ABSTRACT

SAULS, DERRICK LAMONTE’ A Rabbit Model of Hyperhomocysteinemia: The Effect of Homocysteine on Blood Clot Structure and Stability. (Under the direction of Dr. Leon Carl Boyd and Dr. Jonathan Cutler Allen.)

Hyperhomocysteinemia (Hhcys) is a condition that several epidemiological studies have shown to be associated with atherosclerosis and thrombosis, due to an elevation of plasma homocysteine levels. Plasma homocysteine(hcys) levels have a tendency to rise with age and changes in nutrition. Hcys can affect coagulation proteins, altering the formation of blood clots. The mechanism(s) by which hcys might cause modification of coagulation proteins in vivo is not understood. My hypothesis is that adult and juvenile animals could respond differently to chronic administration of hcys, and elevated plasma levels of hcys might lead to modification of fibrinogen in vivo.

Methodology: Six month old (juvenile, n=6) and 12 month old (adult, n=6) New Zealand White rabbits were divided into control (n=3) and homocysteine-treated (hcys-trt) (n=3) groups and injected for seven weeks; afterwards, they were given a bolus injection of hcys. Blood was drawn to evaluate plasma clearance of hcys. At the end, rabbits were exsanguinated by cardiac puncture and blood was collected for coagulation studies. Results: Juvenile hcys-trt rabbits adapted to chronic administration of hcys, however, adult hcys-trt rabbits developed Hhcys. Adult hcys-trt rabbits had higher levels of malonaldehyde in liver tissue, which is evidence of oxidative stress. Juvenile hcys-trt rabbits had similar malonaldehyde levels as juvenile control rabbits. Plasma elimination of hcys was impaired in adult hcys-trt rabbits. Adult hcys-trt rabbits had increased fibrinogen levels, longer reptilase times, and shorter thrombin clotting times versus adult control rabbits. Clots formed from purified fibrinogen obtained from hcys-trt rabbits
lysed slower than comparable clots formed from control rabbits purified fibrinogen.

Some congenital dysfibrinogenemias have clots that are abnormally resistant to fibrinolysis due to alterations in fibrinogen structure, and lead to recurrent thrombosis. Clotting results for adult heys-trt rabbits suggest that hyperhomocysteinemia leads to a similar aquired dysfibrinogenemia. Therefore, the prolonged reptilase times and formation of clots that are abnormally resistant to fibrinolysis could directly contribute to increased risk of thrombosis in hyperhomocysteinemia.
A RABBIT MODEL OF HYPERHOMOCYSTEINEMIA: THE EFFECT OF HOMOCYSTEINE ON BLOOD CLOT STRUCTURE AND STABILITY

by

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APPROVED BY:

_________________________________  _______________________________________

Co-chair of Advisory Committee----Co-chair of Advisory Committee
DEDICATION

*I dedicate this dissertation to my mother Beatrice, who has inspired me academically from my toddler days; to my father William, whose philosophical wit echoes continually in my daily walk in life; to my grandmother Lola Bea Sauls, who has been a comfort in my life; to my wife Vera Mae Sauls, whose love has been helpful and reassuring in my professional student endeavors; to my children who have taught me that life can be so simple through the eyes of a child; to my father-in-law, Bishop Joe L. Smith whose friendship taught me the meaning of spiritual perseverance; to my Godfather, Dr. Augustus McIver Witherspoon, whose friendship guided me in my early college years; to Herbert H. Henderson, a lifelong friend who encouraged me to go back to college; to all family and friends, whose unconditional love has nurtured me; and last but not least, to God who has made it all possible by giving me life, health, and strength......*
BIOGRAPHY

Derrick Lamonte’ Sauls was born on January 29th, 1958 to father William Sauls and mother Beatrice Marie Sauls in Goldsboro, North Carolina. He lived in Goldsboro for the first four years of his life. At the age of four his parents moved to Boston, Massachusetts were he was enrolled in Public School. He attended the David A. Ellis Elementary School and attended the Thomas A. Edison Middle School. He spent two years at the Boston Technical High School and then transferred to Goldsboro High School, where he received his High School diploma in June 1975.

During the fall of 1975, he enrolled in North Carolina State University and upon completion obtained a Bachelor of Science degree in Zoology in 1981. In January of 1985, he enrolled in a Graduate School program at Northeastern University in Boston, Massachusetts, and upon completion he obtained a Master of Science degree in Health Science in 1990. After completing his degree at Northeastern University, he moved back to North Carolina and began employment at Duke University Medical Center, and has worked there for the past twelve years. For several years, he worked with Dr. Brice Weinberg in the area of HIV research. He has co-authored numerous research papers and abstracts, and has submitted for a clinical application patent for treatment of HIV. Under the mentorship of Dr. Maureane Hoffman, his research interests have evolved into the area of studying homocysteine and its effect on coagulation proteins.
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Introduction

Historical Perspective

There has been a considerable growth in interest in the role of homocysteine as a risk factor for atherosclerotic vascular disease – usually a condition of older adults. Ironically, a syndrome related to elevated levels of plasma homocysteine was first described in an eight year old child who died in 1933. This boy developed a stroke, accompanied by weakness, abnormal reflexes on the left side of the body, and an elevated blood pressure which led to his death. The autopsy results established that the cause of death was arteriosclerosis of the carotid artery with cerebral infarction (case 19471, NEJM 1933). The final diagnosis of this young boy’s condition didn’t occur till 32 years later, when a nine year old girl was admitted to Massachusetts General Hospital (1965) with symptoms of slow mental development, dislocated lenses of the eyes, and a ruddy flushed complexion because of dilated vessels. In studying her condition, it was noted that it was similar to several cases of children with elevated levels of homocysteine in the urine. This young girl was the niece of the boy who died in 1933 (case 19471). After further investigation McCully concluded that her uncle who died of a stroke must also have had homocystinuria (McCully, 1990). The young girl was found to have a mutation of the enzyme cystathionine beta synthase (CBS). CBS is a key enzyme in one of the three pathways of homocysteine metabolism. Deficiency of this enzyme leads to the massive accumulation of homocysteine in the plasma, with spillover into the urine.

Homocystinuria is characterized by mental retardation, tall stature, dislocation of the lenses of the eyes, ruddy flushed complexion, and light-colored hair. There is rapid growth of the long bones due to the excessive homocysteine leading to long legs, arms, fingers and toes. Sadly, many of the children with homocystinuria die from the development of blood clots in the
brain, heart or kidneys, resulting in heart attacks, stroke or kidney failure related to premature arteriosclerosis.

HOMOCYSTEINE METABOLISM:

Homocysteine is a non-protein amino acid that is primarily derived from dietary methionine. Methionine is an essential sulfur-containing amino acid. Homocysteine, under certain dietary conditions, can be utilized to replace methionine since the two are freely interconverted. Homocysteine and methionine are linked to a surprising number of intracellular metabolic pathways via their roles in one-carbon metabolism. There are three metabolic pathways that directly metabolize homocysteine. Any interference in these pathways can cause an increase in the plasma levels of homocysteine, which is called hyperhomocysteinemia (Hhcys).

![Homocysteine Metabolism Diagram](image)

Fig. 1 Pathways of Homocysteine metabolism from (Mangoni and Jackson, 2002)
The intake of methionine begins the process of metabolism. In the transmethylation pathway methionine is converted to adenosylmethionine by the addition of adenosine, catalyzed by the enzyme adenosylmethionine synthetase (also called methionine adenosyltransferase). Adenosylmethionine is an important cellular methyl donor, participating in a variety of methylation reactions. Loss of a methyl group converts adenosylmethionine to adenolsylhomocysteine. The enzyme adenolsylhomocysteinase then converts adenolsylhomocysteine back to homocysteine.

Homocysteine can be remethylated to methionine by two different enzymatic pathways. In the folate-dependent cycle methyltetrahydrofolate serves as the methyl donor. This reaction is catalyzed by methionine synthase and also requires vitamin B_{12} as a cofactor. The resulting tetrahydrofolate is remethylated using serine as a methyl donor to form methylene-tetrahydrofolate. The action of methylene tetrahydrofolate reductase (MTHFR) converts this back to methyltetrahydrofolate which can then methylate another molecule of homocysteine.

An alternative pathway utilizes choline as the methyl donor to remethylate homocysteine to methionine. Through choline this pathway links homocysteine to lipid metabolism.

Homocysteine can also undergo transsulfuration to produce cysteine, a metabolite that is needed for the synthesis of sulfur proteins. The transsulfuration pathway starts with an irreversible reaction condensing serine with homocysteine to form cystathionine. The is catalyzed by the enzyme cystathionine-Beta-synthase (CBS), which requires vitamin B_{6}. The cystathionine is then hydrolyzed to cysteine and 2-oxoglutarate by the enzyme gamma-cystathionase, which is also a vitamin B_{6} dependent enzyme. Cysteine can be used for protein synthesis or oxidized to taurine and inorganic sulfates.
The pathways of remethylation and transsulfuration are intricately controlled. The transsulfuration pathway is regulated by the concentration of S-adenosylmethionine (SAM), a substantial increase in SAM causes an increase in CBS activity, and this is important in getting rid of toxic levels of homocysteine. However, if there is a decrease in methionine the remethylation pathways are upregulated (MTHFR) to salvage homocysteine for remethylation (Finkelstein, 1990). Deficiencies of any of the vitamin cofactors involved in homocysteine metabolism can significantly affect the activities of the different metabolic pathways.

**Genetic Causes of Hyperhomocysteinemia:**

Homocystinuria was extensively studied in the 60’s. It was discovered in Belfast, Northern Ireland by Carson (Carson NAJ, 1962). It was later reported simultaneously by Gerritsen in Madison, Wisconsin, and George Spaeth, an ophthalmologist in Philadelphia (Gerritgsen T, 1962). Spaeth was able to correlate lens dislocation and levels of homocysteine in the urine of his patients. Carson and associates studied ten cases of children with homocystinuria and noticed that many of them had died from blood clots in the brain, heart and kidneys. Pathological findings also included fibrous plaques formations on the arteries and loss of artery elasticity (Carson et al., 1965). This disease was interesting due to the severe loss of elasticity and the thickening of the artery walls. These findings were verified in a study by Schimke and colleagues at John Hopkins Hospital in which many of their patients with homocysteinuria died with heart attacks and strokes (Schimke, 1965). After analyzing the pedigrees of the families involved, the cases were found to be genetically related. Further investigation by Mudd and colleagues showed that in cases of homocystinuria the liver was unable to dispose of homocysteine by the normal metabolic pathway due to a genetic error in the
liver enzyme cystathione beta synthase (Mudd, 1964). This malfunction of the transsulfuration pathway led to severely elevated levels of blood homocysteine, which can be 100 μM or higher.

A milder form of homocystinuria is caused by mutations that cause deficiency of methyltetrahydrofolate reductase (MTHFR) (Mudd, 1969). Subjects with MTHFR mutations are usually classified as mild-moderate hyperhomocysteinemia, because the plasma levels of homocysteine are usually greater than 15 μM but less than 50 μM.

In addition to overt deficiency of MTHFR, a common variant of MTHFR has been recognized in apparently healthy people. This is a C to T substitution at nucleotide 677 (C677-T) in the open reading frame of the MTHFR gene, thereby causing a substitution of valine for alanine in the protein. This gives rise to a thermolabile variant of the enzyme with decreased activity at body temperature (Jacques et al., 1996). The defect is an autosomal recessive mutation and individuals that are homozygous (T/T) also have low plasma folate with slightly higher plasma homocysteine values than subjects with the normal form of the enzyme (Gemmati et al., 1999). Recent studies have shown a variation in the frequency of the polymorphism among racial and ethnic groups; 10-13% T/T homozygous and 50% C/T heterozygous among Caucasian and Asian populations and a lower incidence of the T-allele in African Americans (Bailey and Gregory, 1999).

