ODANACATIB AS A POTENTIAL DRUG TO REDUCE THE RISK

OF STROKE IN THE CONTEXT OF SICKLE CELL DISEASE

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[To my friends and family who have motivated to complete this work.]

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LIST OF SYMBOLS AND ABBREVIATIONS

AA	normal hemoglobin
AS	heterozygous hemoglobin
DAPI	4',6-diamidina-2-phenylindole
JNK	c-jun N-terminal kinase
MRI	magnetic resonance imaging
NBF	neutral buffered formalin
PBS	phosphate buffered saline
SEM	standard error of the mean
SS	sickle hemoglobin
TNFα	tumor necrosis factor α

SUMMARY

Sickle cell disease is a genetic mutation of hemoglobin in red blood cells that causes them to become more rigid, aggregate and block blood vessels, and also to prematurely lyse and die releasing inflammatory contents into the blood. Unfortunately, 11% of children with sickle cell disease suffer a stroke before the age of 20, with children between the ages of 2-5 being the most vulnerable. Upon clinical examination of these stroke lesions, there are signs of arterial remodeling through enzymatic degradation of the elastic lamina. Our lab has shown previously that TNFα stimulation or peripheral blood mononuclear cells from people with sickle cell disease can induce the expression and activity of the potent elastase and collagenase cathepsin K in human aortic endothelial cells. Given these promising results, here we present data on cathepsin K inhibition as a novel therapeutic target for the reduction of the elastic lamina degradation in arteries in a mouse model of sickle cell disease.

SS and AS mice were bred using the offspring of an SS male and an AS female Towne's transgenic mouse model. After weaning, three week old AS and SS mice were given 6mg/kg intraperitoneal injections of odanacatib daily for 10 weeks. Mice were euthanized, and the carotid arteries, aorta, and brain were isolated. The carotid arteries were used for histological studies of elastin by Van Giesen staining, and immunostaining for cathepsin K. Cathepsin K staining was elevated in the SS mice receiving saline injections compared to littermate AS controls, and treatment with cathepsin K inhibitor did not change the intensity of the staining. However, elastin staining showed that cathepsin K inhibition significantly reduced the number of fragmentations in the elastin within the arterial wall. This suggests that odanacatib is effectively reducing cathepsin K

activity in the arterial wall, and this is correlated with a significant reduction in elastic lamina fragmentation in these mice.

Proteolytic degradation of elastic lamina is a key signal for smooth muscle cell migration and luminal narrowing, and our demonstration here that elevated cathepsin K in sickle cell transgenic mice can be reduced by cathepsin K inhibition presents a novel mechanism to target accelerated arterial remodeling in children with sickle cell disease at high risk for strokes at such early ages.

CHAPTER 1

INTRODUCTION

Sickle cell disease is a genetic mutation of the hemoglobin in red blood cells that causes them to become more rigid and crescent shaped. Although the mutation only directly affects red blood cells, sickle cell disease negatively impacts nearly every tissue system in the body. Specifically, 11% of children with sickle cell disease suffer a stroke before the age of 20, with children between the ages of 2-5 being the most vulnerable[1]. Upon clinical examination of these stroke lesions, signs of arterial remodeling, similar to the type of remodeling seen in atherosclerosis, are apparent.

Remodeling is initiated when circulating inflammatory factors promote adhesion of circulating monocytes and their subsequent extravasation, or movement beneath the endothelial layer, cause destruction of the elastic lamina, which eventually results in the narrowing of the artery[2, 3]. Enzymatic degradation of the elastic lamina is accomplished through powerful proteases, especially cathepsin K, which plays a particular role in the realm of arterial remodeling as it represents the most powerful mammalian elastase yet identified having the ability to degrade the elastin within the arterial wall[4].

People with sickle cell disease are especially at risk for cathepsin-mediated arterial remodeling because they have chronically elevated plasma levels of tumor necrosis factor (TNFα) and other inflammatory factors which leads to increased monocyte adhesion to the endothelial surface throughout the vasculature[5]. In the field of biomedical engineering, our lab has shown that TNFα stimulation upregulates cathepsin K in human aortic endothelial cells (HAECs) through the JNK/c-Jun signaling axis as evidenced by reduction in cathepsin K activity when the kinase, JNK, was inhibited[6]. Due to the major

role that cathepsin K plays in arterial remodeling, we want to investigate its potential as a novel therapeutic target for the reduction of sickle cell stroke risk in vivo.

