DISSERTATION

A SPONTANEOUS HEREDITARY COAGULOPATHY IN RAMBOUILLET SHEEP

Submitted by

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WE HEREBY RECOMMEND THAT THE DISSERTATION PREPARED UNDER OUR SUPERVISION BY JEREMY S. JOHNSON ENTITLED "A SPONTANEOUS HEREDITARY COAGULOPATHY IN RAMBOUILLET SHEEP" BE ACCEPTED AS FULFILLING IN PART REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

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ABSTRACT OF DISSERTATION

A SPONTANEOUS HEREDITARY COAGULOPATHY IN RAMBOUILLET SHEEP

An inbred flock of Rambouillet sheep was investigated because of increased lamb mortality due to ineffective periparturient hemostasis. Affected, term, neonatal lambs had extensive subcutaneous, umbilical, and intra-cavitary hemorrhage that resulted in hypovolemia and death. Affected lambs were identified by prolongation of the activated clotting time, one-stage prothrombin time (PT), and activated partial thromboplastin time (aPTT). Decreased activity of vitamin K-dependent coagulation factors II, VII, IX, and X was present in all affected lambs while non-vitamin K-dependent coagulation factor activities were similar between affected and unaffected lambs. The hemorrhagic diathesis could not be corrected by supraphysiologic administration of vitamin K_1; however, vitamin K_1 did prolong the required transfusion interval for maintaining adequate hemostasis. Defective γ-glutamyl carboxylase was considered the most likely cause of the heritable coagulopathy because of the inability to correct the hemorrhagic state with supraphysiologic vitamin K_1, and the lack of reported skeletal abnormalities observed in human cases of epoxide reductase

deficiency or *in utero* warfarin exposure. To confirm the suspected underlying cause of abnormal coagulation, crude liver microsomes were isolated from affected lamb liver and evaluated for hepatic γ-glutamyl carboxylase and vitamin K-epoxide reductase activities. Hepatic γ-glutamyl carboxylase activity was significantly decreased compared to age and sex matched control lambs while vitamin K 2,3 epoxide reductase activity was similar between affected and control lamb liver microsomes. We sequenced cDNA and genomic DNA from affected lambs, carrier sheep, and control lambs, and identified four single nucleotide polymorphisms (SNPs) of the γ-glutamyl carboxylase gene allowing for haplotype and genotype characterization. Two of these SNPs were located within the coding sequence of the γ-glutamyl carboxylase gene, specifically in exon 10 (R486H) and exon 14 (R686Stop). Enzyme kinetics demonstrates that neither mutation R486H nor R686Stop significantly impacts the interaction of the enzyme for the synthetic substrate Phe-Leu-Glu-Glu-Leu (FLEEL), nor vitamin K-dependent propeptides of coagulation factor II, IX, and X. Therefore these reactions occur nearer the NH_2-terminus than residue 686. Through comparisons of these SNP frequencies within our inbred sheep flock and the larger U.S. sheep population we determined the likely causative mutation resulting in the fatal coagulopathy to be the result of a premature truncation of the γ-glutamyl carboxylase protein (R686Stop), $p<0.001$. This sheep flock represents the only viable animal model of hereditarily defective γ-glutamyl carboxylase. Two human case reports of γ-glutamyl carboxylase deficiency and site directed mutagenesis have laid the foundation for determining

critical amino acids necessary for enzymatic function, and propagation of this animal model will increase our understanding of the impact of vitamin K metabolism on various systems in the body.

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DEDICATION

This work is dedicated to Stephanie Johnson for always believing in the dream and helping me to live the dream. To Brenna and Colton for allow me to see the world, if only a glimpse, through their eyes.

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CHAPTER 1

Introduction

The work presented in this dissertation represents the scientific investigation of a spontaneous heritable coagulopathy in an inbred Rambouillet sheep flock. These animals were initially examined because of increased periparturient lamb mortality as the result of blood loss and subsequent hypovolemia. The fatal coagulopathy could be corrected with periodic plasma transfusions, but appeared refractory to supraphysiologic vitamin K_1 administration. The pattern of affected offspring suggests that the trait is inherited in an autosomal recessive manner.

This investigation included the genetic and biochemical characterization of the observed coagulopathy, ultimately leading to the discovery of a mutation in the ovine γ-glutamyl carboxylase gene that results in the bleeding phenotype. Currently, no suitable animals model exists to study the clinical implications of abnormal γ-glutamyl carboxylase biology. This animal model of defective γ-glutamyl carboxylase allows for the investigation of impaired vitamin K metabolism due to γ-glutamyl carboxylase dysregulation in multiple systems including coagulation, bone metabolism, and cellular signaling.

Although γ-glutamyl carboxylase has been studied and reviewed,[22,28,29,90] no animal model has been available for the study of this important process. Attempts to develop a knock-out mouse model resulted in the death of all homozygous offspring at birth due to massive intra-abdominal hemorrhage.[107] Interestingly, there was an apparent 50% *in utero* loss of homozygous null pups which is similar to the partial loss at mid-embryogenesis and postnatal hemorrhage reported for prothrombin and factor V deficient mice.[85,106] This sheep[3] flock represents the only viable, naturally occurring animal model of γ-glutamyl carboxylase deficiency, making it an extremely valuable research tool. Sheep have been used as models of bone growth, the effects of estrogen deficiency on bone metabolism, and steroid-induced bone loss.[15,42,64,65] They have been characterized by densitometry, histomorphometry and biochemical markers of bone formation and resorption.[15,65,67,76] Sheep serve as a good model of defective γ-glutamyl carboxylase because of similarities in coagulation parameters with humans and human neonates.[2]

Collaborative investigations stemming from this work will increase our understanding of the role of vitamin K in bone metabolism, with particular emphasis on the association, if any, with osteoporosis. Propagation of this animal flock will provide a resource to increase the scientific community's understanding of the implications of γ-glutamyl carboxylase on intra- and extra-cellular signaling, including pathways of apoptosis, in addition to new insight about the structure and functional relationship of γ-glutamyl carboxylase with procoagulant and anticoagulant vitamin K-dependent proteins.

General Hypothesis

This dissertational work concerns characterization and propagation of a unique animal model of defective γ-glutamyl carboxylase via the specific aims stated below. Based upon the clinical manifestations, pedigree analysis, and preliminary findings I propose the following general hypothesis.

The coagulopathy in this inbred flock of Rambouillet sheep is transmitted in an autosomal recessive manner. This mutation impairs the biological function of the microsomal enzyme γ-glutamyl carboxylase in multiple organ systems of homozygous animals.

Specific Aims

The first specific aim is to biochemically characterize the defect resulting in a fatal hemorrhagic diathesis in affected newborn lambs through the evaluation of coagulation parameters and activity of microsomal enzymes involved in vitamin K metabolism.

The second specific aim is to genetically characterize the ovine model of defective gamma carboxylation via RT-PCR, cDNA sequencing, DNA sequencing, and animal genotyping, and develop a restriction fragment length polymorphism (RFLP) based assay for detecting the carrier state.

The third specific aim is to evaluate γ-glutamyl carboxylase kinetics by determining K_m and V_{max} values in terms of enzyme interaction with the synthetic substrate FLEEL, vitamin K, and vitamin K dependent propeptides.

Coagulation Overview

Normal hemostasis is the result of a finely balanced process that maintains blood in a fluid state within normal vessels while maintaining the ability to respond to injury and induce a rapid and localized response culminating in the formation of a hemostatic plug or clot to limit blood loss from the vasculature. Hemostatic disorders result from shifting this balance to either a hypercoagulable state or a hemorrhagic state. Excessive bleeding, or abnormal hemostasis, may result from 1) acquired or heritable disease of vessels; 2) acquired or congenital platelet deficiency or dysfunction; 3) derangements in the coagulation cascade, or combinations of the three.[8,58]

The Vasculature

Intact vessels, more specifically endothelial cells, serve primarily to inhibit platelet adherence through antithrombotic properties including: 1) the antiplatelet factors-endothelial prostacyclin (PGI_2), CD39, and nitric oxide; 2) anticoagulants - including heparin-like molecules, the protein C pathway, and the tissue factor pathway inhibitor; and 3) fibrinolysis via tissue plasminogen activator.[46,53,54,59] Endothelial cells, through injury, excessive shear force, or increased turbulence become prothrombotic[32,74] via the production and secretion of von Willebrand's factor, expression of surface tissue factor, secretion of platelet activating factor, secretion of thrombin, and secretion of inhibitors of plasminogen activator.[47,59,69,88]

Platelets

Following endothelial injury, platelets encounter and bind, via guanosine triphosphate binding proteins (G proteins),[34] components of the extracellular matrix normally sequestered beneath the endothelium. These include collagen, fibronectin, proteoglycans and other adhesive glycoproteins, which results in platelet adhesion, shape change, secretion, and aggregation.[54,59] Platelet adhesion to the extracellular matrix, specifically collagen, is mediated through von Willebrand's factor and its glycoprotein receptor GpIb.[7,17] Genetic deficiencies of these occur in either von Willebrand's disease or Bernard-Soulier syndrome respectively.[4,7,51]

Following platelet G protein-receptor agonist binding, platelets secrete the contents of platelet α- and dense-granules, generate thromboxane, and activate coagulation reactions leading to the production of thrombin.[51] The release of granule contents provides required calcium for the coagulation cascade, as well as ADP, serotonin, and platelet activating factor (PAF) resulting in platelet aggregation and formation of a primary hemostatic plug[33,59](Fig 1.1). This primary hemostatic plug is unstable and soluble[51,89] until factor XIII is activated by thrombin resulting in cross-linking of fibrin strands, and stabilization of the fibrin/platelet clot.[33,51,89] Finally, platelet activation leads to the exposure of a phospholipid complex on the platelet surface, which provides a surface for binding of coagulation factors of the coagulation cascade (Fig. 1.2).[59]