The enzyme methionine synthase is responsible for the conversion of homocysteine to methionine (methyl group exchange). Deficiency of this enzyme can also cause homocystinuria, however it is rare. The rare forms of homocystinuria are also associated with arterial vascular disease, similar to CBS deficiency (Kanwar et al., 1974).
Determination of Normal Levels of Plasma Homocysteine

While it is fairly easy to identify massive elevation of plasma homocysteine as being abnormal, it is not so straightforward to determine what constitutes a “normal” level of homocysteine. Several labs have tried to determine a reference range for normal individuals; however, the normal ranges varied widely in different populations because they are affected by a number of genetic and non-genetic determinants. The cutoff for hyperhomocysteinemia is usually, but arbitrarily, set at the 95th percentile of the homocysteine distribution in healthy subjects. In fasting subjects this corresponds to a total homocysteine level of approximately 15 uM. However, when a population is studied with an adequate vitamin intake the upper limit of the normal range may be as low as 12 uM (Ubbink, Becker et al., 1995). Normal levels of fasting plasma homocysteine are usually considered to be between 5 to 15 uM with a mean of 10 uM. An increase of plasma homocysteine of 2 uM over the established upper normal value would generally be classified as hyperhomocysteinemia (Ubbink et al., 1995; Rasmussen et al., 1996; Cramer, 1998). A range for the different levels of hyperhomocysteinemia were established with 16 to 30 uM being classified as moderate hyperhomocysteinemia, 31 to 100 uM is called intermediate hyperhomocysteinemia and greater than 100 uM is severe hyperhomocysteinemia (Kang et al., 1992).

The methionine load test has been used to help to improve the ability to distinguish between normal individuals and subjects with mild abnormalities of homocysteine metabolism (Selhub and Miller, 1992). Similar to a glucose tolerance test, a standard oral dose of methionine is administered and the plasma homocysteine levels are monitored. Abnormally high homocysteine levels following a dose of methionine indicate an abnormality of metabolism that might increase the risk of cardiovascular disease.
Non-genetic factors in Homocysteinemia

A number of non-genetic factors can affect the metabolism of homocysteine and cause moderate to intermediate hyperhomocysteinemia, with levels of plasma homocysteine in the range of 15 to 30 uM. Dietary factors most frequently associated with hyperhomocysteinemia include deficiencies of folate, vitamin B₁₂, or vitamin B₆ (Selhub et al., 1999). In some of the epidemiological studies of Hyperhomocysteinemia and vascular disease there was an inverse correlation between plasma levels of homocysteine and folic acid levels. Folic acid is intricately involved in the remethylation pathway. There are other vitamins involved in the metabolism of homocysteine, including vitamins B₆ and B₁₂. Folic Acid, and Vitamin B₁₂, regulate metabolic pathways that are catalyzed by MTHFR, and methionine synthase. The vitamin B₆ is a cofactor for CBS. Taking into consideration that all three vitamins could possibly be involved in controlling the elevation of plasma levels of homocysteine, Boushey and colleagues did a quantitative assessment of the interactions of vitamins and homocysteine levels (Boushey et al., 1995). They discovered that when they compared control subjects with no history of heart problems, to subjects with myocardial infarction, stroke, and peripheral disease, the blood homocysteine values of the cardiovascular disease subjects were higher than controls, and the vitamin B₁₂, B₆, and folic acid levels were lower than the controls. An earlier paper by Pancharuniti investigated the possibility of a interrelationship among plasma vitamin B₁₂, folate, and homocysteine levels, and development of coronary artery disease (CAD) (Pancharuniti et al., 1994). There was an inverse correlation of plasma homocysteine and folate and between plasma homocysteine and vitamin B₁₂ concentrations. The researchers concluded that there was an increased risk of CAD with the lower folate concentration, yet there wasn’t an increased risk of
CAD with the lower vitamin B\textsubscript{12} concentration, suggesting that in the population the folates levels are below where the homocysteine levels increase. This is important because it suggests that normal individuals can easily become sufficiently folate deficient to cause a rise in plasma homocysteine. However, it is much more unlikely that normal individuals will become sufficiently B\textsubscript{12} deficient to cause hyperhomocysteinemia. Fifty percent of the control population had a plasma folate concentration < 12.5 nM, compared to 6 percent of CAD patients. Thirteen percent of both CAD patients and control had plasma vitamin B\textsubscript{12} levels <225 pM (Pancharuniti et al., 1994). Verhoef found that patients who had a myocardial infarction were deficient in folate and vitamin B\textsubscript{6}, and there was an inverse correlation with blood homocysteine concentrations (Verhoef et al., 1996). There was no clear association of vitamin B\textsubscript{12} with myocardial infarction, and folate status was clearly the most important determinant of plasma homocysteine levels. The previous studies used methodologies to correlate vitamin status with plasma homocysteine levels and the risk of cardiovascular disease. The conclusion was that a decrease in folic acid, B\textsubscript{12} or B\textsubscript{6} could possibly increase the risk of vascular disease. A nutritional study by Appel, sought to determine whether one’s dietary habits could control or reduce plasma homocysteine levels (Appel et al., 2000). The experiment analyzed various dietary patterns and established a nutritional assessment that showed, by altering the intake of fruits, vegetables, dairy products, poultry, fish, sweets, and sugar containing beverages, the plasma homocysteine could be influenced. It was determined that, diet modifications can alter homocysteine levels, and there is an inverse association between serum folate levels and plasma homocysteine values. In contrast to folate, it would take a severe and prolonged deficiency of vitamin B\textsubscript{6} or vitamin B\textsubscript{12} to cause hyperhomocysteinemia. This is because the requirements for
these vitamins are lower than the requirement for folate and B₁₂ which can be stored in the liver (Brattstrom, 1996), whereas the body has no significant store of folate.

Out of the three vitamins involved in homocysteine metabolism, folic acid has clearly emerged as a major factor involved in determining plasma homocysteine levels. In one nutritional study, the addition of folic acid by supplements, folate rich foods, or folic acid fortified cereals has been explored as a way of increasing folic acid intake. The subjects were given a folic-acid fortified breakfast cereal (350-400 ug folate), a folic acid supplement (≥437ug), or folate-rich foods (≥600 ug/d). A control group was given canola oil. The human subjects were selected randomly, had to have homocysteine values ≥ 9 μM, and were separated to conform to a four day dietary regimen. Serum folate levels increased in every group relative to control, the fortified cereal and the supplements had serum folate values > 20nM and a > 20 percent reduction in plasma homocysteine. The subjects on folate-rich foods only had a serum folate value of 7 nM and 9 percent reduction in homocysteine. Supplements and cereals seem to be the most effective means to administer folic acid (Riddell, 2000). Malinow also discovered that the fortified folic acid cereal diet could decrease the homocysteine levels in blood, but the homocysteine levels began to increase again when the cereal was decreased or stopped (Malinow, 2000).

Homocysteine levels may also be influenced by drugs that interfere with the metabolism of folate (methotrexate, trimethoprim; and anticonvulsants); vitamin B₁₂ (nitrous oxide); or vitamin B₆ (theophylline) (Joosten et al., 1993; Ubbink et al., 1996; Smulders et al., 1999). Several diseases can contribute to elevated levels of plasma homocysteine, such as chronic renal failure due to the involvement of decreased metabolic functions in the kidney, diabetes mellitus due to impairment of amino acid metabolism in setting insulin resistance, and inflammatory
bowel disease where there is an insufficient uptake of nutrients needed for methionine metabolism (Bostom et al., 1995; Fenton and Rosenberg, 1995; Munshi et al., 1996). Smoking, lack of exercise, hypertension, obesity, and alcohol use have been implicated in the Hordaland homocysteine study as factors that could cause an increase in plasma levels of homocysteine (Nygard et al., 1995).

Hormonal status may also affect plasma homocysteine levels. One study outlined a difference in levels of pre and post menopausal women, which showed women had lower levels of plasma homocysteine before menopause and the levels increase 1 to 2 uM after menopause. Giles (1999), found that young females (15-29 years of age) had plasma homocysteine levels of 7.1 uM and older females (30-39 years of age) had homocysteine levels of 11.6 uM (Giles, 1999). However, before menopause, the women’s overall homocysteine values were still lower than men but gradually increased when women reached their 50’s and 60’s to levels comparable to men. This implies that hormones are involved in controlling homocysteine metabolism (Nygard et al., 1995).

It has become evident that there is also an increase of the plasma homocysteine levels as one ages, and there is a significant difference in values when children are compared to adults. Dietary habits and lifestyles could cause one’s plasma homocysteine levels to elevate over the years, so it is important to do nutritional assessment and correlations of lifestyle activities as people age (Bates, 2002). One also has to consider the excess methionine in an animal protein diet and the resulting acute hyperhomocysteinemia, which may be caused by saturation of homocysteine catabolism. In some people this condition may be prolonged and could lead to chronic hyperhomocysteinemia. This could increase the concentration of adenosylmethionine, triggering an increase of CBS activity, thus increasing homocysteine catabolism which helps rid
the system of excess sulfur amino acids (Ueland and Refsum, 1989). Overall, there are many environmental factors that could influence plasma homocysteine levels, and it is possible that these factors could be responsible for the increase in plasma homocysteine levels noted with age.

**The Role of Homocysteine in Cardiovascular disease**

Hyperhomocysteinemia has been implicated as a risk factor for ischemic heart disease (IHD), stroke, and peripheral artery disease, with the involvement of vascular pathology leading to atherosclerosis or thrombosis (McCully, 1990). There is a correlation of increased coronary risk with homocysteine levels greater than 15 uM. These subjects have an adjusted relative risk 1.4 times higher than subjects with homocysteine levels below 10 uM (Boushey, Beresford et al., 1995). A follow-up study by Graham using control subjects comparing them with patients with vascular disease determined that patients with plasma homocysteine levels above 12 uM had double the risk for cardiovascular disease (Graham, 1997). Nehler conducted a review of several studies, examining plasma homocysteine levels as an indicator of risk for arterial disease. He found that the mean plasma homocysteine values were 25-40% higher in patients with symptomatic atherosclerotic disease compared with controls (Nehler, 1997). Wilcken showed that patients with vascular-disease were predisposed to having elevated plasma levels of homocysteine (Wilcken, 1976). He used a methionine-load test, this test measures homocysteine before and after the intake of 100 mg/ kg body weight of methionine and multiple sampling is done to monitor the plasma homocysteine. It was concluded that patients with vascular-disease reached higher plasma homocysteine levels than controls, therefore demonstrating abnormal homocysteine metabolism. Wald and colleagues did an analysis of several studies on homocysteine and the risk of ischemic heart disease and compared them to their study. They
found a dose-response relationship between serum homocysteine and the odds ratio of ischemic heart disease (Wald, 1998).

Hyperhomocysteinemia has also been implicated in the risk of arterial and venous thromboembolism (Boers, 1997). Hyperhomocysteinemia was established as being associated with both arterial and venous premature thromboembolic events in subjects less than 45 years old and in patients with arterial occlusive disease. The risk of these events was twice as high when the methionine load test was used for analysis (Fermo, 1995; D'Angelo, 1997). Petri found that plasma homocysteine concentrations higher than 14.1 μM were significantly associated with arterial thrombotic events with an odds ratio of 3.74, and stroke with an odds ratio of 2.24. Hyperhomocysteinemia remained an independent risk factor after taking into consideration all other risk factors (Petri, 1996).