Our lab uses the Townes knock-out/knock-in transgenic mouse model of sickle cell disease that expresses human hemoglobin with the sickle mutation. One specific point of interest in vivo is the branching between the left and right carotid arteries because this is where arterial remodeling is observed in people with sickle cell disease[7]. Excessive remodeling of these arteries can be fatal, as it would cut off circulation to the brain. Consequently, it is important to be able to apply the correlation between TNFα and cathepsin K to restore normal arterial structure in the context of sickle cell disease. Currently, the only known method to reduce the risk of stroke is routine blood transfusion; however, this is not a solution, a quick fix that causes these children great distress. Consequently, this study aims to identify a potential drug target to provide a better treatment option for these children. In general, Odanacatib is a newly developed cathepsin K inhibitor currently in phase III trials to treat osteoporosis[8]. This drug will be used in vivo on sickle transgenic mice to inhibit cathepsin K activity within the arterial walls, which we hypothesize will decrease elastin degradation. This is important because by successfully abrogating cathepsin activity and restoring arterial structure, the risk of stroke should drastically decrease indicating a possible therapeutic target.

CHAPTER 2

BACKGROUND

Sickle cell disease is a genetic disorder characterized by "crescent" shaped red blood cells. The genetic code is altered by a point mutation changing an adenosine to a thymine, consequently replacing a glutamic acid with a valine in the sixth position of the amino acid sequence of each beta hemoglobin[9]. This is a dangerous substitution because glutamic acid and valine have very different properties. Glutamic acid is negatively charged and hydrophilic while valine is neutral and hydrophobic. While oxygenated, this mutation is irrelevant; however, upon deoxygenation there is a conformational change that exposes the valine to the hydrophilic environment[10]. Due to hydrophobic interactions, the exposed valine finds other exposed valines to reduce energy within the system. This is what leads to the formation of sickle hemoglobin polymers that are stiff, which distorts the biconcave disc shape of red blood cells[11]. This process repeats every cardiac cycle, and the periodic stress that this puts on the red blood cell significantly reduces its life span.

Although the mutation is of the beta hemoglobin in red blood cells, there are several repercussions that affect most organs and tissues in the body. One such repercussion is elevated inflammatory and adhesion factors. Adhesion factors include intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), and E-selectin while inflammatory factors include tumor necrosis factor alpha (TNFα) and interleukin-1 (IL-1)[12]. The combination of these factors lead to red blood cell adhesion to the endothelium and vaso-occlusions in the micro-vasculature[13]. This is the cause of the painful crisis that many sickle patients experience in their life time.

However, these vaso-occlusions do not explain the fact that there is over a 200 percent increase in risk of stroke, which is caused by impaired blood flow in the carotid arteries[14]. The risk of stroke is greatest in children between 2 and 5 year old[14]. Normally, strokes

have physical symptoms such as face drooping, muscle weakness, and impaired speech. However, about 17-35% of children who experience strokes, instead experience silent strokes[15]. These types of strokes don't have the same physical symptoms, and instead result in the death of parts of the brain, which may manifest itself in learning disabilities later in life[16].

When examined clinically, these stroke lesions indicate the presence of extensive arterial remodeling, which cuts off blood supply to the brain[17]. Loss of blood flow to the brain is caused by a blockage in the carotid arteries leading to ischemia, or low oxygen supply, in parts of the brain. These blockages primarily develop in three ways: aggregations of blood cells, lack of vasodilation, and arterial wall thickening. Aggregations of blood cells noticeably impair blood flow only in the microvascular where vessels are a few cells wide. That is why this does not explain impaired blood flow in the larger carotid arteries, which are about 6mm in diameter. Lack of vasodilation is promoted by hemoglobin's affinity for nitric oxide (NO) in the blood. Since sickle cells are constantly undergoing cyclic deformation, they tend to lyse and spill their intracellular into the bloodstream. The free hemoglobin then is able to bind to NO, preventing it from binding to the endothelium and subsequent signaling for vasodilation[14]. While this accelerates the impairment of blood flow, the main contributor is arterial remodeling[18]. Particularly, intimal hyperplasia, or arterial wall thickening, reduces the cross-sectional area of the lumen, or open space for blood to flow through. This type of arterial remodeling indicated in sickle cell is seen in other diseases such as atherosclerosis, except instead of plaques being filled with fatty material, it is filled with excess extracellular matrix[18].

