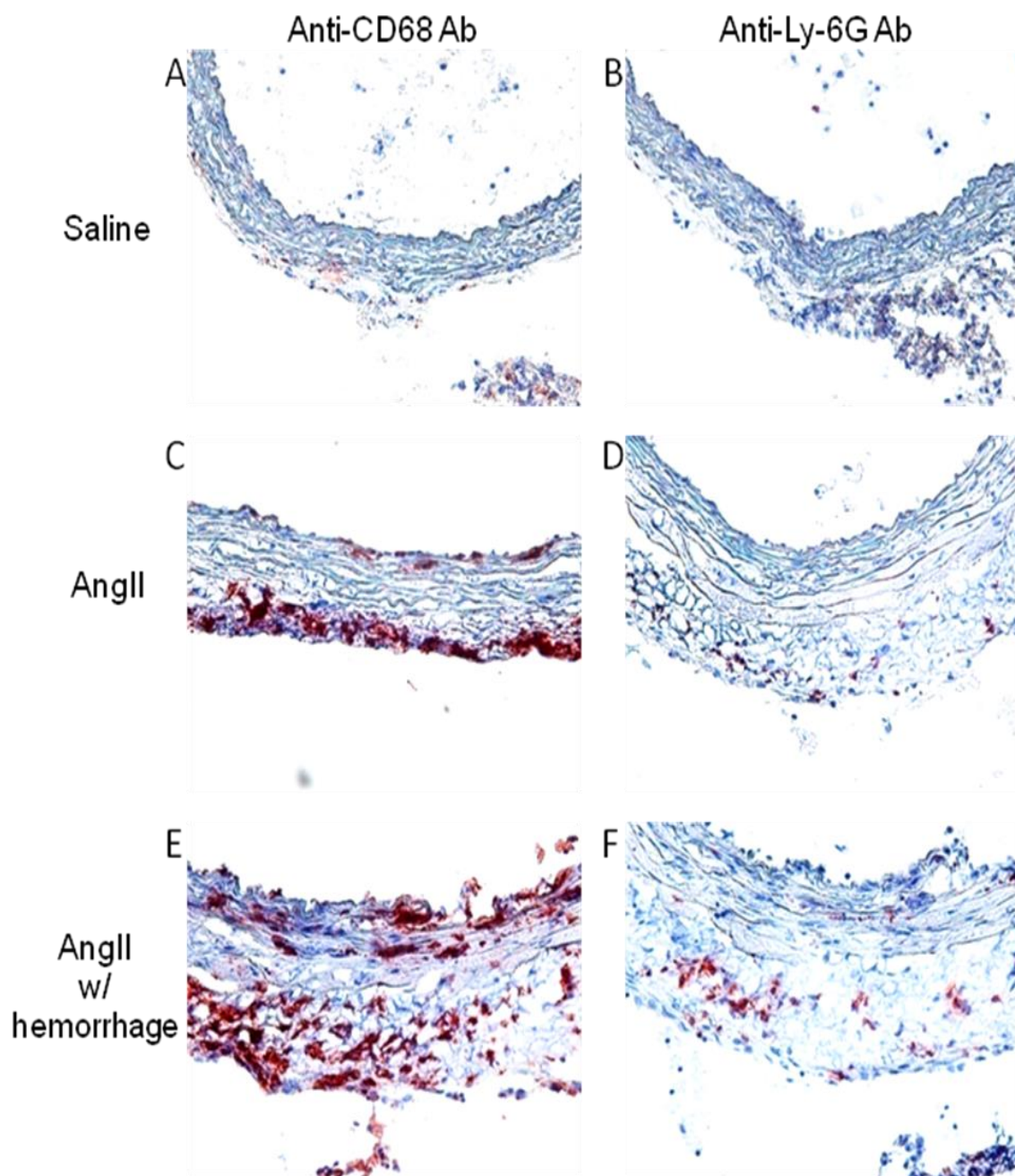


Figure 3.4 Neutrophils were increased in both hemorrhaged and non-hemorrhaged ascending aortas. Immunostaining of macrophages or neutrophils in AngII-infused ascending aortas. Mice were infused by either saline (A, B) or AngII (C, D, E, F) for 5 days. AngII infusion for 5 days promoted intralamellar hemorrhage in the ascending aorta (E, F). Panels (A, C, E) were immunostained using an anti-CD68 antibody for detecting macrophages. Panels (B, D, F) were immunostained using an anti-Ly-6G antibody. Red color indicates positive cells.