Thus, patients with severe hyperhomocysteinemia/homocystinuria clearly have a very high risk of premature atherosclerotic vascular disease. In addition, epidemiologic studies suggest that much less severe homocysteinemia related to non-genetic factors is an important risk factor for thrombosis and atherosclerosis in the general population. However, it is not yet clear whether hyperhomocysteinemia actually causes cardiovascular disease. Yet, with the prevalence of elevated baseline levels of homocysteine in the general population, there seems to be a substantial risk for cardiovascular disease. If homocysteine directly causes vascular disease, there is also substantial potential to decrease the risk by cheap and easy dietary manipulations.

**Cellular and Biochemical effects of Homocysteine**

It is clear that homocysteine levels correlate with cardiovascular risk, but it is not clear if homocysteine actually causes cardiovascular disease. Several studies have investigated the
potential pathogenic effects of hyperhomocysteinemia in animal models. In his pursuit to understand the effects of homocysteine, McCully chronically elevated the plasma homocysteine levels of rabbits, which then showed signs of atherosclerotic development (McCully, 1970). He did a follow-up study, which entailed the study of juvenile and adult animals. The younger animals utilized the homocysteine for growth and never developed increased plasma levels of homocysteine. However, the adults had elevated plasma levels of homocysteine (McCully, 1975). Harker used primates to show that an infusion of L-homocysteine could cause vascular changes, including segments of endothelial cell loss and fibromusculoelastic lesions, similar to atherosclerosis (Harker, 1974).

Many studies have examined the effects of homocysteine on vascular cell functions and suggested that these effects might promote the development of atherosclerosis. Tsai et al (1994), examined the effect of homocysteine on the growth of both vascular smooth muscle cells and endothelial cells in vitro. They showed a 25 percent increase in DNA synthesis in the vascular (rat aortic) smooth muscle cells treated with 0.1 mM homocysteine, and a homocysteine dose dependent inhibitory effect on DNA synthesis in endothelial cell cultures (Tsai, 1994). Homocysteine has been reported to have a number of effects on endothelial cell function, including impaired Nitric Oxide production in vitro and in vivo, activation of factor V, induction of tissue factor, reduced activation of protein C, impaired expression of thrombomodulin, decreased expression of heparin sulfate, inhibition of prostacyclin synthesis, increased synthesis of thromboxane in vivo, reduced t-PA binding to endothelial cells, and derivitization of t-PA receptor, annexin II. These effects could contribute to the development of atherosclerotic vascular disease and arterial and venous thromboembolism (Hajjar and Acharya, 2000).
Homocysteine becomes chemically reactive when it is converted to homocysteine thiolactone (homocysteine-thiolactone), which contains a reactive intramolecular thioester (McCully, 1976).

**Protein + Homocysteine-Thiolactone**

![Diagram of Protein Homocysteinylation](image)

**Homocystamidc Protein Adduct**

**Fig. 2 Protein Homocysteinylration,** *(from Jakubowski, 1997)*

Homocysteine thiolactone is synthesized by methionyl-tRNA synthetase in an error-editing reaction for the prevention of the translational incorporation of Homocysteine into proteins (Jakubowski, 1981). The homocysteine-thiolactone undergoes two major reactions: one involves protein homocysteinylation where asparagine, glutamine, arginine, and lysine residues become acylated at the free amino groups. The other reaction is the enzymatic hydrolysis of homocysteine-thiolactone to homocysteine by a calcium dependent enzyme (homocysteine thiolactonase), a component of HDL. Detection of homocysteine-thiolactone in blood and cells is unlikely (Mudd et al., 1989) because it is rapidly eliminated, and its half-life is 1 hour or less (Jakubowski, 2000). Several studies have investigated the reaction of homocysteine-thiolactone
with proteins. Vidal (1986) highlighted the possible toxic effects of homocysteine-thiolactone, when the thiol group is introduced into low density lipoprotein by reacting with free amino groups of apoB protein, conceivably promoting lipid peroxidation. A polyclonal antibody was generated in rabbits to detect homocysteine-thiolactone-LDL adduct, and the specific binding at apoB lysyl residues. The antibody was able to detect not only the homocystamide-LDL adduct, but homocystamide-lysyl residues of other plasma proteins (Ferguson, 1998). This method of detection is important because of the implication of damage caused by homocysteine thiolactone in promoting atherosclerotic plaque formation in animals. Jakubowski explored the reaction of homocysteine thiolactone with proteins in human serum by using a radio-labeled Homocysteine-thiolactone and incubating homocysteine thiolactone in serum for two hours. The proteins in the serum were all labeled and the intensity of the protein bands on the gel were proportional to their abundance in serum. Under physiological conditions the homocysteinylation was complete in a few hours and the major determinant of the reactivity of the protein was dependent on their lysine content (Jakubowski, 1999). Using HUVEC cells, Jakubowski identified that homocysteine incorporation into proteins and homocysteine-thiolactone formation are dependent on extracellular homocysteine, folic acid and HDL (Jakubowski, 2000). Thus, while there is currently no evidence that homocysteinylation occurs in vivo, it represents one biochemical mechanism by which homocysteine could modify critical proteins and lead to vascular dysfunction or thrombosis.
Conclusion:

It was first recognized that patients with inborn errors of metabolism that led to a massive elevation of homocysteine in the plasma and urine suffered premature atherosclerosis. However, in the general population, modest elevations of plasma homocysteine levels have also been correlated in epidemiological studies with cardiovascular disease, including atherosclerosis, myocardial infarction, stroke, and venous thrombosis (Hankey, 1999). Approximately 5 to 10% of the general population, and 30 to 40% of the elderly population have increased plasma levels of homocysteine (Lentz, 1997). Thus, elevated plasma homocysteine could represent a major, modifiable risk factor for cardiovascular disease in the general population. It has been proposed that homocysteine could promote development of cardiovascular disease by altering endothelial function, including promoting oxidative injury (Welch, 1997), inhibiting S-adenosylmethionine-mediated methylation (Fu, 2000), or by direct toxicity to endothelial cells (Lee, 1999). Other proposed effects have involved the enhanced lipid uptake by monocytes in atherosclerotic plaques (de Vries, 1999) and the inhibition of binding of fibrinolytic enzymes to endothelial receptors (Hajjar, 1998). Some in vivo studies have demonstrated endothelial dysfunction in animal models of hyperhomocysteinemia (Lentz, 1996; Durand, 1997). Several in vitro studies have shown a direct correlation of the toxic effects of homocysteine and its metabolites on cells (McCully, 1993; Jakubowski, 1997). However, it is hard to determine whether or not the observations of the in vitro effect of homocysteine on cells has relevance to the in vivo effects of homocysteine. This is mainly due to the administration of non-physiologically high levels of homocysteine in vitro in order to see pathophysiologic changes in vascular cells. Despite the convincing evidence for a correlation between homocysteine and cardiovascular risk, it is not
clear whether homocysteine is a major contributing factor or risk factor to the cause of cardiovascular pathology or is a secondary mechanism.

While numerous studies have focused on the potential effects of homocysteine on cells, there hasn’t been an extensive study of the effect of homocysteine on coagulation proteins. Homocysteine could modify plasma proteins by several mechanisms: 1) homocysteine and other molecules with a free sulfhydryl group can modify proteins by forming a disulfide bond with cysteine residues; 2) homocysteine can promote oxidation of proteins, potentially leading to fragmentation and rearrangement processes involving amino acid side chains; and 3) homocysteine can be metabolized to its cyclic thioester, homocysteine thiolactone, which can react with primary amines in amino acid residues, especially lysine (Leanza, 1992; Olszewski, 1993). Each of these proposed mechanisms is non-enzymatic and the extent of protein modification should be a direct function of the level of the relevant homocysteine metabolite, which should increase as the plasma homocysteine level increases. Overall modification of the protein would be cumulative, increasing over the lifetime of a protein in vivo.

Thus, it is plausible that homocysteine or its metabolites might react with coagulation proteins and affect their functions. Therefore, it is important to study the effects of homocysteine on fibrinogen, a coagulation protein involved in blood clot formation. It has a relatively long half-life (5 days) in the plasma and it also has many cysteine and lysine groups that could be targets of homocysteine modification. Any structural modification of fibrinogen could potentially alter the resulting fibrin clot structure and enhance its resistance to fibrinolysis. In using a rabbit model of hyperhomocysteinemia, the plasma fibrinogen will be exposed to chronically elevated levels of homocysteine and there may be modifications to the fibrinogen.
Therefore, my hypothesis is that elevated plasma levels of homocysteine could have an effect on blood clot structure and stability.
References:

Differences in the Metabolic Response to Exogenous Homocysteine in Juvenile and Adult Rabbits
Abstract

Homocysteine has recently received a lot of attention as an independent risk factor for atherosclerotic and thrombotic cardiovascular disease. Plasma homocysteine levels tend to rise with age, but are also greatly influenced by nutritional factors. Early reports suggested that there were differences in the metabolism of homocysteine in adult and immature animals. The current work tests the hypothesis that adult and juvenile animals respond differently to chronic administration of homocysteine. We have previously found that adult rabbits given homocysteine parenterally twice daily for seven weeks developed progressive folate deficiency and concurrently developed an impairment of homocysteine metabolism. We now report that juvenile rabbits do not develop folate deficiency with chronic homocysteine loading and do not have progressively higher trough levels of homocysteine, as do the adults. In addition, juvenile rabbits that have been chronically pre-treated with homocysteine exhibit a lower peak homocysteine level after a single dose than do juvenile rabbits that have never received homocysteine. This adaptation did not occur in the adult rabbits. In addition, adult homocysteine-treated rabbits had evidence of oxidative stress as evidenced by higher levels of malondialdehyde in liver tissue than adult controls. The homocysteine-treated juvenile rabbits had the same levels of malondialdehyde as the juvenile control rabbits. We conclude that the plasma elimination kinetics are altered in juvenile rabbits in response to homocysteine pre-treatment. The difference in metabolism of homocysteine may protect the juvenile rabbits from the damaging effects of homocysteine. Future studies are planned to elucidate the mechanism of this adaptive response.
**Introduction**

Elevated plasma homocysteine is now recognized as an important risk factor for cardiovascular disease. Homocysteine is not normally present in the diet, but is produced as a normal metabolite of methionine. The metabolism of homocysteine is complex [1]. The metabolic cycle that interconverts homocysteine and methionine also produces S-adenosylmethionine (SAM), a key intermediate in one-carbon metabolism. Homocysteine is converted to methionine by addition of a methyl group, a process called trans-methylation. The methyl group can come from 5-methyltetrahydrofolate in a reaction that depends on the availability of the vitamins folate and B₁₂. Alternatively, betaine can serve as the methyl donor for formation of methionine from homocysteine. Betaine is a metabolite of choline, an intermediate in lipid metabolism. Homocysteine can be removed from the methionine cycle when it is converted to cystathione by the trans-sulfuration pathway. The key enzyme in this pathway is cystathione \( 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>100μM and is classified as severe hyperhomocysteinemia. Normal levels are considered to be <16 μM [5]. Recent works suggest that even within the “normal” range those individuals with higher levels of homocysteine are at greater risk of cardiovascular disease and thrombosis [6].