The major protease that is responsible for arterial remodeling is cathepsin K, a cysteine protease that is capable of cleaving collagen in its native conformation[19]. It is considered to be the most potent mammalian collagenase and a very strong elastase[20]. By degrading the elastin within arterial walls, the structural integrity of the artery is compromised, and a combination of smooth muscle cell proliferation and matrix deposition thickens the

intima layer of the wall leading to a decrease in lumen area[21]. Cathepsin K is known to upregulated in plaques seen in atherosclerosis, and since there are strong parallels between these two types of arterial remodeling, there is a strong suggestion that cathepsin K plays a role in stroke lesion development in sickle cell disease as well[22].

Currently, there is only one drug on the market for alleviating some of the symptoms of sickle cell disease - Hydroxyurea[23]. Still, this drug does not reduce the risk of stroke in these patients. Therefore, the purpose of this study is to understand the mechanistic relationships between the biological, biochemical, and biomechanical factors that regulate cathepsin K, which consequently regulates arterial remodeling. New insights gained from this deeper understanding may then be used to potentially discover new targets for drug therapies that will attenuate the risk of stroke in people with sickle cell disease.

Chapter 3

Materials and Methods

Animal treatment and drug trial

Sickle (SS), sickle trait (AS), and normal (AA) mice were bred using the offspring of an AS male and an AS female mouse. Three week old AA, AS, and SS mice are to be given 50mg/kg injections of the cathepsin inhibitor Odanacatib twice a week for 5 weeks. Mice are going to be euthanized with CO_2 asphyxiation, and the carotid arteries, aorta, and brain are going to be isolated.

Elastin staining on carotid arteries

The carotid arteries are going to be fixed in 4% paraformaldehyde and processed for paraffin sectioning. HistoGel-imbedded carotid arteries will be deparaffinized before staining. Elastin will be visualized using a modified Verhoeff elastic-van Gieson stain. Briefly, sections will be placed in a modified Verhoeff elastic for 7 minutes, and washed with warm water for 1 minute. Samples will then be resolved in 0.4% ferric chloride, rinsed again with warm water for 5 minutes, and counter-stained with Van Geisons solution for 60 seconds. Slides will be covered with a glass plate and imaged.

Immunohistochemistry on carotid arteries for cathepsin K

To visualize cathepsin K expression, deparaffinized sections of carotid arteries will be permeabilized with 0.2% Triton, washed three times with PBS, followed by antigen retrieval using 0.1% trypsin. Samples will be washed again three times with PBS and blocked with 2% bovine serum albumin (BSA) for 1 hour at room temperature. Cathepsin K will be visualized with a rabbit monoclonal anti-mouse primary antibody before tagging with AlexaFluorTM 488 secondary antibody and imaged.

Zymography and Western blots on Aortas for cathepsin K

Isolated and cleaned aortas will be grinded and lysed with a lysis buffer. The aorta lysates will be run through a modified SDS-PAGE protocol using a gelatin-based gel to quantify the activity of proteins, specifically cathepsin K. By using antibodies, specific proteins will be tagged for visualization with fluorescence through western blot. Examples of relevant protein are JNK, cathepsin K, and elastin.

Magnetic Resonance Imaging (MRI) of mice brains

The brains are going to be fixed in 4% paraformaldehyde. The fixed brains will embedded in 2% agarose and imaged using 9.4 Tesla magnet resonance imaging scanner to visualize stroke lesion formation.

Chapter 4

Results

Systemic inhibition of cathepsin K decreases elastin fragmentation in sickle transgenic mice.