4. Discussion

Many studies have determined that macrophages were increased in both patient⁵³ and animal models^{31, 32} of TAAs. My current study demonstrated that in the early stage of TAA formation, both macrophages and neutrophils accumulated.

AngII promotes leukocyte infiltration during inflammation. These leukocytes may interplay to produce pro-inflammatory responses. Neutrophil infiltration is initiated by changes on the surface of endothelium that result from stimulation by inflammatory mediators.⁶¹ Endothelial cells can be directly activated by AngII based on the demonstration that endothelial AT1a receptor deficiency attenuates TAA formation.³³ Neutrophils may promote TAA formation by inducing monocyte recruitment and activating macrophages during inflammation. This could be due to: 1) Granule proteins anchoring endothelial proteoglycan promoting monocyte rolling along the endothelium; 2) Neutrophil granule proteins activating endothelial cells and promoting cytokine and adhesion molecules by activated endothelium; 3) Neutrophil proteins secreted from recruited neutrophils in inflamed tissue activating formyl peptide receptors (FPR) and enabling the rapid recruitment of monocytes; 4) Neutrophils activating macrophages to process phagocytosis, promoting a “pro-resolution program”. During the “resolution program”, macrophages promote cytokines for anti-inflammatory responses.⁶¹ These processes may explain interactions between neutrophils and macrophages in the media and adventitia of AngII-infused

aortas.

5. Future studies

Based on my studies and published literature, I hypothesize that AngII induced inflammation in the ascending aorta recruits neutrophils, which initiate early development of TAAs. (Figure 3.5) To examine this hypothesis, I would suggest two studies listed below.