It has been suggested that fasting levels of homocysteine do not always give a good indication of cardiovascular risk [7, 8]. Some individuals have “normal” levels of homocysteine in the fasting state, but develop abnormally high plasma levels of homocysteine following ingestion of a methionine load. These individuals can be identified with a methionine loading test (MLT). Graham et al. [9] studied patients with arterial occlusive disease (AOD) and compared them with controls. Patients with fasting homocysteine levels in the top fifth compared with the bottom four fifths of the population had a relative risk of AOD of 2.2. An additional 27% of AOD patients had normal fasting levels of homocysteine, but were identified as having abnormal methionine/homocysteine metabolism on the MLT. Thus, it appears that individuals with an increased risk of cardiovascular disease have an impaired ability to metabolize homocysteine formed from dietary methionine that is often, but not always, manifest as an elevated fasting level of homocysteine.

The non-genetic factors that influence homocysteine levels are related to nutritional status, gender and age, as well as drugs, toxins and renal function (reviewed in [10]). Elevated plasma homocysteine can result from deficiencies of vitamins B₁₂, B₆ and especially folate. In fact, one study showed that folate status is the major determinant of fasting plasma homocysteine levels in human subjects [11]. In addition to the levels of B-vitamins in the diet, the composition of dietary protein can influence homocysteine levels. Animal proteins contain three times the amount of methionine as plant proteins and the ingestion of a methionine load results in a
temporary increase in plasma homocysteine levels. Thus, a consistently high intake of animal proteins could potentially lead to a chronic elevation of plasma homocysteine.

Clinical and epidemiological studies have also correlated plasma homocysteine levels with gender and age [12-14]. The blood levels of homocysteine in premenopausal women averaged 2uM less than those in men. With the onset of menopause women’s homocysteine levels begin to increase. Men’s levels progressively increase with age from puberty. Children had significantly lower levels of homocysteine than adults. In spite of these descriptive studies, the mechanisms linking homocysteine levels to age and gender are not understood.

There is some evidence that the relative activities of the different pathways involved in homocysteine metabolism differ in adult and juvenile animals. McCully demonstrated in guinea pigs and rats that the metabolism of homocysteine differs between adult and young animals [15]. The young animals were able to utilize exogenously administered homocysteine for growth, and never developed elevated plasma levels of homocysteine. However administration of exogenous homocysteine to adult animals resulted in an elevation of their plasma homocysteine levels. Studies of juvenile animals fed a methionine-deficient diet showed a decrease in growth rate and premature death, but homocysteine administration could ameliorate the effects of methionine deficiency in growing animals.

We hypothesized that juvenile animals might adapt better to an exogenous homocysteine load than would adult animals. We compared the rate of homocysteine clearance in juvenile and adult rabbits. Half of the animals in each group were injected twice daily with homocysteine for a 7 week period, while the remainder were injected with buffer alone. After this pre-treatment period, the clearance of a single injection of homocysteine was monitored. The plasma levels achieved and rate of clearance of homocysteine were the same in adult and juvenile control
rabbits who were injected with an equal dose of homocysteine on a weight basis. Adult rabbits preconditioned by injections of homocysteine became folate deficient over the course of the pre-treatment period and exhibited a slower clearance of exogenous homocysteine than controls, as we have recently reported [16]. By contrast, juvenile rabbits that had been pre-treated with homocysteine did not become folate deficient and did not develop chronically elevated levels of homocysteine, similar to the results of McCully in other species [15]. The peak plasma level achieved after a dose of homocysteine at the end of the pre-treatment period was dramatically lower than that reached in controls or adult pre-treated animals. Thus, we suggest that juvenile animals can modify their metabolic patterns to cope with or utilize a homocysteine load more effectively than can mature animals. If this adaptive or compensatory mechanism can be understood, it could lead to better management of human patients with elevated levels of plasma homocysteine or abnormal responses to a methionine load.
**Methods and materials**

**Animal Model:**

We used a rabbit model of hyperhomocysteinemia, similar to the protocol used by McCully in his experiments on homocysteine-induced atherosclerosis [17]. Six month old (juvenile, n=6) and 12 month old (adult, n=6) New Zealand White rabbits were obtained from a breeding facility (PSI Robinson Services, Clemmons, NC). Treatment of the rabbits was conducted in accordance with an Animal Research protocol approved by IACUC. Their diet consisted of Prolab Rabbit 5P26 chow *ad libitum* and water (Purina Mills, St. Louis, MO.). An indwelling injection port (Vascular Access Ports, Access technologies, Skokie, IL.) was surgically placed under the skin, attached to the muscle layer and the catheter was inserted into the peritoneal cavity of each animal.

Control rabbits received injections of the diluent (5% dextrose/water) and experimental rabbits received 30mg/kg DL-homocysteine (Sigma, St. Louis, MO) in 5% dextrose/water every twelve hours through an indwelling injection port into the peritoneal cavity. The homocysteine solution was prepared fresh and sterile filtered before each dose. Blood was drawn every two weeks to monitor the homocysteine levels and other blood chemistries.

After seven weeks of the pre-treatment protocol the trough plasma levels of homocysteine became progressively elevated in the homocysteine-treated adult rabbits as we have previously shown [16]. By contrast, the plasma levels of homocysteine did not become elevated in the juvenile rabbits – the trough levels of homocysteine returned back to baseline within the 12-hour period between doses of homocysteine (Table 1).
At the end of the pre-treatment period the clearance of a single 30mg/kg i.p. dose of homocysteine was monitored in the four groups of rabbits. Blood samples were drawn into buffered 3.8% citrate anticoagulant before administration of the homocysteine dose and at 1, 2, 4, 6, 8 and 10 hours after the injection of homocysteine through a 23-gauge catheter in the ear artery. Each sample was immediately spun at 2000 rpm for twenty minutes, and the plasma frozen at \(-80^\circ\)C until analysis.

The plasma homocysteine levels were plotted and fitted to the equation \(C = C_0e^{-kt}\) using Excel (Microsoft Corp). Where \(C\) is the concentration at any given time, \(C_0\) is the concentration at the first time point after dosing, \(k\) is the rate constant for elimination and \(t\) is the time in hours after dosing. Our earliest plasma level was taken one hour after dosing and thus the elimination curve does not reflect the early rapid redistribution phase of the clearance curve. Similar to what has been observed in human subjects [18], the elimination kinetics of plasma homocysteine were first-order (fit the exponential model) over a six hour period. The differences between levels achieved at various times after dosing were tested for statistical significance using a t-test corrected for multiple comparisons. The plasma homocysteine levels and rate of elimination were no different in the control juvenile and adult animals, and those data were combined in the analysis and graphing.

At the end of the clearance study, the rabbits were anesthetized, exsanguinated by cardiac puncture and the blood from each rabbit was collected into a syringe containing buffered citrate. The plasma was immediately aliquotted and stored at \(-80^\circ\)C until assayed. Tissue samples were frozen immediately in liquid nitrogen and stored at \(-80^\circ\)C until analysis.
Blood Chemistries:

Total homocysteine assays were performed in the Clinical Chemistry Laboratory of UNC Hospital (Chapel Hill, NC), using the IMX system (Abbott Labs, Oslo, Norway). Assays of aspartate amino transferase (AST), alanine amino transferase (ALT), calcium, glucose, Vitamin B12, and folate levels were performed in the Clinical Laboratory, Durham VA Medical Center (Durham, NC)

Thiobarbituric Acid Reactive Substances (TBARS) Assay:

The presence of the lipid peroxidation product malondialdehyde was assayed in lipids from liver samples as described [19]. The livers were extracted as described below, and one ml of lipid was put into a preweighed 13x100 screw top tube. 100ul of 0.28M Trichloroacetic acid was added and the tube was vortexed and spun at 4000g for fifteen minutes; 0.3ml of the upper phase was added to a clean 13x100 tube. To this sample was added 1.5ml Glycine HCl, 1.5ml 0.04M Thiobarbituric acid (TBA), and 1.7ml distilled water. The tube was then placed in boiling water for fifteen minutes. The sample was cooled and 1ml of top pink layer was put into a glass cuvette and the absorbance read at 532nm. The assay was standardized by using 1,1,3,3-tetraethoxypropane (TEP) as a standard.

Glutathione Assay:

Total glutathione levels were assayed by using a commercial assay kit (BIOXYTECH GSH 400, R&D Systems, Minneapolis, MN). Red Blood Cells (RBC) were extracted with two volumes of cold metaphosphoric acid and spun at 2000g for thirty minutes. The supernatant was removed and assayed for glutathione content. The liver samples were cut in small pieces, added to cold metaphosphoric acid (2.6mg/1.69mls) and disrupted by sonication. The samples were
then centrifuged at 3000g at 4^0 C for ten minutes and the supernatants used for the assay. The results were compared to a standard curve prepared from a fresh 0.5mM glutathione solution.

*Lipid extraction/Fatty Acid Analysis:*

Fatty Acids were extracted from 1 gram samples of frozen liver by using one ml of BHT chloroform and 9 ml methanol, followed by the addition of 10 ml of chloroform [20]. Tissue residue was removed, the upper phase was aspirated and discarded, lower phase was dried down and lipid weight determined. The lipid samples were subjected to separation over a silica column into non-polar and polar fractions. Further separation using 2% acetic acid in ethyl ether (PE, 98:2, HOAc) yielded the fatty acids from the non-polar fractions. Fatty acids were derivatized using a BF3/Methanol reagent, boiled in water for 30 minutes, dried down and re-dissolved in 200 ul of Iso-Octane. Analysis was done by gas-liquid chromatography as described [21].
Results

Blood Chemistries:

The plasma levels of several analytes were measured after eight weeks of homocysteine treatment (Table 1.1). Blood was drawn for measurement of homocysteine levels immediately before injection of the next dose, and thus represents the trough level. The levels of homocysteine were similar in the control and homocysteine-treated groups in the juvenile rabbits. The adult rabbits had significantly higher trough levels of homocysteine in the HCys (29.8 ± 9.9 uM) compared to the control (17.4 ± 1.7 uM) group (Table 1.1). By contrast, the trough levels of homocysteine were not different in the HCys and control groups of juvenile rabbits, and were not different from the control adult rabbits (Table 1).

AST, ALT, calcium, glucose, and Vitamin B12 levels were similar between the control and HCys groups of both the adult and juvenile rabbits. This is evidence that there is no damage to the hepatic cells.

After eight weeks of homocysteine administration the total plasma folate levels were significantly lower in the adult HCys group than the adult control group (16.2 ng/ml ± 3.7 versus 24.8ng/ml ± 5.9) in the adult rabbits (Table 1). However, the total plasma folate levels were not significantly different in the HCys and control groups of the juvenile rabbits. While not statistically significant, there was a tendency for the homocysteine-injected juvenile rabbits to have higher folate levels than the controls. We speculate that the depletion of folate in the adult HCys rabbits led to an impairment of homocysteine metabolism, thereby leading to the elevation of the plasma homocysteine levels.
None of the other blood chemistries differed significantly between the treatment groups as shown in Table 1.1.

**Homocysteine Clearance:**

The plasma clearance of a single dose of homocysteine was compared in rabbits that had been chronically administered homocysteine and those that had not previously received homocysteine. The results are shown in figure 1. While the pre-dose level of homocysteine was higher in the adult HCys rabbits than in the controls, the peak level after dosing was not as high as in the controls. This finding suggests that there is some degree of adaptation induced by chronic homocysteine exposure. However, the plasma homocysteine levels did not return to baseline as rapidly in the adult HCys rabbits as in the controls and the elimination rate constant (k) was 0.281 in the controls and 0.178 in the adult HCys animals. The elimination half life was 2.47 hours in the controls and 3.89 hours in the adult HCys animals. This is consistent with the significant degree of folate deficiency that developed in the HCys rabbits over the course of the pre-treatment period. Folate deficiency would be expected to impair homocysteine metabolism by transmethylation to methionine.