Elastin staining showed that SS mice treated with odanacatib had reduced elastin fraying and fewer elastin breaks compared to SS mice treated with vehicle (Fig 1A and C). Elastin fragmentation of SS mice treated with odancatib was equivalent to AS mice treated with vehicle (Fig 1A and B). In addition, there was no effect of odanacatib on elastin fragmentation on AS mice because there was no difference in elastin breaks and fraying with the two groups (Fig 1B and D).
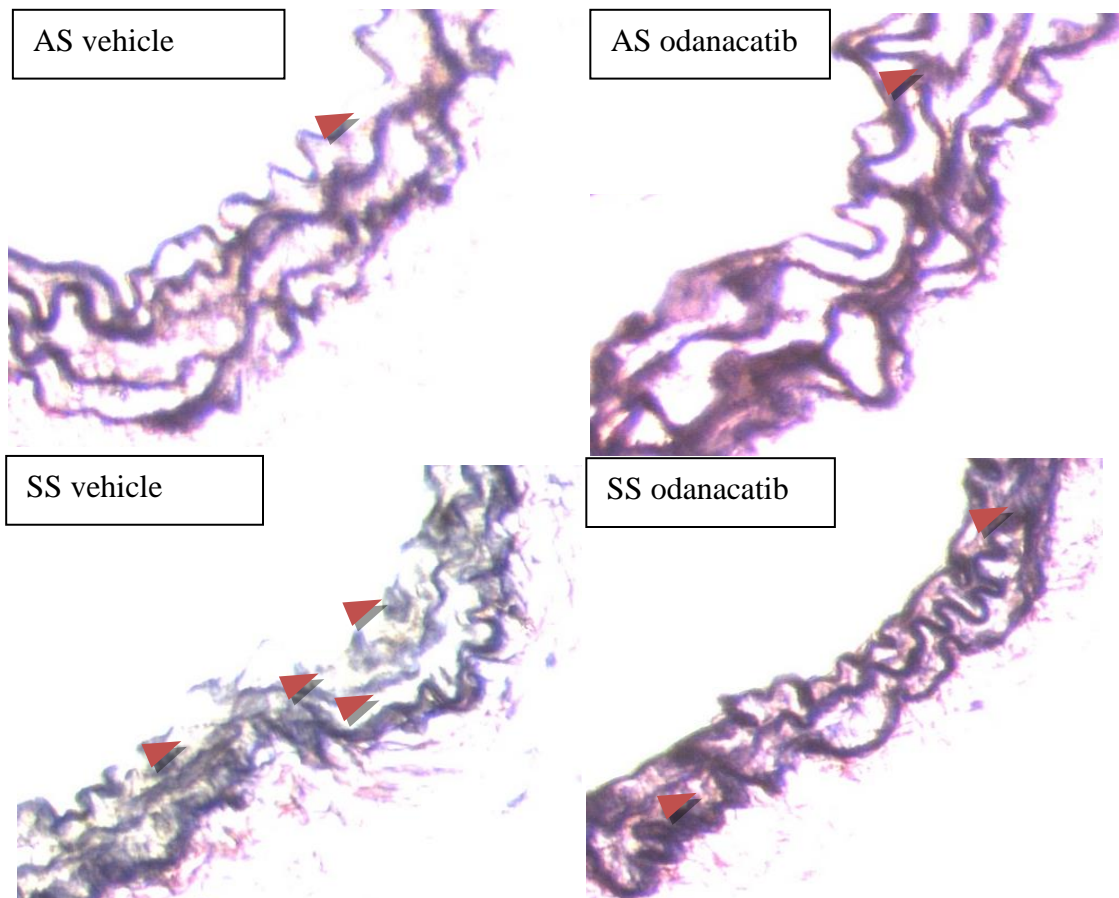

Figure 1 Odanacatib preserves elastin integrity in sickle transgenic mice. Carotid aortas were isolated from drug and placebo group animals, sectioned, and histologically stained for elastin morphology. Sickle transgenic animals receiving daily injections of odanacatib had substantially reduced incidence of elastin fragmentation compared to both trait and normal animals of sickle transgenic animals.

Systemic inhibition of cathepsin K activity has no effect on cathepsin K expression.