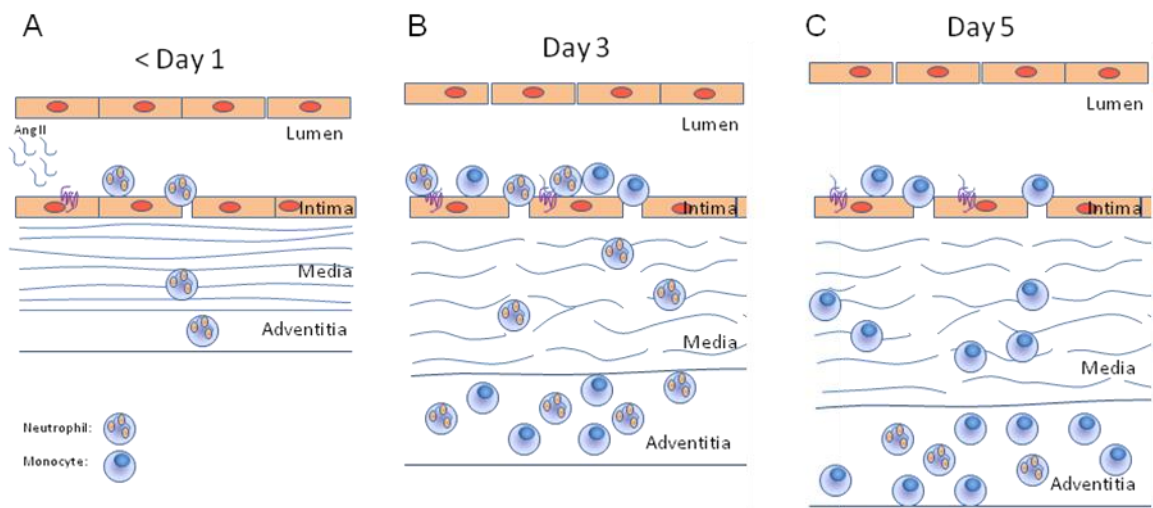


Figure 3.5 A potential pathway: Neutrophils promote infiltration of macrophages during AngII-infused TAA formation. A potential pathway in which neutrophils play a role in promoting monocyte infiltration into aortic tissue of the ascending aorta. (A) AngII binds to angiotensin II type I receptors and activates endothelium to initiate neutrophil infiltration into the media and adventitia within 24 hours of infusion. (B) Based on the increase of neutrophil life span, this cell type will be present in maximum numbers in the media and adventitia by day 3 of AngII infusion. As neutrophils die, this promotes infiltration of macrophages into the media and adventitia. Apoptotic neutrophils are phagocytized by macrophages. (C) Within days through clearance of apoptotic neutrophils, the presence and infiltration of neutrophils decreases in the media and adventitia, while the number of macrophages increases.

Study 1: The signaling pathway of TAA formation is initiated when AngII binds to AT1a receptors. This triggers inflammatory responses in endothelial cells. AT1a receptors on endothelial cells, not leukocytes and SMCs, may be important for TAA formation based on evidence that AT1a receptor deficiency in endothelial cell attenuates AngII-induced TAAs.³³ Endothelial cells activate adhesion molecules and cytokines to recruit leukocytes including neutrophils. In addition, neutrophils are increased in patients with acute aortic dissection,⁵¹ and my data also demonstrated that neutrophils were present at day 5 of AngII-infusion in hemorrhaged ascending aortas. Moreover, macrophage accumulation by infiltrating monocytes is also important in the TAA formation, and infiltrated neutrophils may be responsible for the recruitment of macrophages into the ascending region during inflammation.⁶¹ To distinguish contributions of neutrophils, a time course study of 5 days will be performed because the plateau of luminal diameter dilation is reached at day 5.

To determine whether infiltrating neutrophils mediate AngII-induced TAA formation, C57BL/6 mice will be infused with either saline or AngII, and will be analyzed for their responsiveness to AngII-infused TAA formation.

Experimental groups: Eight week old, male C57BL/6 mice will be infused by either saline or AngII (1000 ng/kg/min) for day 0, 1, 2, 3, 4 and 5. I will use 10 mice per group infused with AngII. Six mice infused with saline for each time point because I am expecting that there will be no TAAs formed in mice infused with saline.

I will measure lumen dilation of ascending aorta by ultrasound at each time point. I will use a half of each group to detect neutrophils and macrophages. The remainder will be dissected with elastase and collagenase and immune cells will be collected using a 70 μm strainer. Numbers of neutrophils and macrophages will be measured using flow cytometry.

Study 2: Many studies have demonstrated that macrophages are detected in patients with TAAs. The findings from my studies demonstrated that AngII infusion augments the population of neutrophils and macrophages in the aortic medial and adventitia at an early time point in TAA formation. Neutrophils recruit macrophages during the pro-inflammatory response triggered by AngII infusion. Thus, I propose to determine the effect of neutrophil depletion, using a neutralizing anti-Ly-6G antibody, related to macrophage accumulation in the aortic media during early TAA formation.

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Publications

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Post presentations

1. **Kyung Sik Jung**, Debra Rateri, Deborah Howatt, Anju Balakrishnan, Jessica Moorlegghen, Alan Daugherty. Distribution of Macrophages in the Medial Hemorrhage Phase During Development of AngII-induced Thoracic Aortic Aneurysms. Gill heart Institute 15th Annual Cardiovascular Research Day. 2012 Oct, Lexington KY.
2. **Kyung Sik Jung**, Debra Rateri, Deborah Howatt, Anju Balakrishnan, Jessica Moorlegghen, Alan Daugherty. Distribution of Macrophages in the Medial Hemorrhage Phase During Development of AngII-induced Thoracic Aortic Aneurysms. South East Lipid Research Conference. 2012 Sept, Pine Mountain, GA.
3. **Kyung Sik Jung**, Debra Rateri, Deborah Howatt, Anju Balakrishnan, Jessica Moorlegghen, Alan Daugherty. Cellular Heterogeneity of the Ascending Aorta: Relationship to AngII-induced Medial Hemorrhage. Vasculata. 2012 July, Nashville, TN.
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