Our findings were quite different in the juvenile rabbits. In the juvenile rabbits there was a much greater difference in the peak level of homocysteine achieved in the HCys and control rabbits. Because of the lower initial recovery of the dose in the plasma, the homocysteine level returned to baseline much more quickly in the HCys pre-treated juveniles than in the juvenile controls. However, the elimination rate constants and elimination half lives were not significantly different in the juveniles Hcys rabbits (0.266 and 2.61 hours) and juvenile control rabbits (0.281 and 2.47 hours).
**Lipid Peroxides:**

Homocysteine has been hypothesized to cause cellular dysfunction contributing to the development of atherosclerosis by promoting oxidative stress [22]. Therefore, we hypothesized that administration of homocysteine would promote oxidation of polyunsaturated fatty acids to their lipid hydroperoxides. These hydroperoxides break down to form malondialdehyde, which is measured as thiobarbituratic acid-reactive substances (TBARS) and is used as an indicator of oxidation *in vivo*.

The amount of oxidation products in the liver extracts of the adult HCys rabbits was significantly higher than the adult control rabbits (table 1.2). However, the levels of lipid oxidation products in juvenile HCys rabbits were comparable to the juvenile control rabbits (table 1.2). The lack of increase of lipid peroxidation products in the juvenile HCys rabbits seems to reflect an adaptation to homocysteine treatment that could be related to age.

**Fatty Acid analysis:**

It is possible that homocysteine treatment might affect lipid metabolism, because one important pathway of homocysteine metabolism is transmethylation by betaine homocysteine methyl transferase. Betaine is derived from choline, a major precursor of the fatty acid-containing lipids, phosphatidylcholines. The fatty acid compositions of rabbit livers reflected the dietary fatty acid pattern, with the most abundant polyunsaturated fatty acid being linoleic acid.
No significant differences in the fatty acid composition of livers from HCys and control rabbits of either age group were found (data not shown).

**Glutathione analysis:**

Glutathione is both a potential metabolite of homocysteine (through the cystathione synthase pathway) and an important intracellular antioxidant. Therefore, we expected its concentration to be altered by homocysteine treatment. Total glutathione content in liver from juvenile homocysteine-treated rabbits was significantly higher than controls (42 ± 3 vs 36 ± 5 nmol/mg protein, p<0.05). By contrast, liver glutathione levels were comparable in control and homocysteine-treated adult rabbits (49 ± 7 versus 52 ± 13 nmol/mg protein) (Table 1.3).
Discussion

The elevation of plasma levels of homocysteine has been associated with an increased risk of atherosclerosis and thromboembolism. Therefore, it is of interest to evaluate the factors that play a role in determining the plasma homocysteine level. This is not a straightforward problem, since homocysteine is involved in several metabolic cycles. In addition, there is a continual flux of homocysteine between the tissues and plasma. It is not known whether the pool of homocysteine that is most directly related to cardiovascular disease is intravascular or intracellular. However, the plasma level of homocysteine is at least a reflection of the pathophysiologically important pool.

In our study, we examined the plasma homocysteine levels achieved in response to a dose of homocysteine in adult and juvenile rabbits. A subset of each age group was chronically administered homocysteine. After seven weeks of injections we compared the clearance of a single test dose of homocysteine. We found two major differences in the elimination of homocysteine from plasma in the adult and juvenile rabbits.

First, the homocysteine-treated adults developed persistent elevation of plasma homocysteine levels over the course of the study as we have described [16]. Early in the study period each homocysteine-injected animal had cleared a dose of homocysteine before the subsequent dose was administered 12 hours later. By the end of the study, the adult animals no longer completely cleared each dose of homocysteine by the end of the 12-hour dosing interval and the elimination half-life was prolonged compared to controls. By contrast, the juvenile homocysteine-treated animals continued to clear each dose of homocysteine within a 12-hour period throughout the entire pre-treatment period. The defect in homocysteine metabolism in the adult animals was associated with the progressive development of folate deficiency, even though
the animals were fed a standard folate-replete diet. This is a novel observation, since we are not aware of previous reports suggesting that homocysteine loading can cause folate deficiency. In contrast to the adult animals, the juvenile rabbits did not become folate deficient in the face of chronic homocysteine administration.

We do not know why homocysteine administration led to folate deficiency in the adult, but not the juvenile rabbits. It has been suggested that oxidative stress may lead to inactivation of folate cofactors, resulting in folate deficiency [23]. We speculate that the oxidative stress induced by homocysteine in the adult animals led to their folate deficiency. The juvenile animals have less evidence of oxidative damage, which might have allowed them to maintain sufficient folate stores.

The second major difference between the handling of homocysteine in adult and juvenile animals was that the peak plasma levels achieved after a dose of homocysteine were significantly lower in the homocysteine-treated juvenile animals than in the homocysteine-treated adult animals or control animals. This finding is of interest because it suggests that juvenile individuals have the ability to metabolize or assimilate a homocysteine or methionine load in a fashion that is lost in adults. While we do not yet know the nature of this adaptive response, it may be a major factor in the progressive rise in homocysteine levels that is associated with aging.

While the difference in folate status in the adult homocysteine-treated rabbits explains the longer elimination half-life in this group, it does not explain why the juvenile homocysteine-treated rabbits had much lower peak plasma levels of homocysteine in response to a standardized test dose. It is possible that these animals developed an enhanced ability to metabolize
homocysteine to methionine or cysteine. It is also possible that they sequestered homocysteine into the intracellular space much more efficiently than did the other groups of animals.

Our data suggests that impaired homocysteine elimination in the adult HCys-treated rabbits had potentially significant pathologic effects. The homocysteine-treated adult rabbits had elevated levels of lipid peroxidation products, as has previously been reported in a rat model of hyperhomocysteinemia [24]. The homocysteine-treated juvenile rabbits, even though they had been given equivalent doses of homocysteine over the 8-week treatment period, did not have elevated levels of lipid peroxidation products.

It has been reported that glutathione levels tend to decline with age [25] while homocysteine levels tend to rise. Reduced glutathione is a major intracellular anti-oxidant. We hypothesized that glutathione levels would be higher in juvenile rabbits than in adults and that the levels might change in response to the oxidant damage induced by homocysteine. However, the glutathione level in liver from the juvenile control rabbits was lower than adult controls (on a per mg protein basis). The glutathione level in adult liver did not change with homocysteine loading, while the glutathione level increased significantly in the homocysteine-treated juvenile rabbits. This suggests that perhaps the juveniles were able to metabolize homocysteine via the cystathione beta synthase pathway more efficiently than were the adults, leading to enhanced levels of glutathione in the homocysteine-treated juveniles. This could possibly be an adaptive response of the young animal versus the old.

Differences in the plasma recovery and clearance of a dose of homocysteine in adult and juvenile rabbits could be due to any of several mechanisms and our current study does not definitively distinguish among them. Since only about 2% of a homocysteine load is excreted unchanged in the urine [18], it is unlikely that a difference in renal function is a major
contributor. Important factors could include the rate of redistribution of homocysteine between intravascular, extravascular and intracellular compartments; the activity of key enzymes in the trans-methylation and trans-sulfuration pathways; and the utilization of methionine derived from homocysteine for growth and protein synthesis.

From our studies we conclude that juvenile rabbits adapt to chronic exposure to homocysteine in a manner that effectively decreases the plasma level of homocysteine in response to a homocysteine load. This adaptation is functionally important since it is associated with a reduction in evidence of oxidative stress, as noted by lower TBARS and increased glutathione in the juvenile rabbits compared to similarly treated adults. Defining the mechanism of this adaptation may allow improved understanding and treatment of the progressive hyperhomocysteinemia that develops in humans with ageing.
References

### Table 1.1 – Selected Blood Chemistries

<table>
<thead>
<tr>
<th></th>
<th>Juvenile Control</th>
<th>Juvenile HCys treated</th>
<th>Adult Control</th>
<th>Adult HCys treated</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Homocysteine (μM)</strong></td>
<td>12.7 ± 4.45</td>
<td>13.8 ± 4.94</td>
<td>13.5 ± 1.67</td>
<td>29.8 ± 9.88*</td>
</tr>
<tr>
<td><strong>AST (U/L)</strong></td>
<td>25 ± 2.5</td>
<td>19 ± 7.2</td>
<td>23.8 ± 10.3</td>
<td>22.6 ± 10.8</td>
</tr>
<tr>
<td>(aspartate transaminase)</td>
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<td></td>
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</tr>
<tr>
<td><strong>ALT (U/L)</strong></td>
<td>57.7 ± 15</td>
<td>55 ± 39</td>
<td>53.1 ± 25.4</td>
<td>51.7 ± 22.3</td>
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<tr>
<td>(alanine transaminase)</td>
<td></td>
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<tr>
<td><strong>Calcium (mg/dl)</strong></td>
<td>12.8 ± 0.25</td>
<td>12.9 ± 0.3</td>
<td>13.04 ± 0.99</td>
<td>11.75 ± 1.0</td>
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<tr>
<td><strong>Glucose (mg/dl)</strong></td>
<td>141.7 ± 8.96</td>
<td>171 ± 51</td>
<td>136 ± 49</td>
<td>106 ± 10.4</td>
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<tr>
<td><strong>B12 (ng/ml)</strong></td>
<td>37 ± 8.9</td>
<td>35.2 ± 4.8</td>
<td>65.1 ± 26</td>
<td>83.7 ± 11.2</td>
</tr>
<tr>
<td><strong>Folate (ng/ml)</strong></td>
<td>16 ± 5.29</td>
<td>26.7 ± 15.5</td>
<td>24.8 ± 5.98</td>
<td>16.2 ± 3.7**</td>
</tr>
</tbody>
</table>

---

1 Serum samples were collected at the time of sacrifice and kept frozen at -80°C until the time of assays. Data are shown as the mean ± standard deviation. * p<0.05; ** p<0.01 compared to age-matched controls
### Table 1.2 Lipid Peroxidation Products (TBARS) in Liver

<table>
<thead>
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<th></th>
<th>Control</th>
<th>HCys treated</th>
</tr>
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<tbody>
<tr>
<td>Adult</td>
<td>1.72 ± 0.47</td>
<td>2.41 ± 0.46*</td>
</tr>
<tr>
<td>Juvenile</td>
<td>3.97 ± 2.25</td>
<td>1.83 ± 0.58</td>
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</tbody>
</table>

2 Liver tissue was collected at the time of sacrifice, snap frozen in liquid nitrogen and kept at –80°C until the time of assay. Results are expressed as the mean ± standard deviation in mg Thiobarbituric acid (TBARS) per kg liver tissue. * p<0.05 compared to age-matched controls.
**Table 1.3 Total Glutathione content in Liver\(^3\)**

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>HCys treated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adult</td>
<td>49 ± 7</td>
<td>52 ± 13</td>
</tr>
<tr>
<td>Juvenile</td>
<td>36 ± 5</td>
<td>42 ± 3*</td>
</tr>
</tbody>
</table>

\(^3\) Liver tissue was collected at the time of sacrifice, snap frozen in liquid nitrogen and kept at −80\(^\circ\)C until the time of assay. Results are expressed as the mean ± standard deviation in nmoles of glutathione per mg protein. * p<0.05 compared to age-matched controls.
**Figure 1.**