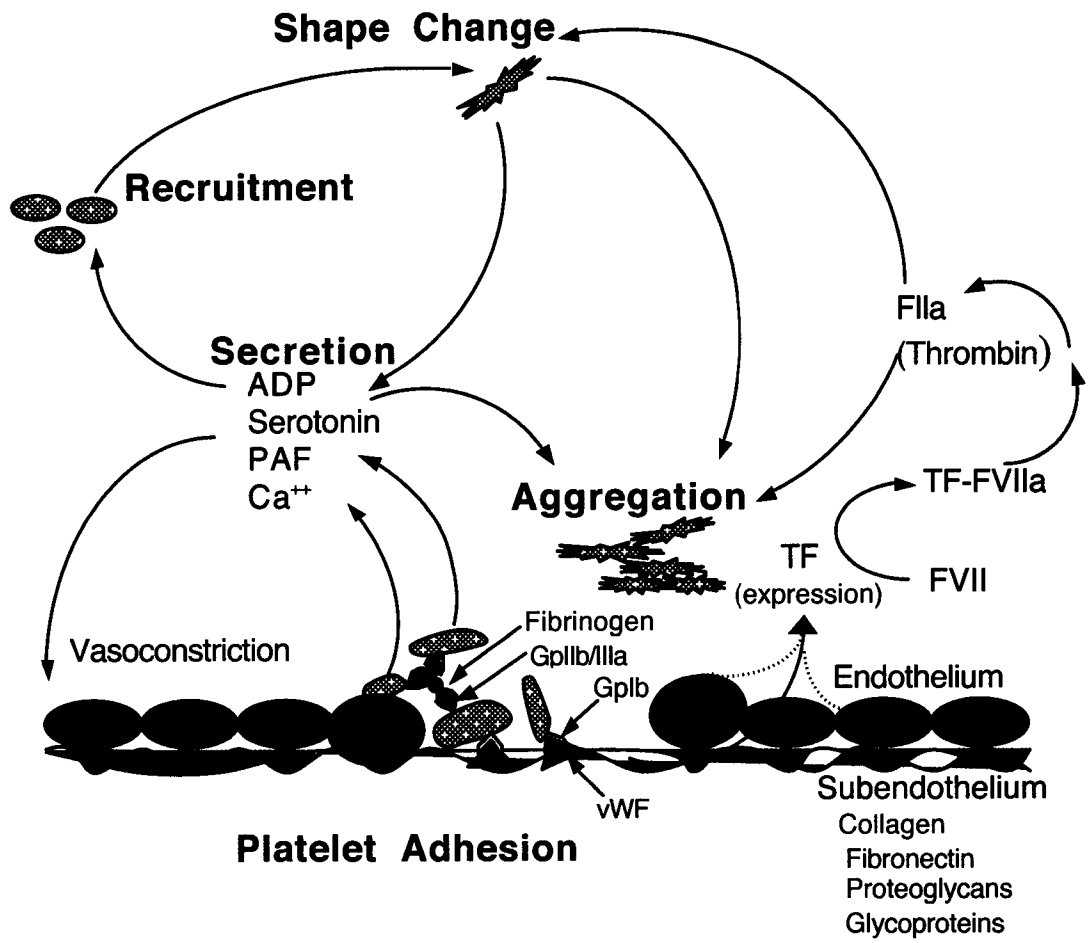


Fig. 1.1 Platelet activation and the initial stages of hemostasis. Damage to endothelial cells exposes subendothelium initiating platelet adhesion via GpIb/vWF and activation resulting in release of α and dense granule contents that recruit additional platelets, cause vasocontriction, and platelet shape change. The generation of thrombin via the extrinsic pathway accelerates the process. ADP=adenosine diphosphate, PAF=platelet activating factor, Ca^{++}=calcium, TF=Tissue factor/Thromboplastin, GpIb & GpIIb/IIIa=glycoprotein platelet receptors, vWF=von Willebrand factor.

The Coagulation Factors

The coagulation cascade comprises the third component of the hemostatic process. The coagulation cascade is a series of steps in which inactive proenzymes are converted to active enzymes, culminating in the formation of thrombin and the conversion of soluble fibrinogen to insoluble fibrin.[8]

The coagulation cascade has traditionally been divided into the intrinsic and extrinsic pathways converging with both pathways activating coagulation factor X (Fig. 1.2). This division is not real because tissue factor/factor VIIa complex is a potent activator of factor IX as well as factor X, and the concept of separate cascades activating coagulation is a result of early testing methods commonly used to evaluate coagulation factors.[9,44,45,78] The principal initiating event in normal coagulation is tissue factor complexed with factor VII exposed to blood flow following vascular injury.[14] Tissue factor is normally expressed on vascular smooth muscle and adventitial fibroblast cell membranes complexed with factor VII. The complex becomes activated on exposure to plasma and binds either factor IX or X, activates IX or X, then releases the factor to repeat the process until inactivated by tissue factor pathway inhibitor.[25,61](Fig. 1.2). The intrinsic pathway is essentially an amplification loop that functions to form more thrombin after small amounts of thrombin are generated; thrombin activates factor XI, which then completes the loop of activation (Figure 1.2). Each reaction in the pathway results from the assembly of a complex comprised of an enzyme (activated coagulation factor), a substrate

(proenzyme form of coagulation factor), and a cofactor, typically assembled on a phospholipid complex and held together by calcium ions.[59]

Once the coagulation cascade has been activated it is important for the reaction to remain restricted to a localized site to prevent pathologic clotting of the systemic vasculature. Localization of clot formation is maintained by restricting factor activation to sites of phospholipid complex formation (activated platelets and endothelium) and by three types of anticoagulants: Antithrombins, Proteins C and S (vitamin K-dependent anti-coagulant proteins), and Plasmin.[59]

The biological function of platelets and the coagulation cascade may be assed by various laboratory methods. Platelet numbers and function may be evaluated by a complete blood count, *in vitro* platelet aggregometry with various platelet agonists, and the bleeding time test, which has also been used as a means of assessing platelet and injured vessel wall interaction.[55,57,72] Unfortunately, it is relatively insensitive and, in many cases, nonspecific,[72] but does lend insight into the biological function of the platelets.[89] Coagulation factors are assessed by the one-stage prothrombin time (PT) which tests the function of the extrinsic and common pathways, and the activated partial thromboplastin time (aPTT) which evaluates the intrinsic and common pathways (Fig. 1.3). A similar, albeit crude, evaluation of the intrinsic and common pathways may also be performed by the activated clotting time (ACT), which also takes into account platelet number and function.[58] The common pathway may be assessed independent of either the intrinsic or extrinsic systems via the Russell viper venom test.[24]

Fig. 1.2 The *in vivo* Clotting Cascade. The central precipitating event is considered to involve tissue factor (TF), which under physiologic conditions is expressed on the membrane surface of vascular adventitial fibroblasts and smooth muscle cells, and is not exposed to the blood. With vascular or endothelial cell injury, TF and already bound factor VII becomes activate to convert factor IX to IXa and factor X to Xa. The "intrinsic pathway" includes "contact" activation of factor XI by the XIIa/activated high-molecular-weight kininogen (HKa) complex. Factor XIa also converts factor IX to IXa and factor IXa in turn converts factor X to Xa, in concert with factor VIIIa and PL (the "tenase" complex). Factor Xa is the catalyst of the "prothrombinase" complex, which converts prothrombin to thrombin. Thrombin cleaves fibrinopeptides from fibrinogen, allowing the resultant fibrin monomers to polymerize, and converts factor XIII to XIIIa, which crosslinks (XL) the fibrin clot. Thrombin accelerates the process (interrupted lines) by its potential to activate factors V and VIII, Natural plasma inhibitors retard clotting: C1-inhibitor (C1 INH) neutralizes factor XIIa, tissue factor pathway inhibitor (TFPI) blocks factor VIIa/TF, and antithrombin III (AT-III) blocks factors IXa and Xa and thrombin. Thrombosis and Hemostasis, Coleman et al 2001

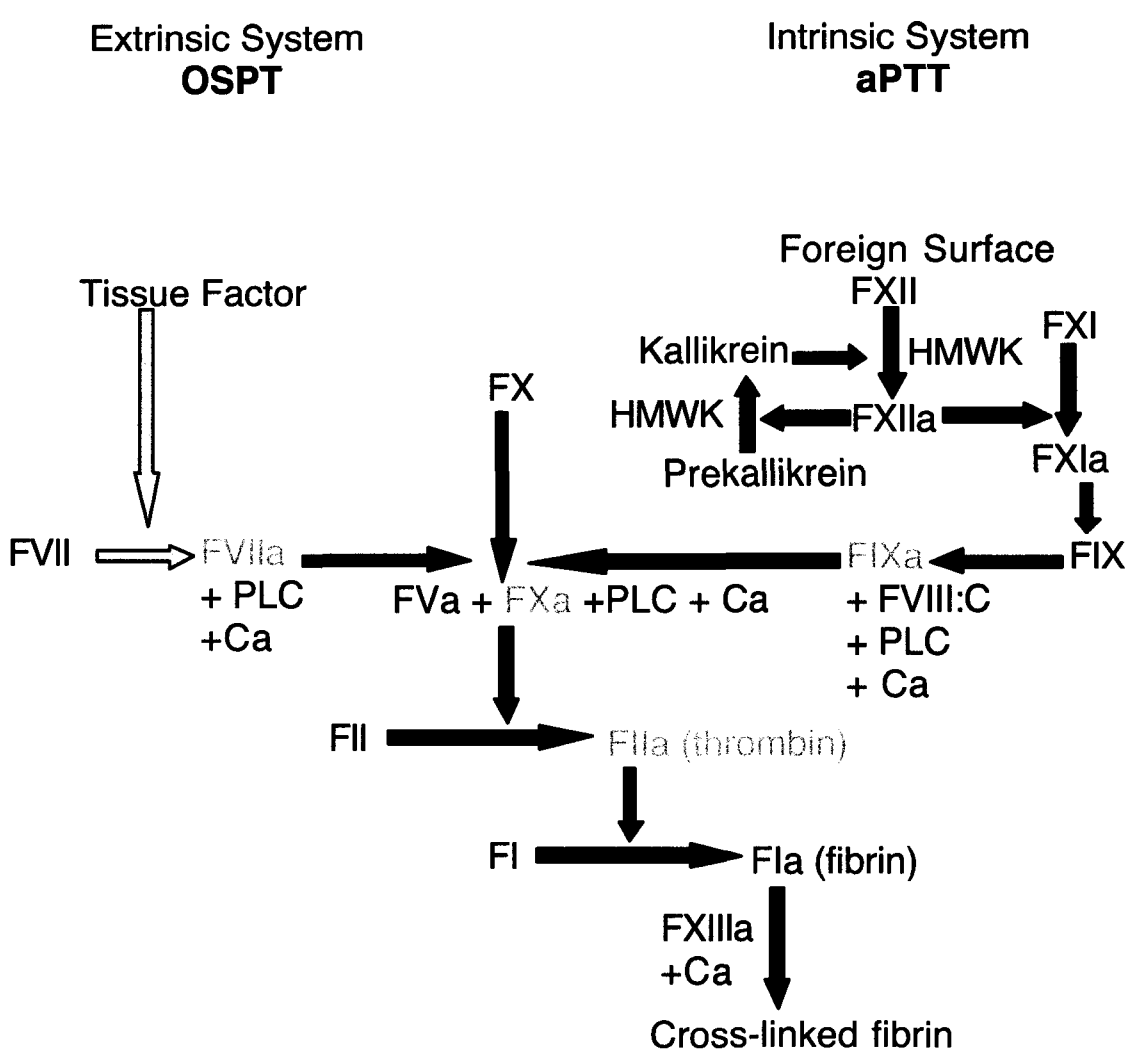


Figure 1.3 Traditional Coagulation Cascade. Yellow arrows designate the extrinsic pathway, Green arrows designate the common pathway, Blue arrows designate the intrinsic pathway. HMWK=High Molecular Weight Kininogen, PLC=Phospholipid Complex, Ca=Calcium ions, Coagulation factors designated by pink coloring represent the vitamin K-dependent procoagulant factors.