Immunohistochemical staining shows that odanacatib treatment has no effect on cathepsin K expression compared to vehicle in the carotid artery wall of sickle transgenic mice (Fig 2C and 2D). Contrarily, cathepsin K expression was not affected by odanacatib treatment in AS mice (Fig 2A and B). The reduction of cathepsin K expression by odanacatib on SS mice made the expression comparable to AS mice with and without odanacatib treatment (Fig 2A and C).
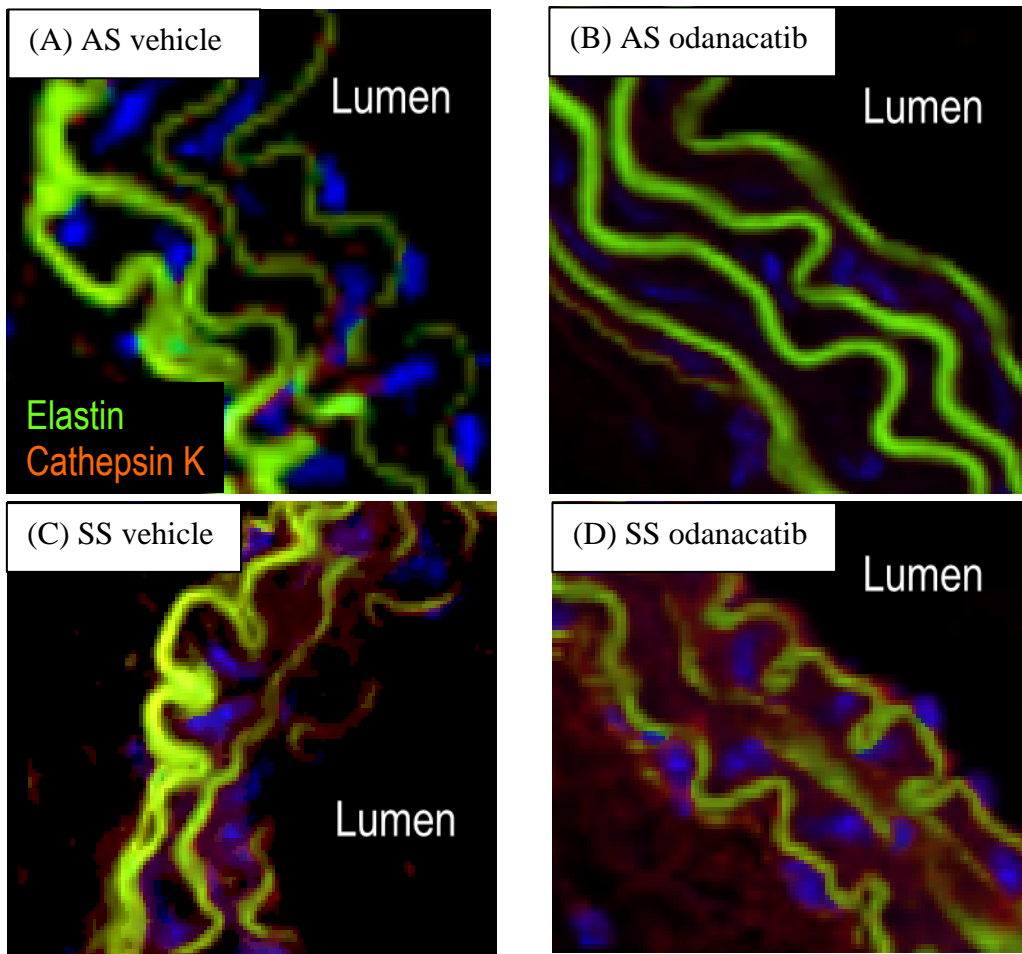

Figure 2 Odanacatib decreases cathepsin K expression in sickle transgenic mice. Carotid aortas were isolated from drug and placebo group animals, sectioned, and immunostained for cathepsin K protein. Sickle transgenic animals receiving daily injections of odanacatib had similar staining for cathepsin K, compared to the placebo group of sickle transgenic animals.

Chapter 4

Discussion

The present study evaluates the use of odanacatib for reduction of sickle cell-mediated arterial pathologies. It has been shown that elastin fragmentation in sickle transgenic mice can be reduced with treatment with odanacatib treatment. It is interesting to see that while elastin fragmentation is decreased, cathepsin K expression levels are the same, if not slightly increased, in sickle cell transgenic mice treated compared to untreated. The reason cathepsin K expression is not decreased is because odanacatib is an activity-based inhibitor, so even though the protease is present, it is not active. In order to confirm this hypothesis, a western blot and zymography will be done to measure the amount and activity of cathepsin K present quantitatively. The reason why cathepsin K expression is increased in odanacatib treated sickle transgenic mice may be that the endothelial cells may be trying to compensate for the lack of perceived cathepsin K activity by upregulated the expression. In order to confirm this hypothesis, PCR would need to be done to measure the amount of cathepsin K mRNA present. Further studies must also be done on the mice brains to test the hypothesis that odanacatib reduces the number of stroke lesions through MRI scans.

Chapter 5

Conclusion

This study shows that odanacatib is effective in reducing the amount of elastin fragmentation in the carotid arteries. By preserving the structural integrity of the carotid arteries, the risk for strokes is believed to be reduced. While the expression of cathepsin K is shown to be similar to the control, odanacatib is an activity-based inhibitor, so it is suggested that although the protease is present, it is in its inactive form. With further research and validation, odanacatib may prove to be a great therapeutic drug to reduce the risk of stroke in people with sickle cell disease.

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VITA

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