**Effect of homocysteine pre-treatment on the elimination of a dose of homocysteine.** Adult and juvenile (weanling) New Zealand White rabbits were pre-treated with twice-daily administration of 30 mg/kg homocysteine (HCys groups) or buffer only (Controls) for seven weeks. Each animal was then given a 30 mg/kg dose of homocysteine and serial plasma samples collected for assay. The mean and standard deviation of the values are shown. The values obtained for adult and juvenile controls were not statistically different, so data from these six animals was pooled to generate the “controls” curve. The “adult HCys” and “juvenile HCys” curves are from three animals each. Statistically significant differences from the control values (p<0.05) are indicated by *. Note that the initial value for the HCys controls was elevated. This value represents the trough value after the last pre-treatment dose.
Elevated Plasma Homocysteine Leads to Alterations in Fibrin Clot Structure and Stability: Implications for the Mechanism of Thrombosis in Hyperhomocysteinemia*

Abstract

Background: Elevated plasma homocysteine is associated with an increased risk of atherosclerosis and thrombosis. However, the mechanisms by which homocysteine might cause these events are not understood. Objectives: The hypothesis is that hyperhomocysteinemia might lead to modification of fibrinogen in vivo, thereby causing altered fibrin clot structure. Methods: New Zealand White rabbits were injected i.p. every 12 hr through an indwelling catheter with homocysteine or buffer for 8 weeks. Results: This treatment raised the plasma homocysteine levels to about 30 μM compared to 13.5 μM in control rabbits by the end of the treatment period. The fibrinogen levels were 3.2 ± 0.6 in homocysteine-treated and 2.5 ± 1.1 mg/ml in control rabbits. The Reptilase time was prolonged to 363 ± 88 for plasma from homocysteine-treated rabbits compared to 194 ± 48 seconds for controls (p<0.01). The thrombin clotting time (TCT) for the homocysteine-treated rabbits was significantly shorter, 7.5 ± 1.7 compared to 28.6 ± 18 seconds for the controls (p<0.05). The calcium dependence of the thrombin clotting time was also different in homocysteinemic and control plasmas. Clots from plasma or fibrinogen of homocysteinemic rabbits were composed of thinner fibers than control clots. The clots formed from purified fibrinogen from homocysteine-treated rabbits were lysed more slowly by plasmin than comparable clots from control fibrinogen. Conclusions: Congenital dysfibrinogenenemias have been described that are associated with fibrin clots composed of thin, tightly-packed fibers that are abnormally resistant to fibrinolysis, and recurrent thrombosis. The results suggest that elevated plasma homocysteine leads to a similar acquired dysfibrinogenemia. The formation of clots that are abnormally resistant to fibrinolysis could directly contribute to the increased risk of thrombosis in hyperhomocysteinemia.
Introduction

Hyperhomocysteinemia is associated with the development of atherosclerosis as well as venous and arterial thrombosis in numerous epidemiologic studies. However, the mechanism(s) by which elevated plasma homocysteine leads to cardiovascular disease has not been clearly determined. Most studies have focused on the effects of homocysteine on vascular cells. Endothelial dysfunction induced by hyperhomocysteinemia very likely contributes to the enhanced risk of atherosclerosis. However, the effects of homocysteine on plasma proteins, especially coagulation proteins, have not received much attention.

The adverse effects of homocysteine have been hypothesized to be related to its chemical reactivity. Homocysteine can undergo either oxidation or reduction and react with a variety of cellular and plasma components. Since homocysteine has a free sulfhydryl group, it can form disulfide bonds with cysteine residues in proteins. Plasma albumin enhances formation of disulfide forms of homocysteine by promoting disulfide exchange. Homocysteine can be metabolized to homocysteine thiolactone, although there is debate about the extent to which this occurs in vivo. Homocysteine thiolactone can react with and covalently modify free amino groups, especially the epsilon amino group of lysines.

We hypothesized that homocysteine or its metabolites react with plasma coagulation proteins and alter their function in vivo. Since these reactions are presumably non-enzymatic, the extent of protein modification will be proportional to the plasma level of homocysteine and the period of time the protein was in contact with homocysteine. Therefore, we focused on the effects of hyperhomocysteinemia on fibrinogen. Fibrinogen is a key coagulation protein because it forms the structure of the fibrin clot and it has a relatively long half-life of 3-5 days. It can be covalently modified by homocysteine thiolactone in vitro. However, the exact sites and nature of the modifications are not known. Fibrinogen has many functionally important lysine residues that could be modified by homocysteine thiolactone. It also has several cysteine residues that could react with reduced forms of homocysteine or be modified by disulfide exchange.
Activation of the fibrinolytic enzyme, plasminogen, by its activator, tissue plasminogen activator (tPA) is enhanced when both are bound to fibrin. Lysine residues mediate the binding of plasminogen and tPA to fibrin. Once activated, plasmin cleaves fibrin at lysine residues, as well. Thus, modification of lysine residues in fibrin(ogen) could hamper fibrinolysis both by impairing activation of plasminogen and by impairing fibrin cleavage by plasmin.

Modifications at sites other than lysines could also alter fibrinogen function in ways that promote thrombosis. For example, in the congenital dysfibrinogenemias fibrinogenDusart and fibrinogenChapel Hill III, a point mutation converts an arginine to a cysteine residue in the A-alpha chain of the molecule. The sulphydryl group of the cysteine mediates disulfide bond formation between fibrinogen and albumin in the circulation. The cross-linking of albumin to fibrinogen alters polymerization of fibrin monomers as clots form. The resulting clots are composed of thinner and more tightly-packed fibers than normal and are unusually resistant to fibrinolysis. The resistance to lysis appears to account for the thrombotic tendency associated with this dysfibrinogenemia.

We hypothesize that elevated plasma levels of homocysteine could enhance resistance of fibrin clots to fibrinolysis and promote thrombosis by two mechanisms. First, modification of lysines in fibrinogen could impair binding and activity of fibrinolytic enzymes. Second, structural modification of fibrinogen could alter the rate and pattern of fibrin polymerization and, thereby, alter fibrin clot structure.

In this paper we show that hyperhomocysteinemic rabbits develop an alteration of fibrinogen function as evidenced by altered snake venom and thrombin clotting times. The plasma and fibrinogen from hyperhomocysteinemic rabbits form clots with thinner fibers than normal that are abnormally resistant to plasmin lysis. While we do not yet know the exact chemical nature of the fibrinogen modification(s) induced by hyperhomocysteinemia, our results suggest that an acquired dysfibrinogenemia could contribute to the thrombotic tendency associated with hyperhomocysteinemia.
Materials and Methods

Animal Model:

We used a rabbit model of hyperhomocysteinemia similar to that used by McCully in studying homocysteine-induced atherosclerosis.\textsuperscript{20} New Zealand white rabbits weighing 3.5-4.0 kg (from PSI Robinson Services, Clemmons, NC) were fed Prolab Rabbit 5P26 chow (Purina Mills, St. Louis, Mo.) and water \textit{ad libitum}. The control rabbits received injections of the diluent (5% dextrose/water) and the treatment group received 30mg/kg DL-homocysteine (Sigma, St. Louis, Mo.) in 5% dextrose twice daily for eight weeks into the peritoneal cavity through an indwelling injection port (V-A-P, Access technologies, Skokie, IL). The homocysteine solution was prepared fresh immediately before each dose. Blood was drawn every two weeks from the central artery of the ear to monitor homocysteine levels and blood chemistries. After eight weeks of treatment the rabbits were anesthetized, exsanguinated by cardiac puncture and the blood collected into citrate anticoagulant or red top (no anticoagulant) tubes. Plasma and serum were stored at \textminus 80\degree\textdegree C until used for assays. A total of 12 rabbits (6 control and 6 experimental) were treated in two separate experiments.

Blood Chemistries

Serum homocysteine assays were done in the Clinical Chemistry Laboratory, UNC Hospital (Chapel Hill, NC), using the IMX system (Abbott Labs). Assays of serum aspartate amino transferase (AST), alanine amino transferase (ALT), alkaline phosphatase, calcium, glucose, potassium, sodium, creatinine, vitamin B12 and folate were performed in the Clinical Chemistry Laboratory, Durham VA Medical Center (Durham, NC).
Clotting Studies.

Prothrombin times (PT) and activated partial thromboplastin times (aPTT) were performed in the Durham VA Medical Center Clinical Hematology Laboratory on an MDA180 automated analyzer (Organon Teknika, Durham, NC).

Reptilase and Ancrod clotting times were performed as follows. Plasma samples (200 μl) were pre-warmed, then 100 μl of Reptilase (Atroxin, Sigma) or Ancrod (Sigma) was added, and clot formation monitored on a FibroSystem Fibrometer. Thrombin times were similarly determined by adding 100μl of thrombin solution (final concentration 7 nM) to 200 μl of pre-warmed plasma sample. In some cases CaCl$_2$ was added to the thrombin solution to give a final concentrations of 10 mM.

Fibrinogen Purification

Fibrinogen was purified from plasma by glycine precipitation.$^{21}$ Twenty μl of a protease inhibitor cocktail containing 10 mM Phe-Pro-Arg chloromethyl ketone (PPACK), 10 mM phenyl-methyl sulfonyl fluoride (PMSF), 10 mM tosyl-lysyl chloromethyl ketone (TLCK), 100 mM benzamidine, and 165mg of glycine (all from Sigma Chemical Co, St Louis, MO) were added to one mL of plasma. The sample was rocked at room temperature and then spun for 30 minutes at 14,000rpm in a tabletop centrifuge. The pellet was resuspended in one ml of 0.055M sodium citrate in HBS, pH 7.4, rocked for 20 minutes, then again centrifuged. The supernatant was removed and the pellet resuspended in 250 ul of 0.055M sodium citrate in HBS. The purity of the preparation was checked on SDS-gel electrophoresis.

Fibrinogen Assays

The amount of fibrinogen antigen in plasma samples and fibrinogen preparations was measured in an ELISA assay. A sheep anti-rabbit fibrinogen antibody (Enzyme Research Laboratories, South Bend, Indiana) was used as the capture antibody. Diluted samples and standards were added to the plate and allowed to bind. Fibrinogen binding was detected by anti-
fibrinogen antibody conjugated to horseradish peroxidase and cleavage of TMP Substrate (KPL, Gaithersburg, MD).

**Measurements of Relative Mass/Length Ratios (\(\mu\)) of Fibrin Clots.**

The relative \(\mu\) of fibers in fibrin clots were determined using a plate-reading spectrophotometer\(^{22}\) by a modification of the method of Carr & Gabriel.\(^{23}\) Fibrin clots were formed from plasma or purified fibrinogen solution adjusted to 1 mg/ml. Samples were recalcified by addition of CaCl\(_2\) to a final concentration of 5mM and clotted with thrombin at a final concentration of 6.25nM.

The absorbance of the samples was read monitored for two hours in a Molecular Devices SPECTRAMax PLUS384 UV/VIS Microplate Spectrophotometer (Sunnyvale, CA). The optical density was then scanned from 400 to 800nm. This data was used to calculate relative \(\mu\) values using the following equation:\(^{23}\)

\[
\tau=\frac{(88/15)\pi^3 n(dn/dC)^2 C\mu}{N\lambda^3}
\]

Where \(\tau\) is the turbidity, \(n\) the refractive index, \(dn/dC\) the refractive index increment, \(\lambda\) the wavelength, \(C\) the concentration of fibrinogen in gm/ml, \(N\) is Avogadro’s number, and \(\mu\) is the mass/length ratio. The calculated values for \(\mu\) can be used to compare the structure of fibrin clots, i.e. clots with a higher \(\mu\) value have thicker fibers than clots with a lower \(\mu\) value.