Vitamin K-dependent Proteins, and γ-Glutamyl Carboxylase

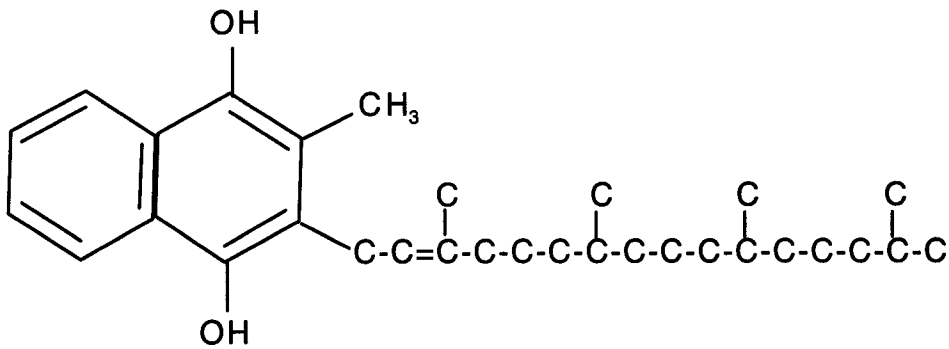
Vitamin K was first discovered in 1929 by Henrik Dam[19] via an observed association between a dietary deficiency and bleeding in sterol metabolism studies of chicks.[20] Dam proposed the antihemorrhagic factor was a new fat soluble vitamin he termed "Koagulations vitamin" or vitamin K.[18,21] Green plants make Vitamin K_1, or phylloquinone. The term vitamin K_2 includes a groups of compounds made by bacteria that are termed menaquinones (MK-n) in which n is the number of isoprene groups in the side chain.[90] Dietary vitamin K is absorbed in the proximal small intestine in a chemically unchanged form,[77] it is incorporated into chylomicrons, secreted into lymph, and delivered to the liver and other tissues in the form of chylomicron remnants.[43]

Initially, the only known biological function of vitamin K was as a cofactor for the microsomal enzyme γ-glutamyl carboxylase.[5] The coagulation factors II, VII, IX, X, protein C, protein S require vitamin K for normal biosynthesis[84,87] and are termed vitamin K-dependent (VKD) proteins. The NH_2 terminal of these proteins contains 9-12 glutamate amino acids (Glu) which are converted to γ-carboxyglutamate (Gla) by the addition of a COOH group in a post-translational modification on the inner surface of the endoplasmic reticulum.[29,30,38,63,84,87,94,103] If Glu residues are not carboxylated, the factors are synthesized and secreted to circulate in the plasma but lack biological function. These proteins are termed PIVKA (proteins in vitamin K absence or antagonism).[92] Additionally, there are two VKD proteins essential for normal bone metabolism-osteocalcin and matrix Gla protein.[37] Other VKD proteins include the Gla containing ligand/receptor tyrosine kinase complex and Axl/gas6

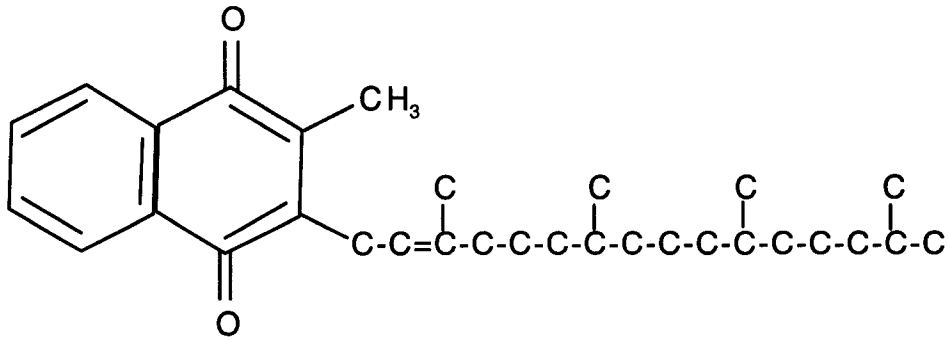
(growth-arrest-specific gene 6), which are shown to regulate cell growth, inhibit apoptosis, and participate in cell transformation.[1,5,28,52,62,75,91] Thus the biological role of vitamin K or vitamin K inhibitors can impact many important biological processes.

Gamma-glutamyl carboxylase is a trans-membrane protein that faces the inner surface of the endoplasmic reticulum and exhibits carboxylase activity, resulting in the conversion of Glu residues to calcium binding Gla. The carboxylase is highly conserved between species and is present in numerous tissues.[27,39,93] The enzyme has a coupled epoxidase function, which converts vitamin K_1H_2 hydroquinone (K_1H_2) to vitamin K_1 2,3 epoxide (Fig. 1.5).[31,86,90] This coupled reaction provides the energy for the carboxylase residue conversion.[10,22,23] The vitamin K_1 2,3 epoxide is recycled to vitamin K_1H_2, in a two-step process by vitamin K epoxide reductase, an enzyme sensitive to inhibition by warfarin[100] (Figs. 1.4 &1.5). This complex process of γ-carboxylation allows for coagulation proteins to function normally and participate in a sequential reaction process culminating in the formation of a stable fibrin clot.[22]

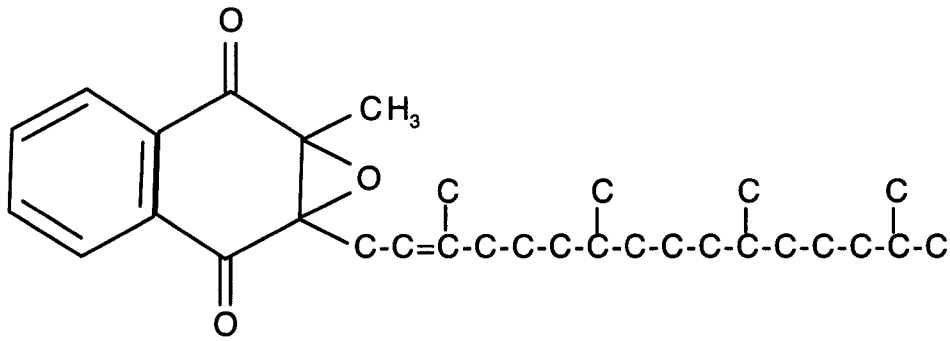
An additional enzyme, DT-diaphorase, an NAD(P)H dehydrogenase, reduces the quinone form of vitamin (K_1) to the hydroquinone form, but not the vitamin K_1 2,3 epoxide.[87] This enzyme requires supraphysiologic concentrations of vitamin K_1[97] and is unlikely to play a role in vitamin K recycling at physiologic tissue concentrations of vitamin K. This enzyme does play an important role when vitamin K_1 is used to reverse the effects of warfarin, or other vitamin K antagonists.



Vitamin K_1H_2 Hydroquinone



Vitamin K_1 Phylloquinone



Vitamin K_1 2,3 Epoxide

Figure 1.4 Chemical Structures of Vitamin K

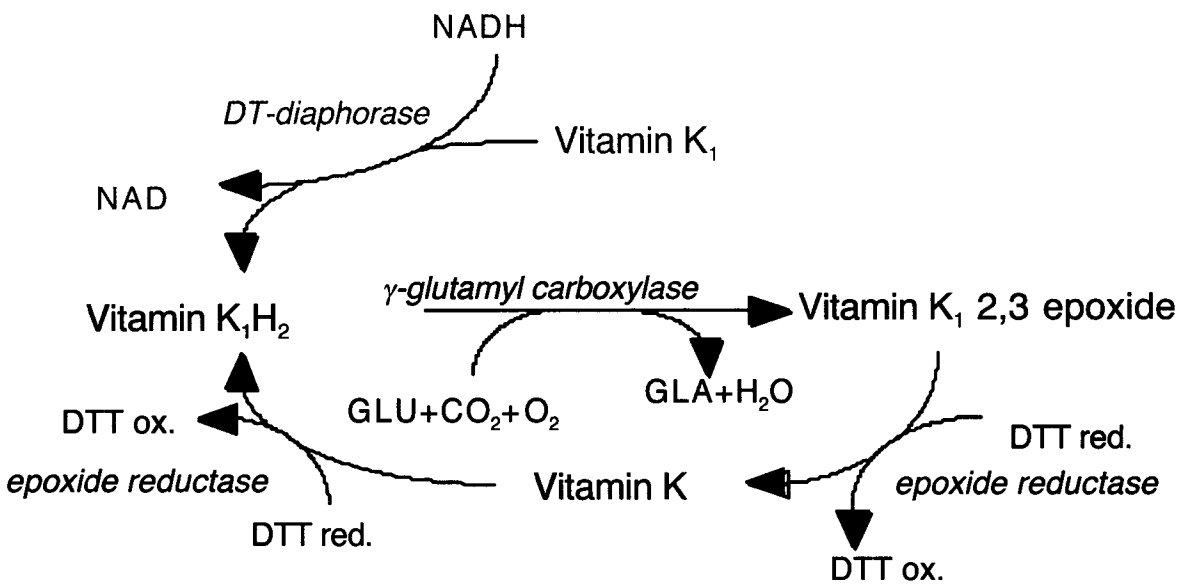


Figure 1.5 Vitamin K Cycle. DTT=dithiothreitol. Glutamic acid (Glu) is carboxylated to γ-carboxyl glutamic acid (Gla) at the expense of CO_2, O_2, and vitamin K_1H_2 to yield H_20 and vitamin K_1 2,3 epoxide. Vitamin K_1 2,3 epoxide is reduced to vitamin K_1H_2 by pathway #2 at physiologic concentrations of vitamin K by epoxide reductase possibly using DTT as an electron donor, and by pathway #1 (blue font) by an NADH reductase at supraphysiologic concentrations of vitamin K.[96] Pathway #2 is inhibited by warfarin, pathway #1 is not.

Gamma carboxylation is the addition of a carboxyl group to the gamma carbon of a Glu amino acid in a peptide substrate,[22] and occurs on the inner surface of the endoplasmic reticulum.[13] Gamma-glutamyl carboxylase itself has 3 Glu residues and converts these residues to Gla prior to carboxylating the NH_2-terminal Glu residues of VKD peptide sequences.[6] Subsequently, the enzyme binds K_1H_2, oxygen, carbon dioxide, and the propeptide sequence of vitamin K-dependent proteins.[70,83] Upon binding the carboxylase, the propeptide assumes a helical conformation facilitating binding of K_1H_2 to the enzyme complex.[29,101] After processively (the carboxylase does not release until the protein is fully carboxylated[60]) carboxylating all the Gla residues, the peptide chain is then released and transported to the trans-golgi where the propeptide sequence is cleaved off. Subsequently, the carboxylated protein is secreted to the plasma for circulation.[29,30] Variations in the respective propeptide sequences result in different binding affinities between the various VKD proteins[81,82] and γ-glutamyl carboxylase. Currently, the propeptide recognition site of γ-glutamyl carboxylase is controversial and has been mapped to different regions of the peptide sequence;[75,103,104] however, most studies strongly support a putative binding site at residues 495-513 of γ-glutamyl carboxylase.[48,71]

Occasional spontaneous defects of gamma-carboxylation in humans[12,16,26,35,36,41,56,68,80,95] and Devon Rex cats[49,50,79] have been reported, but explanations of the underlying cause are mostly limited to studies involving enzyme kinetics and response to vitamin K supplementation. The reported carboxylase defects involving Devon Rex cats could be corrected with supraphysiologic vitamin

K_1, and the authors speculate this was due to a heritable mutation involving the propeptide binding region of the γ-glutamyl carboxylase gene;[79] however, this hypothesis was never confirmed and these cats were lost to further studies.

Two spontaneous cases of defective carboxylation in humans have been examined in sufficient detail to determine the underlying genetic defect that resulted in abnormal carboxylation and have defined important biologic binding sites in the γ-glutamyl carboxylase gene.[11,80,102] Additionally, site-directed mutagenesis studies, using human recombinant γ-glutamyl carboxylase, followed by examination of the resulting enzymatic activity have been helpful in determining the structure and functional relationships of γ-glutamyl carboxylase.[30]

The most important physiologic defect associated with defective γ-glutamyl carboxylase pertains to coagulation; however, the role of γ-carboxylation in bone formation, vascular mineralization, cell signaling, and embryonic development are active topics of investigation.[40,62,66,73,75,98,99,105] Until this time there has not been a suitable animal model to study the clinical implications of γ-glutamyl carboxylase dysregulation. This model will provide a unique and valuable resource with which to evaluate many aspects of normal γ-glutamyl carboxylase biology, and help to elucidate the function of more recently discovered VKD proteins.

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CHAPTER 2

Defective γ-glutamyl Carboxylase and Bleeding in Rambouillet Sheep

Abstract

A flock of Rambouillet sheep was examined because of increased lamb mortality due to ineffective hemostasis at parturition. Neonatal affected lambs were distinguished by inadequate hemostasis at the umbilicus, pale mucus membranes, and markedly prolonged activated clotting time (ACT). Affected lambs had consistently prolonged one-stage prothrombin times (PT) and activated partial thromboplastin times (aPTT) supporting a defect in both the intrinsic and extrinsic system of the coagulation cascade or a common pathway defect. Decreased activity of vitamin K-dependent procoagulant factors II, VII, IX, and X in male and female lambs suggested either a defect of the hepatic enzyme γ-glutamyl carboxylase, or vitamin K_1 2,3 epoxide reductase. Affected lamb hepatic γ-glutamyl carboxylase activity was markedly decreased compared to that of age and sex-matched control lambs while vitamin K_1 2,3 epoxide reductase was similar between the two groups, and supraphysiologic vitamin K_1 supplementation did not resolve clinical symptoms or significantly increase vitamin K-dependent procoagulant activities ruling out involvement of epoxide

reductase. Therefore, these findings confirming defective γ-glutamyl carboxylase activity as the cause of impaired coagulation. This herd represents the only naturally occurring viable animal model of hereditarily defective γ-glutamyl carboxylase.

Introduction

Vitamin K is a required cofactor for the post-translational enzymatic reaction converting glutamic acid residues (Glu) to γ-carboxylated glutamic acid (Gla).[27,28] This reaction (gamma carboxylation) is catalyzed by the enzyme γ-glutamyl carboxylase. Specifically, gamma carboxylation is the addition of a carboxyl group to the gamma carbon of glutamic acid[10] and occurs on the inner surface of the endoplasmic reticulum.[6] Proteins that share this processing before being secreted are referred to as vitamin K-dependent (VKD) proteins. Post-translational modification occurs for VKD proteins involved in hemostasis, bone metabolism, mineralization, growth control, signal transduction, and cell survival.[2,11,21] Gamma carboxylation allows calcium binding and activation of VKD proteins II, VII, IX, X, protein C, protein S[11] and osteocalcin-hydroxyapatite binding in bone.

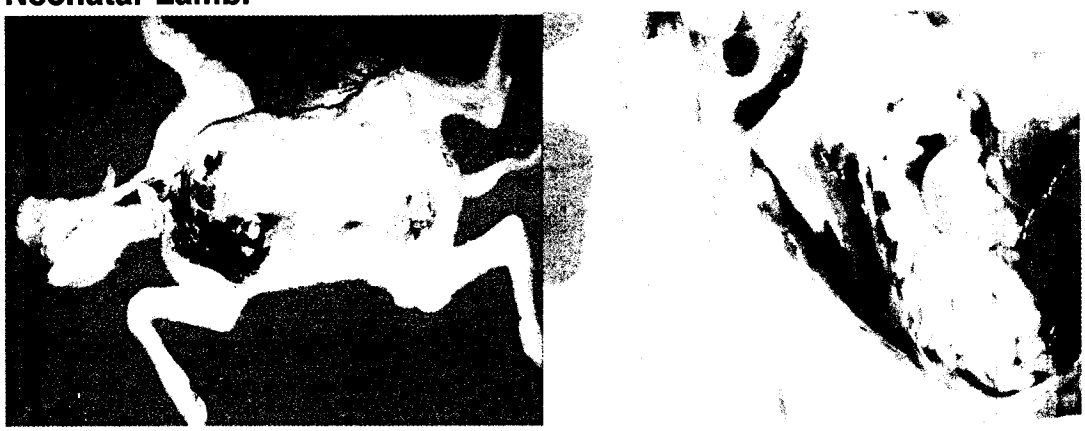
The energy for this reaction is provided through the oxidation of K_1H_2 subsequent to binding the propeptide region of VKD proteins.[3,10] Calcium binding Gla residues are formed and K_1H_2 is converted to vitamin K_1 2,3 epoxide. The vitamin K_1 2,3 epoxide is recycled to vitamin K_1H_2, in a two-step process by vitamin K epoxide reductase, an enzyme sensitive to inhibition by

warfarin and other vitamin K antagonists. This complex process allows for coagulation proteins to fold, bind receptors, and participate in a sequential reaction process culminating in the formation of a stable fibrin clot.[9]

We report a coagulopathy in Rambouillet sheep similar to man.[5,8,13,20] A preliminary report investigating this sheep flock eliminated vitamin K antagonism, or the presence of a circulating inhibitor as a cause for bleeding.[1] Affected lambs are born alive but lack the ability to achieve hemostasis of the umbilical artery and vein. Without intervention, newborn lambs continually bleed from the umbilicus and have extensive subcutaneous and body cavity hemorrhage resulting in death (figure 1). Breeding data suggests the coagulopathy is inherited as an autosomal recessive trait in sheep as in man because approximately equal numbers of male and female affected lambs are observed. While only approximately 10% of offspring are affected, no variation in either clinical presentation or enzyme activity is present between affected lambs. While this flock has a lower than expected percentage of affected lambs, when considering an autosomal inheritance pattern, we observe early *in utero* loss (via ultrasound) and resorption similar to that reported in γ-glutamyl carboxylase knock-out mice,[32] prothrombin deficient mice,[26] and factor V deficient mice,[31] which may explain the lower than expected percentage of affected offspring.

The purpose of this study was to determine the presence or absence of aberrant enzymatic activity involving normal cycling of vitamin K in clinically affected lambs, and to establish an association between abnormal vitamin K biology with the observed heritable coagulopathy.

Figure 1. Subcutaneous and Umbilical Hemorrhage in an Affected Neonatal Lamb.



Materials and Methods

All ewes were synchronized with vaginally inserted progesterone implants (Eazi-Breed CIDR®) followed by removal at 17 days and injection of 500Units of pregnant mare serum (Souix Biological). The ewes were turned into a pen with a known heterozygous ram fitted with a marking crayon. The carrier ram was determined by previous trail breeding resulting in affected offspring. All ewes were observed daily for marking, and delivery dates calculated from the last known breeding date. Ewes were monitored 24 hours a day near the expected parturition dates.

Coagulation Parameters

Activated clotting time (ACT) was determined for every newborn lamb. Tests were performed within 1 hour of birth using a Vaccutainer® containing 6-10mg of siliceous earth. Individual tubes were prewarmed to 37°C using a heating block. Lambs with an ACT longer than 5 minutes were considered affected and transfused with 120mls freshly thawed sheep plasma. Plasma was

prewarmed to approximately 37°C in a water bath prior to administration. Affected lambs were sedated with 0.1mg/kg subcutaneous xylazine and the jugular vein catheterized with a 22 gauge Insyte® catheter (Becton Dickinson). Plasma was slowly administered using a 60cc syringe attached to an extension set. Blood was collected in sodium citrate anticoagulant tubes for use in coagulation studies prior to transfusion. These plasma samples were either immediately frozen and stored at -80°C, or stored on ice and coagulation studies performed within 2 hours of blood collection. Prothrombin and partial thromboplastin times were determined with an MLA Electra 700 coagulation analyzer (Pleasantville, NY).

Coagulation Factor Activities

Individual coagulation factor activities were determined by plotting respective times against a standard curve generated for each coagulation factor. Briefly, blood was collected from 20 unrelated adult Rambouillet ewes into sodium citrate anticoagulant tubes (Becton Dickinson). Plasma samples from all 20 ewes were pooled, frozen and stored at -80°C prior to testing. Pooled sheep plasma was serially diluted with Owren's buffer (Sigma) at 1:2, 1:4, 1:8, 1:16, 1:32, 1:64, and 1:128. Standard curves for coagulation factors were determined by combining 50μl of each serial diluted plasma sample from pooled normal sheep plasma with 50μl of specific factor deficient plasma (George King Biomedical) using an MLA 700 coagulation analyzer. Times for each dilution were plotted on semi-logarithmic paper with 2 cycles X 70 divisions. A straight line was drawn through all dilution time points. Plasma samples from

affected and control lambs were serially diluted as above. Activity of coagulation factors were determined by combining 50μl of each serial diluted plasma sample with 50μl of specific factor deficient plasma using an MLA 700 coagulation analyzer and plotted against standard curves for each respective coagulation factor.

CBC, Clinical Chemistry, and Necropsy

Blood was collected at the time of parturition in affected and control lambs. A complete blood cell count was performed from EDTA anticoagulated whole blood with an ADVIA 120® (Bayer). A differential count of the leukon was determined by manually counting a freshly prepared blood film stained with Wright-Giemsa. A routine clinical chemistry panel was performed from serum collect from a clot tube using a Hitachi 917® (Roche Diagnostics) automated chemistry analyzer. Lambs were euthanized with an intravenous overdose of sodium pentobarbital. Liver was snap frozen in liquid nitrogen for evaluation of microsomal activity. All routine organs were sampled and collected in 10% neutral buffered formalin. Routine 5μm histologic sections were prepared from paraffin embedded blocks, stained with hematoxylin and eosin, and evaluated under light microscopy.

Preparation of Microsomes and Determination of Activity

Hepatocellular microsomes were prepared according to Kotkow *et al.*[15] using either fresh or snap frozen liver obtained from affected and control lambs. Briefly, liver samples were divided into ≤1cm cubes, weighed to approximately twenty-five grams, and combined with twenty-five mls of

homogenization buffer (150mM NaCl, 50mM Tris-HCl, 5% (v/v) glycerol, 1mM EDTA, 1mM benzamidine, 1mM phenylmethysufonyl fluoride, pH 7.5). Liver samples in homogenization buffer were disrupted using a commercial blender and subsequently homogenized using a 300A® homogenizer fitted with a 7mm saw-toothed generator (Pro Scientific Inc.). The homogenate was strained through gauze into a 25 X 89 mm centrifuge tube. Centrifuge tube weights were equilibrated with homogenization buffer and centrifuged at 4°C for ten minutes at 10,000 g. The supernatant was filtered through gauze into a second centrifuge tube and weight equilibrated. Tubes were centrifuged at 4°C for one hour at 130,000 g. The supernatant was discarded and the pellet resuspended in 3 mls of homogenization buffer. Microsomal protein concentration was determined according to the Lowry Method (Sigma Diagnostics). All microsomal assays were performed at a concentration of 10mg/ml unless stated otherwise.

Carboxylase activity was determined from standard reaction mixtures (125 μl) containing: 250 μg of microsomal protein, 0.8M $(NH_4)_2SO_4$, 28mM MOPS @ pH 7.5, 0.5M NaCl, 20μl 1% CHAPS, 3.6mM Phe-Leu-Glu-Glu-Leu (FLEEL), 8mM DTT, 5μl $NaH^{14}CO_3$, 220μM of vitamin K hydroquinone (K_1H_2), and 16μm proIX. The propeptide was generated by Macromolecular Resources (Colorado State University, Ft. Collins, CO, USA) based on residues –18 to –1 of the human proIX sequence.[24] The mixture was incubated at 25 °C for 30 minutes in sealed tubes. One milliliter of 10% trichloroacetic acid was added to stop the reactions. Unbound $^{14}CO_2$ was removed by gently boiling the mixture for 10 minutes, or until the volume was reduced by

approximately 90%. Total incorporation of $^{14}C\,O_2$ was determined by adding 6mls of Ecolyte scintillation cocktail, and counted for 5 minutes/sample on a Beckman LS 1801 scintillation counter. Data are expressed as dpm (degradation per minute) $^{14}C\,O_2$ incorporated per minute/ 250μg microsomal protein.