**Assay of Clot Lysis.**

The stability of fibrin clots made from purified fibrinogen was assessed by their susceptibility to plasmin lysis. Fifty \(\mu\)l of a 0.1 \(\mu\)M solution of plasmin was gently layered on top of a fibrin gel that had been formed from purified fibrinogen. The decrease in absorbance was monitored in a plate-reading spectrophotometer at 405nm every minute for 5 hours. The time required for a 50% reduction in clot turbidity (time to half-lysis) was determined as an indication of the susceptibility of the clots to lysis.\(^{24}\)
Scanning Electron Microscopy (SEM) of Fibrin Clots.

Clots were prepared for SEM as follows. They were fixed with fresh 2% glutaraldehyde for 1 hour, washed and post-fixed for 15 minutes in 0.05% OsO4, then washed in distilled water. The clots were dehydrated through a graded series of ethanol and dried by the critical point procedure (Tousimis, Samdri 790), using sieve dried ethanol as the intermediate fluid and liquid CO2 as the transitional fluid. The dried samples were mounted on aluminum stubs, sputter-coated with platinum, and examined in a JEOL JSM 6400 SEM. Photographs of representative fields from each sample were taken by a technician who did not know the identity of the samples. Photographs were digitized and Adobe Photoshop was used to measure the diameter of fibers. The fiber diameter was measured at its widest point where there was not a branch point.

Deglycosylation

All carbohydrate was enzymatically removed from the samples of purified fibrinogen using a deglycosylation kit (Prozyme, Inc. San Leandro, Ca.). Approximately 200μg of purified fibrinogen in a volume of 0.4ml of was reprecipitated with saturated ammonium sulfate, washed, then the pellets dissolved in 0.2ml 8M urea, 0.1M Tris/HCL pH 8.0, 10mM dithiothreitol. After four hours at 37°C, the samples were diluted 1:4 to reduce the urea concentration to 2M. The reagents from the kit (PNGase F, Sialidase A, and Endo-O-Glycosidase) were incubated with the reduced fibrinogen chains at 37°C for 12 hours. Deglycosylation was verified by a shift in mobility of the B-beta and gamma chains on SDS gel electrophoresis (the A-alpha chain is not glycosylated).

SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS-PAGE and Western blotting was performed using a PhastGel System (Pharmacia, Piscataway, NJ).
Statistical Analysis

Homocysteine-treated and controls groups were compared using a Student’s t test.
RESULTS

Biochemical Measurements

The serum levels of AST, ALT, alkaline phosphatase, calcium, glucose, potassium, sodium, creatinine, and vitamin B₁₂ were similar in the control and homocysteine-treated groups throughout the treatment period (data after eight weeks shown in Table 2.1).

As expected, homocysteine levels were elevated in the treated animals. Blood was drawn for measurement of homocysteine levels immediately before injection of the next dose, and thus represents the trough level. For the first seven weeks of treatment the homocysteine levels (which peaked at about 100 μM within 30 minutes of injection) fell to levels indistinguishable from controls after six hours (data not shown). However, at the end of the eighth week of treatment the trough homocysteine levels of the homocysteine-treated group remained significantly elevated (29.8 μM ± 9.88, compared to levels of 13.5 μM ± 1.67 in the control group, p<0.05).

After eight weeks of homocysteine administration the plasma folate levels were significantly lower in the homocysteine-treated group than the control group (16.2 ng/ml ± 3.7 vs 24.8 ng/ml ± 5.9). Folate levels were not different at the start of the treatment period. Therefore, it appears that depletion of folate in the homocysteine-treated rabbits impaired homocysteine metabolism and allowed the plasma homocysteine levels to rise.

Coagulation Assays.

The amount of immunoreactive fibrinogen in plasma tended to be higher in the homocysteine-treated rabbits (3.2 ± 0.6 vs 2.5 ± 1.1 mg/ml in the controls, Table 2.2.) though this difference was not statistically significant.

The PT results were similar in treated and control rabbits. However, aPTT times tended to be longer for homocysteine-treated rabbits, 109 seconds ± 24, vs 80 seconds ± 7.6 for controls (Table 2.2). This difference, however, was not statistically significant.
The thrombin clotting times (at 7 nM thrombin) of plasmas from homocysteine-treated rabbits were significantly different from controls as shown in Table 2. In the absence of added calcium the control plasmas had clotting times of $28.6 \pm 18.0$ seconds, while the homocysteinemic plasmas had significantly shorter clotting times at $7.5 \pm 1.7$ seconds (p<0.05). In the presence of added calcium (10 mM) the control plasmas had faster clotting times, $19.0 \pm 12.1$ seconds, as would be expected. However, the homocysteinemic plasmas did not have faster clotting times at $7.8 \pm 1.3$ seconds. These data suggest that calcium binding by the fibrinogen is altered in homocysteinemic plasma.

Since thrombin clotting times can be influenced by the level of inhibitors in plasma as well as by fibrinogen function, fibrin polymerization was directly assessed in snake venom clotting times. The snake venoms atrocin (Reptilase) and ancred are commonly used to identify and characterize dysfibrinogenemias. Atrocin and ancred clotting times are not affected by antithrombin III, heparin or other common anticoagulants, but are very sensitive to alterations in fibrinogen function.

The homocysteine-treated rabbits had significantly prolonged Reptilase and Ancrod times at the end of the eight-week treatment period. The values for Reptilase were $194 \pm 48$ for controls compared to $363 \pm 88$ seconds for homocysteine-treated (p<0.01). Ancrod times were $6.8 \pm 1.25$ for controls vs $10.0 \pm 2.15$ seconds for homocysteine-treated (p<0.001). Interestingly, the snake venom clotting times became prolonged after seven weeks of homocysteine treatment. Thus, the fibrinogen abnormality developed concurrent with the development of a persistent elevation in plasma homocysteine.

**Fibrin Clot Fiber Structure.**

We next examined the structure of the fibrin clots formed in plasma from control and treated rabbits. Fibrin clots are composed of a meshwork of fibers. We found a statistically significant difference between the fiber structure of clots formed by the addition of thrombin to
the plasma of homocysteine-treated and control rabbits (Table 2.3). The relative $\mu$ was 40% smaller for the plasma clots from homocysteine-treated rabbits compared to clots from controls. Thus, the clots formed from hyperhomocysteinemic plasma were composed of significantly thinner fibers than were those formed from control plasma.

The $\mu$ values of clots formed from purified fibrinogen from treated and control rabbits were determined. As shown in Table 2.3, the average relative $\mu$ of clots formed from fibrinogen purified from hyperhomocysteinemic plasma was significantly lower than that of control fibrin clots. Because the purified fibrinogen retained the functional abnormality seen in plasma, we infer that there has been some modification of the fibrinogen itself.

**Scanning Electron Microscopy (SEM) of Fibrin Clots.**

The meshwork of the fibrin gel can also be evaluated visually by electron microscopy. While it is harder to get quantitative data with this technique than with optical measurements, it is possible to get an assessment of heterogeneity of the fibrin fibers, the packing density and degree of branching. Fibrin gels formed by addition of thrombin to the rabbit plasmas were viewed by SEM. Representative views are shown in figure 1. Consistent with the results of optical measurements, the homocysteine clots appear to have a more tightly packed structure composed of thinner fibers than did the control clots. Measurements of fiber dimensions revealed that the homocysteine clots had a mean fiber diameter of $185\pm14$ nm, compared to $218\pm15$ nm for the controls ($p<0.05$). Thus the observations on SEM are consistent with the optical measurements.

In addition, clots from homocysteine-treated rabbits had a peculiar rough appearance -- as though the fibers had been sprayed with flocking. We do not know the reason for this appearance, but it was a consistent finding. It could represent crosslinking of other plasma proteins to the fibrin
fibers, result from an aberration of polymerization or from fiber breakage and lateral reorganization.

Characterization of Purified Fibrinogen

To determine whether the alterations in fibrinogen function were due to homocysteine-induced alterations in fibrinogen structure or to alteration of some other plasma component, we assessed some characteristics of purified fibrinogen.

We first performed SDS-PAGE under non-reducing conditions and Western blotting of rabbit plasmas using an antibody against rabbit fibrinogen. As shown in figure 2 for six different plasma samples, the plasma from homocysteinemic rabbits had a high molecular weight band that was not found in control rabbit plasma. The relative amount of the band varied between rabbits. Western blotting with antibodies against rabbit albumin or IgG did not react with the high molecular weight band, suggesting that fibrinogen was not covalently attached to these abundant plasma proteins. The high molecular weight band was not visualized on gels run under reducing conditions, suggesting that formation of disulfide bonds mediated formation of this high molecular weight adduct.

SDS-PAGE of purified fibrinogen preparations under reducing conditions showed the expected $\alpha_A$, $\beta_B$ and $\gamma$ chains of fibrinogen from both control and homocysteine-treated rabbits. As illustrated in figure 2.3, the chains had similar mobility on gel electrophoresis. All fibrinogen preparations also showed the expected increase in migration of the $B_B$ and $\gamma$ chains upon enzymatic removal of the carbohydrate from these chains. Thus, there were no gross alterations in the molecular weight of the fibrinogen chains from hyperhomocysteinemic rabbits.

Plasmin Lysis of Fibrin Clots.

Several studies have demonstrated that the fiber structure of fibrin clots is an important determinant of their resistance to normal fibrinolysis. Clots composed of thinner fibers are lysed
more slowly than clots composed of thicker fibers \(^{27}\). Therefore, we next evaluated whether the fibrin clots from homocysteinemic rabbits exhibited an enhanced resistance to fibrinolysis.

We found that the time required for half lysis of fibrin clots was significantly longer for fibrinogen from homocysteine-treated rabbits, 127 seconds ± 32.2, compared to 81.8 seconds ± 32.0 for controls (p<0.009).
DISCUSSION

Much of the information on the relationship between homocysteinemia and thrombosis comes from epidemiologic studies. Little is known about the mechanism(s) by which homocysteine promotes thrombosis. While the presence of atherosclerotic vascular disease could provide a site for initial formation of a thrombus, this is unlikely to be the explanation for the increased risk of venous thrombosis associated with hyperhomocysteinemia. Enhanced platelet reactivity may contribute to an increased risk of thrombosis,28 as may altered endothelial function. Homocysteine at very high levels has also been reported to modify coagulation factor V in vitro and inhibit its cleavage by activated protein C.29

Some studies of the effects of homocysteine in vivo have been done in rabbit,20 monkey,30 and mouse models.31 We selected the rabbit model because rabbits are easier and cheaper to maintain than primates, but enough plasma can be collected to study the effects of homocysteine on plasma proteins. We chose to inject the rabbits directly with homocysteine to ensure that all experimental subjects received the same dose of the agent. However, there are some limitations to this approach, since homocysteine generated metabolically may have different effects than exogenously administered homocysteine. First, the exogenously administered homocysteine is a mixture of D- and L-isoforms, while metabolically generated homocysteine is the L-isoform only. Second, metabolically generated homocysteine might reach high levels in a compartment that is not readily accessible to exogenously administered homocysteine. For example, if most of the metabolically generated homocysteine is formed intracellularly, the cellular levels of homocysteine may be different when homocysteine is generated metabolically than when it is injected – even though the plasma levels are comparable. With these possible caveats, we believe that the rabbit model is a useful one in which to study the effects of elevated levels of plasma homocysteine on plasma coagulation proteins.

Our findings demonstrate that elevated plasma levels of homocysteine induce an acquired dysfibrinogenemia. The alterations in fibrinogen function are reflected in the altered response of
fibrinogen polymerization to calcium, prolonged snake venom clotting times, formation of clots with thinner fibers, and increased resistance of clots to plasmin lysis. The abnormalities in fibrin clot formation, structure and lysis must be due to modification of the fibrinogen protein, since abnormalities were not only observed when testing plasma, but also purified fibrinogen.