Vitamin K epoxide (KO) reductase activity was performed as described previously.[12] Vitamin KO reductase was prepared according to the procedure of Tishler et al.[29] Standard reaction mixtures (125 μl) contained: 250 μg of microsomal proteins, 25 mM Tris-HCl pH 7.4, 150 mM NaCl, 0.28 % CHAPS, 0.12% phosphatidyl choline, 5 mM $NaHCO_2$, 222 μM of vitamin KO and 6 mM DTT. No exogenous substrates were added. The extraction, purification and analysis of plasma samples were performed as reported previously.[12]

Glucose-6-phosphatase activity was determined according to the methods of Burch *et. al.*[7] Enzyme activity was determined by the reduction of NAD^+ in the reserve reaction where hexokinase converts glucose to glucose-6-phosphate. Reaction mixtures contained 100 μg homogenized liver microsomes combined with 100 μl reagent 1 at pH 6.8 (50 mM imidazole base, 50 mM imidazole HCl, 1 mM EDTA, 0.02 % bovine albumin, and 10 mM glucose-6-phosphate. The reaction mixture was incubated at 25°C for 60 minutes and then at 95°C for 2 min. To the reaction mixture was added 1 ml of reagent 2 at pH 7.2 (35 mM imidazole, 15 mM imidazole HCl, 75 mM KCl, 100 μM ATP, 300 μM p-pyruvate, 2 mM $MgCl_2$, 2 mM β-mercaptoethanol, 125 μM NADH, 2 μg/ml rabbit muscle pyruvate kinase, and 2.5 μg/ml lactate

dehydrogenase-beef muscle). The reaction mixture was transferred to a cuvette and read on a spectrophotometer at a wavelength of 340 nm. Subsequently, 0.3 U of hexokinase was added to the reaction, incubated at room temperature for 15 minutes, and read on a spectrophotometer at 340 nm.

Vitamin K administration

Three affected lambs were transfused with ovine plasma, as above, and subsequently administered 35mg vitamin K_1, subcutaneously every 24 hours. The dosage used is in excess of that reported to reverse the effects of vitamin K antagonism from warfarin in neonatal lambs.[19] Blood was collected to assess coagulation parameters and response to treatment.

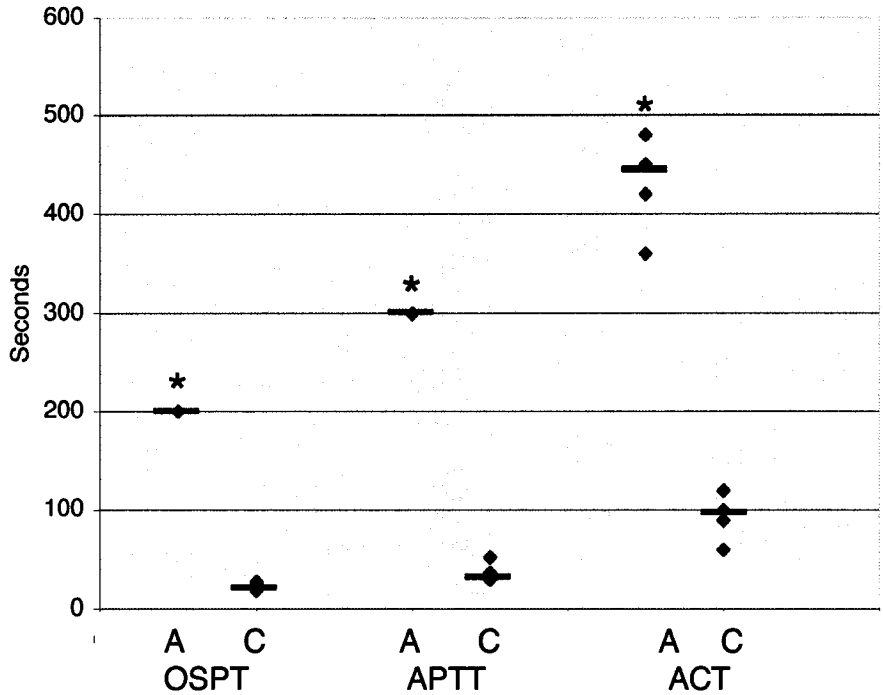
All statistical values were determined using StatView® 5.0.1 (SAS Institute).

Results

Coagulation Parameters.

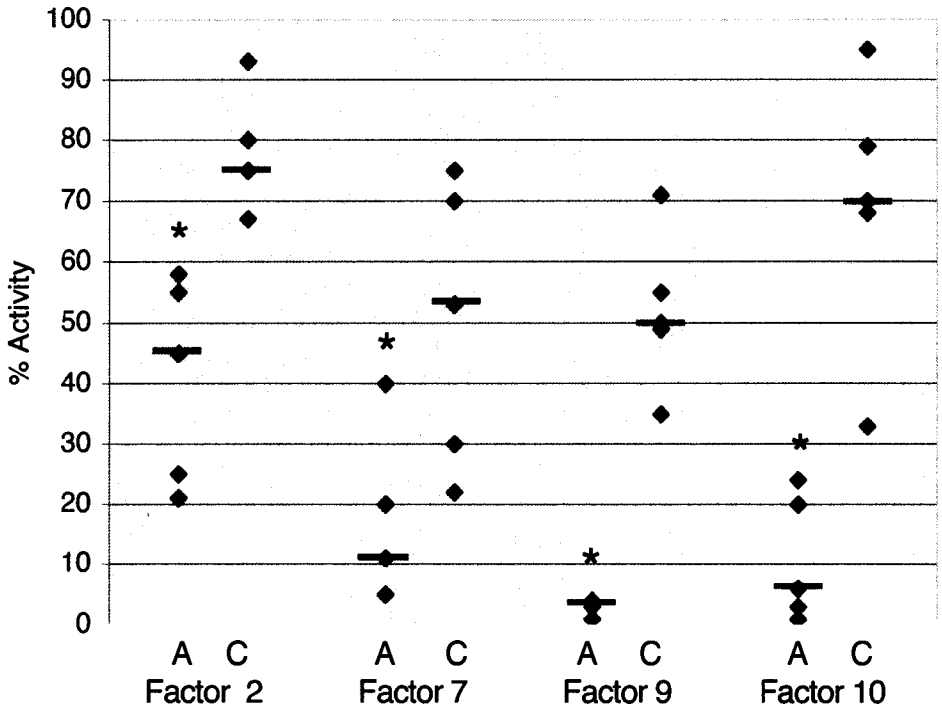
All affected lambs had markedly prolonged prothrombin and activated partial thromboplastin times (Fig. 2.1), decreased vitamin K-dependent procoagulant factor activities (Fig. 2.2), and similar activity of non-vitamin K-dependent coagulation factor activity (Fig. 2.3) compared to age matched control lambs.

Fig. 2.1 Coagulation Parameters. Affected and control lambs, day 0, prior to plasma administration.



A=Affected lambs (N=5)
C=Control lambs (N=5)
OSPT=One-stage prothrombin time
aPTT=Activated partial thromboplastin time
ACT=Activated clotting time
Each blue triangle represents a single data point. The horizontal black bar represents the median of each group.
*Represents statistical significance between affected and control lambs (Mann-Whitney non-parametric analysis).

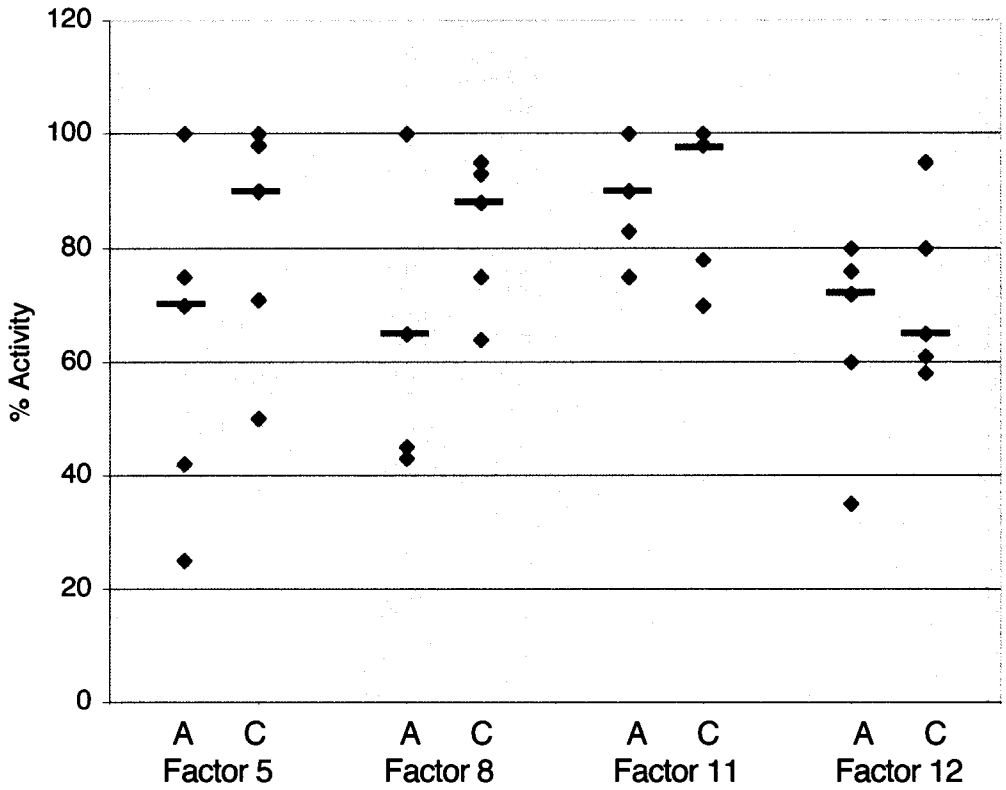
Fig. 2.2 Vitamin K-Dependent Factor Activity. Affected and control lambs, day 0, prior to plasma administration.



A=Affected lambs (N=5)
C=Control lambs (N=5)
Blue triangles represent individual data points as a percentage of coagulation n factor activity. A horizontal black bar represents the median.

* Represents statistical significance (Mann-Whitney non-parametric analysis) between affected and control lambs.

Fig. 2.3 Non-Vitamin K-Dependent Factor Activity. Affected and control lambs, Day 0, prior to plasma administration.



A=Affected lambs (N=5)
C=Control lambs (N=5)
Blue triangles represent individual data points. A horizontal black bar represents the median of each group.
There is no statistical difference between the affected and control lambs for each represented coagulation factor (Mann-Whitney non-parametric analysis).

The results of gross and microscopic examination of clinically affected lambs at necropsy were unremarkable. Serum biochemistry and CBC results, at parturition, were within the reference range for all parameters except for mildly elevated sorbitol dehydrogenase (25-29 IU/L; normal range 10-23 IU/L), which was contributed to mild periparturient hepatocellular injury.

Microsomal Activity.

Because the fatal coagulopathy in affected lambs was related to vitamin K metabolism, we investigated the activity of the enzymes involved in the vitamin K cycle (Fig. 2.4). Microsomes prepared from age-matched control lamb liver had incorporation of $^{14}C\,O_2$ in the substrate, while microsomes from affected lambs had markedly diminished incorporation of $^{14}C\,O_2$ (Fig. 2.5). As a control for biological activity, we tested the DTT dependent epoxide-reductase activity and glucose-6-phosphatase activity, from one of the effected lambs. Activities were similar between the affected lamb and age matched control lamb (Fig. 2.6).

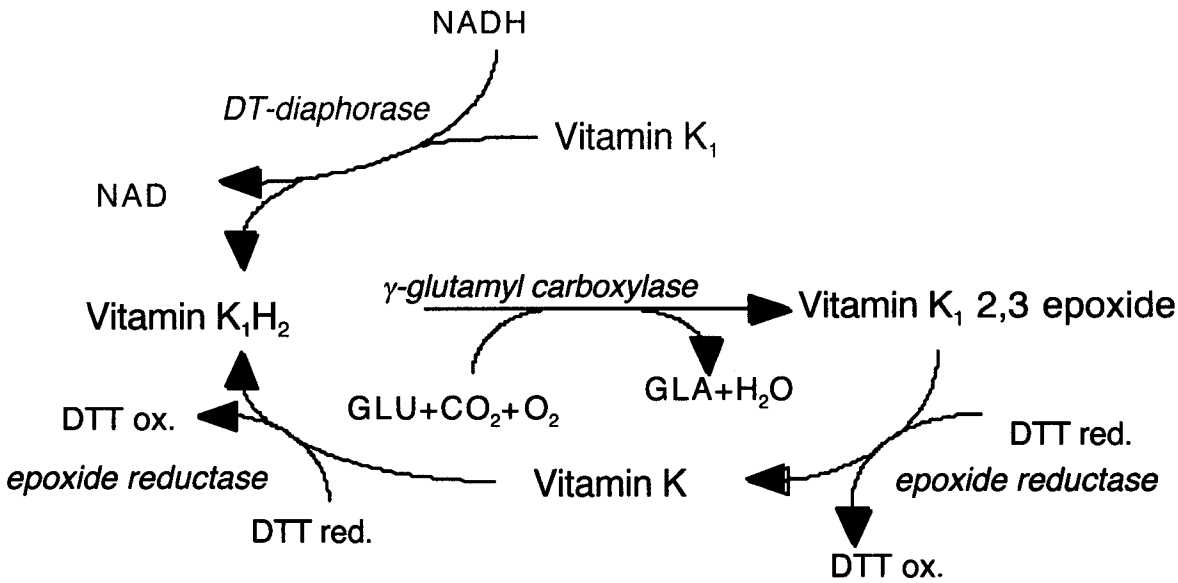
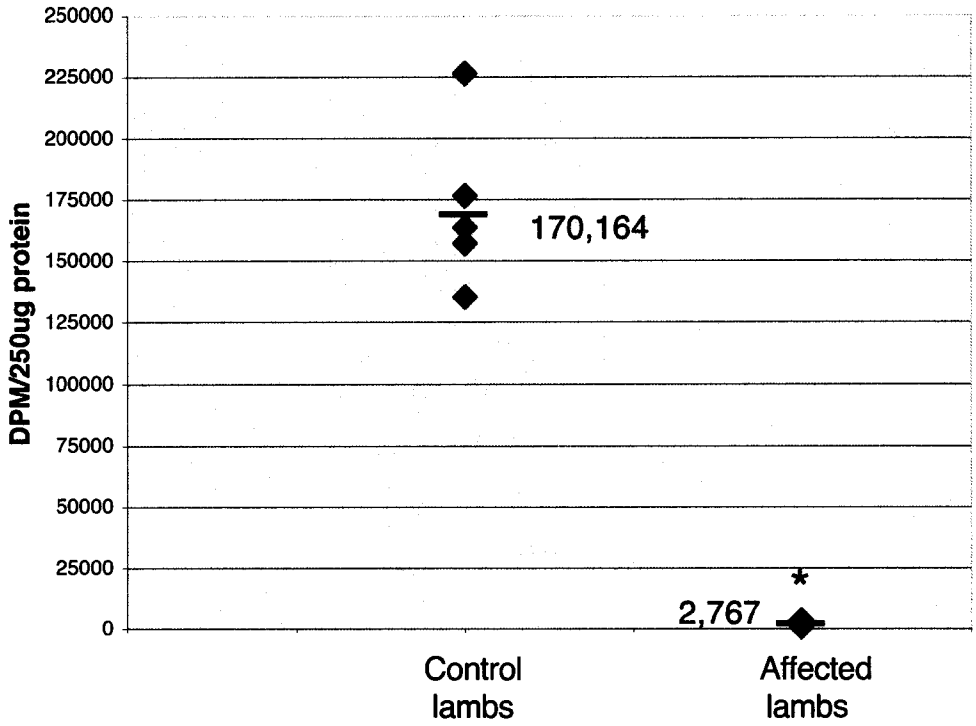


Figure 2.4 Vitamin K Cycle. DTT=dithiothreitol. Glutamic acid (Glu) is carboxylated to γ-carboxyl glutamic acid (Gla) at the expense of CO_2, O_2, and vitamin K_1H_2 to yield H_20 and vitamin K_1 2,3 epoxide. Vitamin K_1 2,3 epoxide is reduced to vitamin K_1H_2 by pathway #2 at physiologic concentrations of vitamin K by epoxide reductase possibly using DTT as an electron donor, and by pathway #1 by an NADH reductase at supraphysiologic concentrations of vitamin K.[30] Pathway #2 is inhibited by warfarin, pathway #1 is not.

Fig. 2.5 Gamma-Glutamyl Carboxylase Activity. Affected and control lambs from crude liver microsomal preparations.



Blue triangle represent individual data points and are expressed as degredation per minute per 250 micrograms protein per reaction well. The median of each group is represented by a horizontal black bar.

*represents statistical significance between the two groups (Mann-Whitney non-parametic analysis).

N=5

Fig. 2.6 Vitamin K epoxide reductase activity. Affected and control lamb from crude liver microsomes.

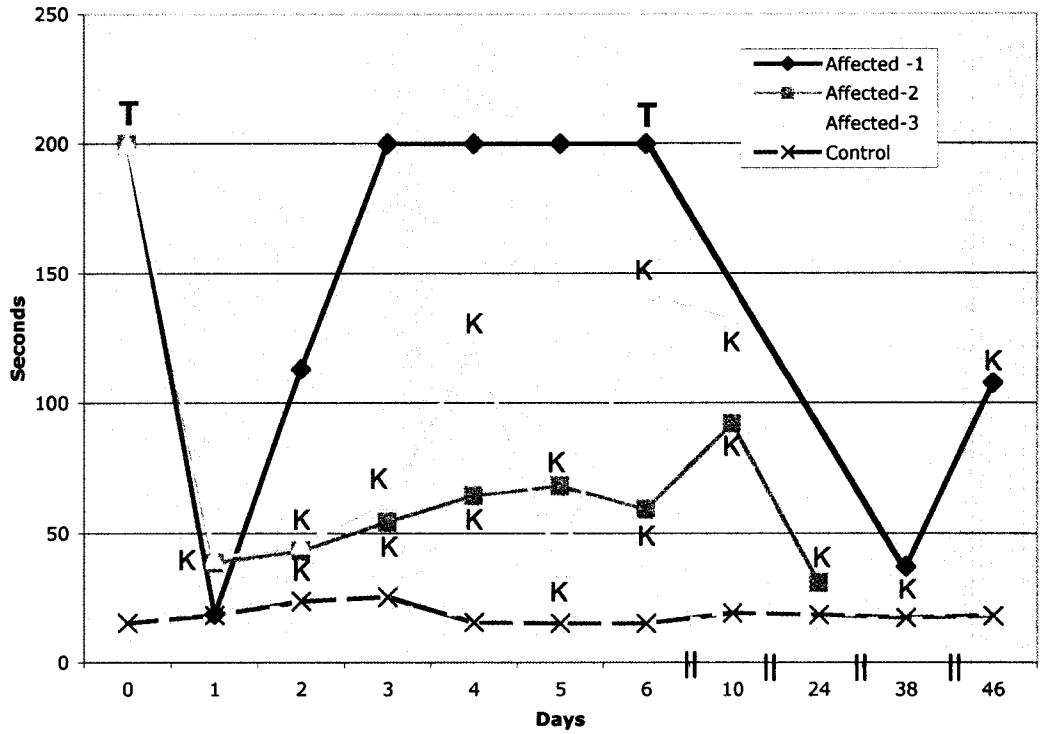
Microsomes	*Epoxide-reductase*			
	t= 0 min		t= 30 min	
	KO (%)	K (%)	KO (%)	K (%)
Control lamb	91	9	18	82
Affected lamb	88	12	39	61

Vitamin K 2,3 epoxide (KO) and vitamin K_1 (K) are expressed as a percentage of the total amount of vitamin K measured (K + KO).

Response to Vitamin K therapy.

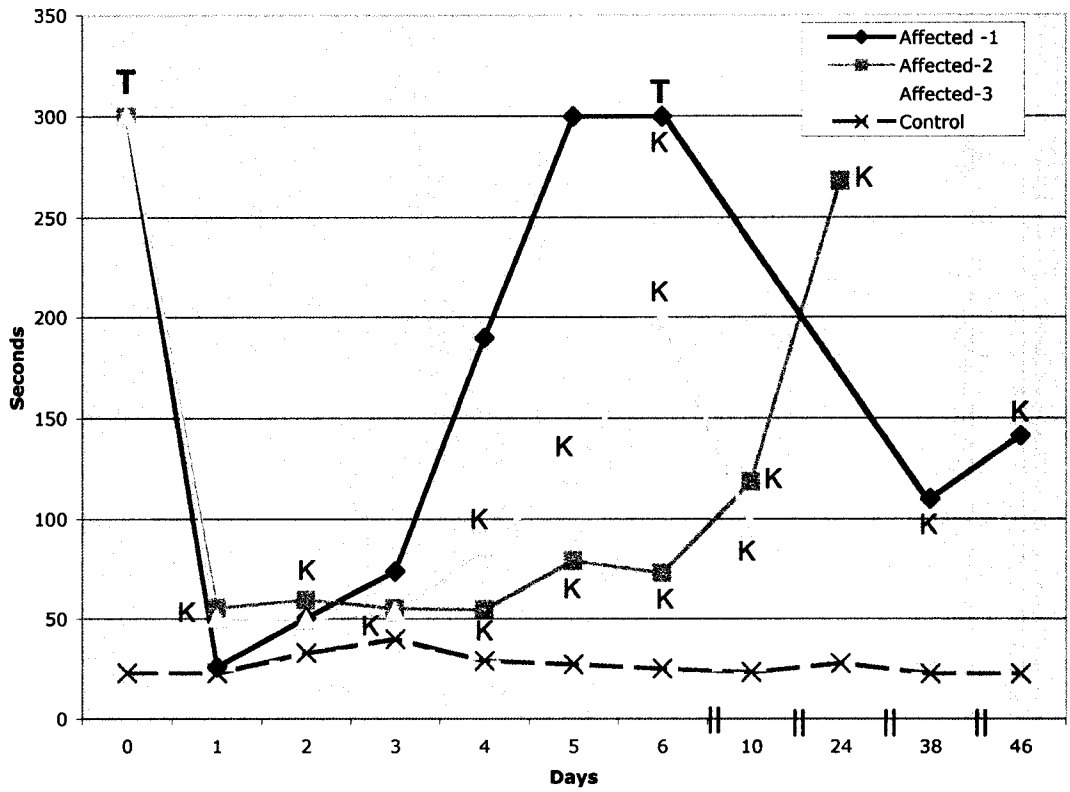
Two lambs (lambs 1 & 2) were administered supraphysiologic vitamin K_1, immediately following plasma transfusion and every 24hours thereafter. These lambs had PT's and aPTT's between 2-8 times that of a normal age-matched control lamb, but did not require additional plasma transfusions for approximately 30 days; however, these lambs were susceptible to life-threatening hemorrhage secondary to trauma suggesting a tenuous state of hemostasis. In contrast, a third affected lamb (lamb 3), which did not receive supraphysiologic vitamin K_1 after plasma administration, reached the cut-off point of the analyzer by day 5 (Fig. 2.7 & 2.8). Subsequently, lamb 3 was again transfused with plasma, administered supraphysiologic vitamin K_1, and demonstrated a similarly prolonged transfusion interval as did lambs 1 & 2. No significant difference between administration, and lack of administration of vitamin K_1 was observed for the vitamin K-dependent coagulation factor activities.