One modification to the plasma fibrinogen that we detected was the presence of high molecular weight forms of fibrinogen in the plasma of homocysteinemic rabbits. At this point we do not know the nature of the modification that results in this high molecular weight form. It seems likely that crosslinking of fibrinogen via disulfide bonds occurs, since the high molecular weight form is not seen on gels under reducing conditions. Also, there are no differences in the mobility of fibrinogen chains under reducing conditions, either with the carbohydrate attached or removed. The precise chemical modification(s) to fibrinogen remains to be determined.

While it may seem paradoxical that prolonged clotting times could be associated with a thrombotic tendency in vivo, there is a clear precedent for this phenomenon. Of the symptomatic congenital dysfibrinogenemias about 45% of them are associated with thrombosis rather than bleeding. In most cases the Reptilase clotting time is prolonged. In many cases calcium binding is affected. The apparent paradox of fibrinogen that clots abnormally, yet is associated with thrombosis, can be explained by analyzing the consequences of the polymerization defect. The dysfunctional fibrinogen forms a clot with an abnormal structure that is resistant to lysis by plasmin. Thus, the clots that are formed cannot be removed by the fibrinolytic system.

The effect of an acquired dysfibrinogenemia may be synergistic in promoting thrombosis with a homocysteine-induced defect in plasminogen activation. Annexin II is a receptor on endothelial cells that promotes activation of plasminogen by tissue plasminogen activator (tPA). Incubation of cultured endothelial cells or purified annexin II with homocysteine resulted in chemical modification of the annexin II. This modification blocks binding of tPA to annexin II and blocks activation of plasminogen by tPA on endothelial cells. Thus, elevated plasma homocysteine may promote thrombosis by causing a defect in plasmin generation as well as rendering clots more resistant to plasmin lysis.
REFERENCES

25. Langer BG, Hong SK, Schmelzer CH, Bell WR. Deglycosylation of a native, protease-sensitive glycoprotein by peptide N-glycosidase F without protease inhibitors. Anal Biochem. 1987;166:212-217
Figure 2.1 Scanning Electron Microscopy (SEM) of plasma clots from a homocysteine treated rabbit (top panel) and a control rabbit (bottom panel). Plasma was clotted with thrombin, prepared for SEM and viewed as described in “Material and Methods”. These views are representative of multiple views of plasma clots from control and experimental plasma. Note that the homocysteine clot appears to be composed of more densely packed fibers. Additionally, the surface of the fibers from homocysteinemic plasma has many small projections. We hypothesize that this could be due to disorganized packing of the fibrin monomers as they polymerize, crosslinking of other proteins to the fibers or their constituent fibrin monomers or fiber breakage and lateral reorganization.
Figure 2.2 Western blot (using an anti-rabbit fibrinogen antibody) of plasma samples from 3 hyperhomocysteinemic and 3 control rabbits separated by SDS-PAGE under non-reducing conditions. Note the high molecular weight band visualized with the anti-fibrinogen antibody in the hyperhomocysteinemic samples, but not in the control samples. The position of the expected fibrinogen band is also indicated. The samples shown in this blot are from a different group of rabbits than those from which the biochemical results were obtained.
**Figure 2.3** Samples of purified fibrinogen from control and homocysteine-treated rabbits separated by SDS-PAGE under reducing conditions. The locations of the constituent fibrinogen chains are indicated. There was no gross difference in electrophoretic mobility of the fibrinogen chains from any of the control and homocysteinemic rabbits. Removal of the carbohydrate chains resulted in the expected increase in mobility of the B-beta and gamma chains. A small amount of residual glycosylated B-beta chain in the control sample makes clear the relative positions of the glycosylated and de-glycosylated bands.
TABLE 2.1

Blood Chemistries of Homocysteine -Treated and Control Rabbits after Eight Weeks of Treatment

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<tr>
<th></th>
<th>Control</th>
<th>HCys</th>
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<td>AST (IU/L)</td>
<td>26.2± 14.5</td>
<td>28.7± 9.2</td>
</tr>
<tr>
<td>ALT (IU/L)</td>
<td>47.0± 10.1</td>
<td>49.0± 34.7</td>
</tr>
<tr>
<td>AlkPhos (IU/L)</td>
<td>31.0± 13.3</td>
<td>18.0± 3.6</td>
</tr>
<tr>
<td>Calcium (mg/dL)</td>
<td>12.6± 0.95</td>
<td>11.3± 0.62</td>
</tr>
<tr>
<td>Glucose (mg/dL)</td>
<td>181 ± 69</td>
<td>108 ± 18</td>
</tr>
<tr>
<td>Potassium (mM)</td>
<td>3.97± 0.54</td>
<td>3.27± 0.08</td>
</tr>
<tr>
<td>Sodium (mM)</td>
<td>145 ± 1.55</td>
<td>145 ± 7.8</td>
</tr>
<tr>
<td>Creatinine (mg/dL)</td>
<td>1.5 ± 0.06</td>
<td>1.25± 0.3</td>
</tr>
<tr>
<td>Folate (ng/mL)</td>
<td>24.8± 5.9</td>
<td>16.2± 3.7*</td>
</tr>
<tr>
<td>Vitamin B12 (ng/ml)</td>
<td>65.2± 26.1</td>
<td>83.8± 11.3</td>
</tr>
<tr>
<td>Homocysteine (µM)</td>
<td>13.5± 1.67</td>
<td>29.8± 9.88**</td>
</tr>
</tbody>
</table>

*p<0.05

**p<0.01
Table 2.2

Coagulation Assays on Plasma
from Homocysteine-Treated and Control Rabbits

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>HCys</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fibrinogen - ELISA</td>
<td>2.5 ± 1.1</td>
<td>3.2 ± 0.6</td>
</tr>
<tr>
<td>(mg/ml)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PT (seconds)</td>
<td>8.3 ± 0.26</td>
<td>8.8 ± 1.8</td>
</tr>
<tr>
<td>aPTT (seconds)</td>
<td>80 ± 7.6</td>
<td>109 ± 24</td>
</tr>
<tr>
<td>Ancrod (seconds)</td>
<td>6.8 ± 1.25</td>
<td>10.0 ± 2.15**</td>
</tr>
<tr>
<td>Atroxin (seconds)</td>
<td>194 ± 48</td>
<td>363 ± 88*</td>
</tr>
<tr>
<td>Thrombin (seconds)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>- Calcium</td>
<td>28.6± 18.0</td>
<td>7.5 ± 1.7*</td>
</tr>
<tr>
<td>+ Calcium</td>
<td>19.0 ± 12.1</td>
<td>7.8 ± 1.3</td>
</tr>
</tbody>
</table>

* **p<0.001
* p<0.05

Clotting times on plasma samples from homocysteine-treated and control rabbits were determined as described in “Methods”. Statistical significance was determined using a Student’s t test.
Table 2.3

Relative Mass per Unit Length ($\mu$) of Fibers in Plasma and Fibrinogen Clots

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>HCys</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma</td>
<td>1.0 ± 0.24</td>
<td>0.601 ± 0.054</td>
<td>0.01</td>
</tr>
<tr>
<td>Purified Fibrinogen</td>
<td>1.36 ± 0.24</td>
<td>1.05 ± 0.37</td>
<td>0.04</td>
</tr>
</tbody>
</table>

Plasma samples or purified fibrinogen was clotted with 6.25 nM thrombin and the relative $\mu$ calculated as described in “Methods”. The mean $\mu$ for plasma clots from control rabbits was set equal to “1” and the $\mu$ values for the other clots are expressed relative to that value. Statistical significance was determined using a Student’s $t$ test.
Conclusion:

Chronic elevations of plasma levels of homocysteine are associated with an increased risk of atherosclerosis and thromboembolism. The mechanism(s) by which homocysteine may cause these effects is incompletely understood. This study was designed to determine whether the coagulation protein, fibrinogen, could be modified by chronic elevations of plasma homocysteine. First, it had to be decided whether a juvenile or adult rabbit model would be suitable for the experiments. In preliminary testing, results showed that prolonged treatment of the juvenile rabbits did not induce hyperhomocysteinemia. After eight weeks of treatment, the baseline levels of the homocysteine injected juvenile rabbits were similar to control injected rabbits. However, the adult homocysteine injected rabbits did develop elevated levels of plasma homocysteine and after eight weeks of homocysteine injections the values were twice the level of control injected rabbits. These levels were in the range associated with an increased risk of atherosclerosis and thrombosis in humans. The juvenile homocysteine-treated rabbits cleared the bolus injections of homocysteine faster than the adult homocysteine-treated rabbits and had higher liver glutathione levels. The adult homocysteine-treated rabbits developed folate deficiency and the juvenile rabbits did not. In addition, the adult rabbits had evidence of pathologic changes accompanying the elevated homocysteine levels, since they had increased lipid peroxidation products while the juvenile rabbits did not. The metabolic differences between adult and juvenile rabbits are a novel finding, and may be related to the progressive elevation in homocysteine levels seen during aging.

The clotting studies performed on adult homocysteine-treated rabbit plasma suggest that clots made from the plasma have an abnormal structure. The evaluation of homocysteine-treated rabbit versus control rabbits plasma treated with atroxin and thrombin showed that there seemed
to be a structural change in the fibrin clot formed. The results for homocysteine-treated plasma were an elongated clotting time for atroxin, decrease clotting time for thrombin, and decreased fibrin fiber diameter. These types of changes are similar to abnormal fibrin fiber assembly seen in certain dysfibrinogenemias. Patients with these dysfibrinogenemias have abnormal fibrin clot structure, which causes the clot to be resistant to normal lysis by plasmin, and is associated with thrombosis. Further study of the plasma of homocysteine-treated rabbits revealed a difference in fibrinolysis times. After the fibrin clot was treated with plasmin, the lysis times were longer in the homocysteine treated rabbits versus control rabbits. This is evidence of a dysfunctional fibrinogen that forms a clot with an abnormal structure and is less susceptible to lysis by plasmin. It is possible that these changes observed in the homocysteine treated rabbits may be analogous to the human dysfibrinogemias and could represent a novel mechanism by which elevated homocysteine leads to thrombosis.

Homocysteine and its metabolites have been shown to non-enzymatically modify proteins. We had hypothesized that homocysteine thiolactone, a reactive metabolite of homocysteine, might modify lysine residues in fibrinogen. While \textit{in vitro} studies have shown modification of protein structure or function by homocysteine or homocysteine thiolactone, they have used higher than physiological concentrations to see an effect. Our studies demonstrate that the fibrinogen protein is modified \textit{in vivo}, but we have not yet determined the exact nature of the modification. Future studies to characterize the biochemical nature of the modification of fibrinogen during hyperhomocysteinemia are planned.

The injection model of hyperhomocysteinemia used in these studies has led us to some novel and exciting observations, and may help to understand the mechanism(s) of atherosclerosis and thrombosis. However, there may be differences in metabolism and pathogenic effects when
homocysteine is injected compared to when it is generated during metabolism. Therefore, we are pursuing studies in which rabbits are made hyperhomocysteinemic by dietary folate deficiency. Preliminary results suggest this model will be useful in pursuing the mechanism of homocysteine-induced dysfibrinogenemia and its relevance to human disease.

In summary, the objective is to understand how elevated plasma levels of homocysteine could have an effect on blood clot structure and stability. Knowing the possible mechanisms could give insight into the involvement of homocysteine in cardiovascular disease and allow development of better tests to predict the risk of cardiovascular events in patients.