Fig. 2.7 Prothrombin Times. Affected and control lambs with vitamin K administration



All lambs received 120mls ovine plasma on Day 0 (T)
Yellow and pink (affected lambs 2 & 3) were administered 35mg vitamin K_1, subcutaneously, every 24 hours (K).
Blue (affected lamb 1) was transfused (T) with 120mls ovine plasma on Day 6, and subsequently administered 35mg vitamin K_1, subcutaneously, every 24 hours (K)

Fig. 2.8 Activated Partial Thromboplastin Times. Affected and control lambs administered vitamin K.



All lambs received 120mls ovine plasma on Day 0 (T)
Yellow and pink (affected lambs 2 & 3) were administered 35mg vitamin K_1, subcutaneously, every 24 hours (K).
Blue (affected lamb 1) was transfused (T) with 120mls ovine plasma on Day 6, and subsequently administered 35mg vitamin K_1, subcutaneously, every 24 hours (K).

Discussion

The fatal coagulopathy observed in the affected lambs in this report was the result of significantly reduced levels of vitamin K-dependent coagulation factor activity. Investigation excluded hepatic disease, fat malassimilation, and vitamin K antagonism as potential causes.[1] We therefore analyzed hepatic enzymes involved in the vitamin K cycle. We successfully demonstrated markedly reduced activity of the enzyme γ-glutamyl carboxylase, while activity of epoxide reductase was similar between affected and control lambs.

Interestingly, administration of vitamin K_1 (35mg/day, a dosage exceeding the reported requirement to counteract the effects of warfarin in newborn lambs[19]) to three of the affected lambs resulted in prolongation of the required transfusion interval to maintain adequate hemostasis; however a significant difference in vitamin K-dependent coagulation factor activity was not observed. Lambs supplemented with vitamin K_1 alone remained in a tenuous state of coagulation and rapidly manifested clinical signs of the described fatal coagulopathy in the face of minor trauma.

While supraphysiologic vitamin K_1 did not correct the observed coagulopathy, as reported in some cases of human GGCX deficiency[4,18,23] and Devon Rex cats,[17,22] we did observe a clinical response to therapy, as the PT and aPTT times could be maintained 2-8X longer than normal, and the required plasma transfusion interval was clearly prolonged.

Interestingly, the response observed in Devon cats was hypothesized to be the result of a mutation in the γ-glutamyl carboxylase gene resulting in

decreased affinity for the propeptide sequences of vitamin K-dependent proteins. Unfortunately, this hypothesis could not be proven as these cats were lost to further investigation; however, comparative substitution studies, at critical amino acid positions can reduce the propeptide binding affinity from 10 to 600 fold.[24]

Comparison between response to vitamin K_1 in these lambs and Devon Rex cats suggests that if a mutation is present in the γ-glutamyl carboxylase gene, it involves more than just the enzyme-substrate active site; therefore, the γ-glutamyl carboxylase defect observed in these lambs may be the result of a larger sequence deletion, translocation, or mutation than reported in human cases of defective γ-carboxylation.[4,23]

The affected lambs described in this report have variably reduced activity of the VKD proteins. While coagulation factors IX and X have minimal activity, the activity of coagulation factors II and VII are consistently higher. Coagulation factor activity in normal sheep relative to humans has been reported and is similar.[14] Standard curves generated from normal sheep did not demonstrate an inhibitory phase for any of the coagulation factors tested, which excludes the notion of inadequate dilution contributing to relatively higher activity levels in coagulation factors II and VII. Interestingly, the prothrombin propeptide reportedly has the lowest affinity of the coagulation factors tested[25] yet maintains the highest relative activity in affected lambs. Lin et al. propose that the rate of release of the propeptide appears to be the rate-determining step in substrate turn-over; therefore, substrates whose propeptides have relatively low affinities

for the carboxylase have increased rates of turnover.[16] This scenerio may explain the higher observed activity for FII, but not the activity of FVII relative to its reported affinity. The FVII propeptide is unique in that in contains a di-glutamate pair and appears to act as a glutamate substrate for the carboxylase. We cannot discount the fact that this glutamate pair may interfere with the relative affinity and subsequent activity of the coagulation factor. Unfortunately, the propeptide sequences of the ovine coagulation factors are not known, it is possible that species variation between the respective propeptide sequences may also be contributing to the reported propeptide affinities and influencing the individual factor activity *in vivo*.

We have determined that the observed coagulopathy in affected Rambouillet lambs is the result of markedly reduced activity of the enzyme γ-glutamyl carboxylase. Determination of the underlying genetic defect will certainly lend insight into the carboxylase active site, and expand our understanding of the structure and functional relationships of γ-glutamyl carboxylase.

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Chapter 3

Truncated γ-Glutamyl Carboxylase and Bleeding in Rambouillet Sheep.

Abstract

A flock of Rambouillet sheep was examined because of increased lamb mortality due to ineffective hemostasis at parturition. Decreased activity of coagulation factors II, VII, IX, and X in affected lambs was observed. We previously eliminated vitamin K antagonism, hepatic insufficiency, and fat malassimilation as potential causes of the fatal coagulopathy. Hepatic γ-glutamyl carboxylase activity was markedly reduced in all affected lambs while vitamin K 2,3 epoxide reductase was equivalent to an age matched control lamb. Parenteral vitamin K_1 supplementation did not correct the fatal coagulopathy in affected lambs, or significantly increase vitamin K-dependent procoagulant activities, but did prolong the required plasma transfusion interval for maintaining adequate hemostasis. Affected lamb γ-glutamyl carboxylase DNA was sequenced, and four single nucleotide polymorphisms (SNP's 2, 3, 4, & 5) of the γ-glutamyl carboxylase gene, were identified. SNP-3, located in exon 10 results in an arginine to histidine substitution at residue

486 (R486H). We have determined that the homozygous state (histidine) of this polymorphism is present in approximately 54% of the U.S. sheep population. Based upon the frequency of this polymorphism and the extremely rare occurrence of this fatal coagulopathy, it is not the causative mutation. Alternatively, SNP-4, located in exon 14 results in an arginine to stop codon (UGA) substitution, which prematurely terminates the peptide at residue 686 (R686Stop), has a strong association with the coagulopathy observed in clinically affected lambs, $p<0.001$. The remaining two SNPs are located within non-coding sequences. Together, these four SNPs allowed for determining the genotype associated with the observed fatal coagulopathy (GATT). Screening for this mutation (SNP-4) based upon the presence of a *Bbv I* restriction site in normal lambs, but not in the affected lambs, allows for detection of the heterozygous state.

Introduction

Vitamin K is a required cofactor for the post-translational enzymatic reaction converting glutamic acid residues (Glu) to γ-carboxylated glutamic acid (Gla).[34] Proteins that share this processing before being secreted are referred to as vitamin K-dependent (VKD) proteins. Post-translational modification occurs for VKD proteins involved in hemostasis, bone metabolism, mineralization, growth control, signal transduction, and cell survival.[2,12,32] Carboxylation allows calcium binding and activation of VKD

proteins II, VII, IX, X, protein C, protein S[16] and osteocalcin-hydroxyapatite binding in bone. This unique gamma carboxylation is catalyzed by γ-glutamyl carboxylase (*GGCX*).

Gamma carboxylation is the addition of a carboxyl group to the gamma carbon of glutamic acid[9] and occurs on the inner surface of the endoplasmic reticulum.[5] Energy is provided through the oxidation of vitamin K hydroquinone (K_1H_2) subsequent to binding the propeptide region of VKD proteins.[3,9] Calcium binding Gla residues are formed and K_1H_2 is converted to vitamin K_1 2,3 epoxide (KO). KO is recycled to K_1H_2, in a two-step process by vitamin K epoxide reductase, an enzyme sensitive to inhibition by warfarin. This complex process allows for coagulation proteins to fold, bind receptors, and participate in a sequential reaction process culminating in the formation of a stable fibrin clot.[8]

We report a coagulopathy in Rambouillet sheep similar to man.[4,7,19,27] Breeding data suggests the coagulopathy is inherited as an autosomal recessive trait. Affected lambs are born alive but lacked the ability to achieve hemostasis of the transected umbilical artery and vein. Without intervention, newborn lambs continually bleed from the umbilicus and have extensive subcutaneous and body cavity hemorrhage resulting in death.

We have previously demonstrated affected lambs from this sheep flock have markedly reduced activity of γ-glutamyl carboxylase. To determine the underlying mechanism of reduced enzymatic activity we have determined the

genetic sequence of the ovine γ-glutamyl carboxylase and the genetic defect associated with the observed coagulopathy.

Materials and Methods

Three Rambouillet lambs were born with an activated clotting time of >7 minutes (normal 2-3 minutes). Blood was collected for coagulation studies prior to transfusion with thawed citrate anti-coagulated ovine plasma. Prothrombin, partial thromboplastin times, and coagulation factor activities were determined with an MLA Electra 700 coagulation analyzer (Pleasantville, NY). Coagulation factor activity was compared with pooled plasma (100% activity) from 20 unrelated adult sheep using human factor deficient plasma (George King Biomedical, Overland Park, KS).

Hepatocellular microsomes.

Microsomes were prepared according to Kotkow *et al.*[21] using fresh liver obtained from lamb red 104 and an age/sex matched control lamb from a separate sheep flock and breed. *GGCX* activity was determined from standard reaction mixtures (125 μl) containing: 250 μg of microsomal protein, 0.8M $(NH_4)_2SO_4$, 28mM MOPS @ pH 7.5, 0.5M NaCl, 20μl 1% CHAPS, 8mM DTT, 10μCi $NaH^{14}CO_3$, 220μM of vitamin K_1H_2. Exogenous substrates added were either the pentapeptide FLEEL (3.6mM) or FLEEL + bovine proPT (8μm), a peptide based on amino acids –18 to -1. The mixture was incubated at 25°C for 30 minutes in sealed tubes. Adding 1 ml of 10% trichloroacetic

acid to the reaction mixture stopped reactions. Unbound $^{14}CO_2$ was removed by gently boiling the mixture for 10 minutes, under a hood vented to the exterior. Total incorporation was determined using a Beckman LS1801® liquid scintillation counter (Beckman Coulter, Fullerton, CA, USA). Background counts per minute (cpm) for assays lacking vitamin K_1H_2 were subtracted from all data points.

KO reductase activity.

This technique was performed as described previously.[13] KO was prepared according to the procedure of Tishler et al.[35] Standard reaction mixtures (125 μl) contained: 40 μg of microsomal proteins, 25 mM Tris-HCl pH 7.4, 150 mM NaCl, 0.28 % CHAPS, 0.12% phosphatidyl choline, 5 mM $NaHCO_2$, 222 μM of vitamin KO and 6 mM DTT. No exogenous substrates were added. The extraction, purification and analysis of plasma samples were performed as reported previously.[13]

Populations and Sampling.

The sheep populations used for single nucleotide polymorphism (SNP) discovery and validation consisted of 24 individuals from the affected flock, including 4 affected individuals, and the Meat Animal Research Center (MARC) Sheep Diversity Panel 1.1 (MSDP 1.1).[11] The MSDP 1.1 was designed to sample the breadth of genetic diversity in U.S. breeds and consists of 90 individuals from 9 popular sheep breeds. This panel provides

sufficient sequence information to estimate the frequency of sheep SNPs and haplotypes in U.S. sheep populations.

Primer design, PCR Amplification and DNA sequencing.

Primers were designed from the ovine *GGCX* cDNA sequence (AF312035, http://www.ncbi.nlm.nih.gov/), using OLIGO 6.0 (National Biosciences Inc., Plymouth, MN). Primers generated a set of seven overlapping amplicons spanning the entire ovine *GGCX* cDNA (15 exons). Human genomic sequence for *GGCX* was used to predict intron-exon junctions in ovine cDNA sequence (HSU65896 and NM 000821).[23] Amplicons spanning introns were targeted since these regions tend to be the most variable.[18,36] Amplification and sequencing reactions were performed using standard procedures.[14,17]

SNP Identification.

DNA segments from animals in the affected flock and MSDP 1.1 were amplified, sequenced, and compared to identify candidate SNPs. Sequences were edited and aligned using Phred and Phrap software.[10] Semi-automated SNP identification was performed using PolyPhred 3.0 and Consed 8.0.[26] Deviation of SNP genotype frequencies from Hardy-Weinberg equilibrium was tested using GENEPOP 3.1.[31] Relationships between haplotypes were determined by median-joining-network analysis using the method of Bandelt et al. and Network 3.1.0.1 software.[1] Comparisons of allele frequencies were performed by Pearson's X^2 test using system 9.01 (SPSS, Inc., Chicago, IL).

RFLP of R686Stop.

We designed a specific PCR approach for the analysis of R686Stop to determine the carrier state in newborn lambs. The mutant allele of SNP-4 results in loss of a *Bbv I* restriction site. First, we amplified a 218-bp fragment, which included part of exon 14 and intron 14 of the ovine GGCX using primers RFLP (5'-AAGGCTCCAAGAGATTGAAC-3') and (5'-AGGGAAAAGTTAGCACTGG-3'). The PCR product was cut into fragments with *Bbv I* (New England Biolabs, Beverly, MA) and subjected to electrophoresis in 3% agarose (fig. 3.2).

Results and Discussion

Coagulation Parameters.

Affected lambs had prolonged prothrombin and activated partial thromboplastin times and decreased vitamin K-dependent factor activities (Table 1).

Enzyme Activity.

Microsomes prepared from affected lamb liver had 158-fold decrease in *GGCX* activity, as measured by incorporation of $^{14}CO_2$ in peptide substrates, compared to age/sex matched control lamb liver when FLEEL alone was supplied to the reaction mixture. Affected lamb microsomes had a 24-fold decrease in *GGCX*-mediated incorporation of $^{14}CO_2$ when bovine

proPT was added to the reaction mixture in addition to FLEEL. DTT dependent KO-reductase activity was similar in affected and normal lamb liver microsomes indicating specific lack of *GGCX* activity in affected lambs (Table1).

GGCX SNP and Haplotype Identification.

The entire GGCX cDNA (2345 bp, less 29 bp of the 5' end and 34 bp of the 3' end that were subject to primer remodeling) was sequenced from 2 affected lambs, one carrier ewe, and one unrelated ewe. Two non-synonymous SNPs, SNP-3 and –4 were identified. Two *GGCX* genomic DNA segments OARGGCXDS6 (AY330326) and OARGGCXDS7 (AY330325), which included these SNPS, were chosen for characterization based on cDNA sequencing results and robust PCR amplification (Fig. 1A). The OARGGCXDS6 DNA sequence was 100% identical with the 3' 28 nucleotides (nt) of exon 10 and the 5' 70 nt of exon 11 of ovine *GGCX* (AF312035). OARGGCXDS7 had 100% DNA sequence homology with the 3' 148 nt of exon 14 and the 5' 150 nt of exon 15 of ovine *GGCX*. Indicating the DNA segments were amplified from the ovine *GGCX* gene locus.

OARGGCXDS6 and OARGGCXDS7, totaling 1245 bp of ovine *GGCX* genomic DNA, were sequenced from MSDP1.1 and 24 members of the affected flock, revealing one SNP in intron 10 (SNP-2), one in exon 11 (SNP-3), one in exon 14 (SNP-4) and one in intron 14 (SNP-5, Fig. 1A). The rare allele of SNP-3 results in an A/G, R486H substitution (exon 11). The rare

allele of SNP-4 results in a C/T, R686Stop substitution (exon 14). The homozygous and heterozygous state of SNP-2, -3, and –5 were present in the MSDP 1.1 panel (Figure 1C). The rare allele of SNP-4 was only detected in the affected flock.

The four observed SNPs defined five haplotypes, all of which were observed in this study (Figure 1B). Haplotype-5 was only observed in the affected flock. Homozygosity for Haplotype-5 was strongly associated with the fatal coagulopathy, $p<0.001$, and only animals homozygous for Haplotype-5 were clinically affected.

These findings are similar to those reported by Roth et al.[30] in which truncation of the peptide to 676 residues resulted in a 28-fold decrease in the carboxylase V_{max}. The theory that the vitamin K epoxidase domain also resides within this truncation[30] is supported by the lack of a clinical response to vitamin K_1 administration in affected lambs.

Based upon the frequency of homozygosity (54.5%) of SNP-3 (R486H) in the MARC 1.1 sheep diversity panel, we conclude it is not associated with the bleeding phenotype. We cannot discount the fact that SNP-3 may alter enzyme kinetics at a level sufficient to further reduce carboxylase activity when combined with the truncation of the peptide, while only sub-clinically affecting GGCX activity in homozygous, R486H, animals that lack premature peptide termination. The mutation R486H is near the putative propeptide binding site and determining enzyme kinetic parameters for the various

haplotypes will certainly lend insight into the impact, if any, of the R486H mutation.

The variable coagulation factor activities pose an interesting dilemma. Propeptide binding regions for *GGCX* are somewhat controversial. According to Wu *et al.*,[38] the binding site lies COOH-terminal to residue 438, while others suggest it is either within the first 314 amino acids of peptide,[22,38] or involves two NH_2-terminal cysteine residues;[29] however, recent evidence suggests that residues 495-513 are involved.[24] These studies suggest the possibility that multiple, non-contiguous regions of the carboxylase may form functionally important propeptide binding sites. The various propeptide affinities have been determined for the human recombinant carboxylase.[33] Evidence suggests that the propeptide release is likely to be the rate-limiting step in factor IX turnover in vitro.[6,15,25,28] ProPT binds 10-fold more loosely to the carboxylase than proIX, and studies evaluating expression of recombinant proPT and FIX showed a greater percentage of fully carboxylated prothrombin.[20] Wallin et al. found that while prothrombin and proX were tightly bound to the carboxylase, in vitro carboxylation of these substrates resulted in release of prothrombin precursors, but not FX precursors.[37] Taken together, these findings support the notion that propeptides with lower affinity for GGCX have higher substrate turnover, and putatively higher biologic activity, and lend insight into the variable coagulation factor activities observed in affected lambs.

We have successfully identified the genotype associated with the coagulopathy in this sheep flock (GATT). This unique flock represents the only known animal model of defective *GGCX* and allows for *in vivo* investigation of the function and importance of various VKD proteins.

Table 3.1 Summary of affected lambs coagulation data, vitamin K-dependent protein levels, and enzyme activity.

Lambs	R 104 (Day 0)	B 139 (Day 0)	R102 (Day 0)	Control 1 (Day 0)	Control 2 (Day 0)
PT (s)	> 200	> 200	> 200	19	24
aPTT (s)	> 300	> 300	> 300	34	33
Factor II	55	21	25	80	68
Factor VII	20	11	5	70	60
Factor IX	3	3	4	49	40
Factor X	20	3	6	70	70

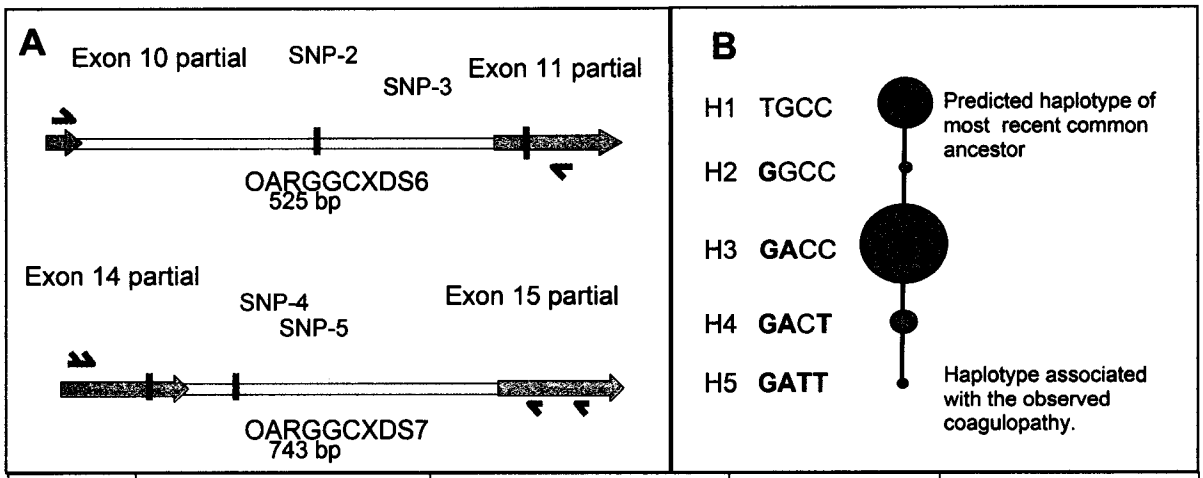
	Carboxylase		**KO-Reductase**			
microsomes	substrate		t= 0 min		t= 30 min	
	FLEEL	FLEEL + proPT	KO (%)	K (%)	KO (%)	K (%)
Control	94,533	141,603	91	9	18	82
Red 104	599	5,783	88	12	39	61

Coagulation factor activity is expressed as a percentage of normal pooled ovine plasma. PT, prothrombin time; aPTT, activated partial thromboplastin time.

$^{14}CO_2$ incorporation in FLEEL and FLEEL + proPT was measured after 30 min (average of duplicate experiments) and expressed as DPM/ 250μg microsomal protein.

Final concentration of substrates was 5 mM and 100 μM respectively.

Vitamin KO and K are expressed as a percentage of the total amount of vitamin K measured (K + KO).

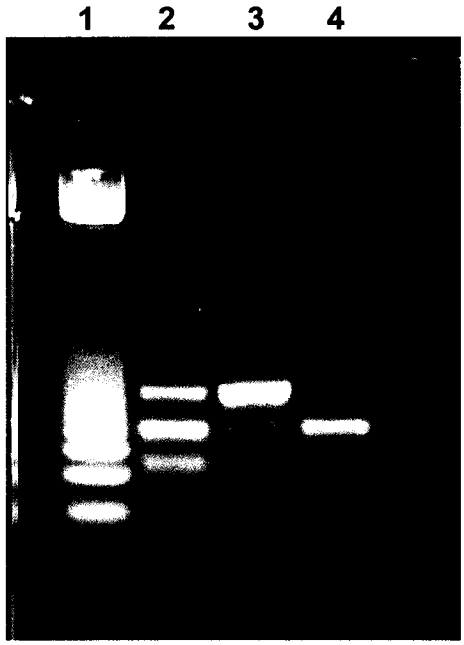


C

Breed	oaGGCX-2					oaGGCX-3					oaGGCX-4					oaGGCX-5				
	G	T	T,T	G,G	T,G	A	G	A,A	G,G	A,G	C	T	C,C	T,T	C,T	C	T	C,C	T,T	C,T
Composite	0.65	0.35	0.40	0.10	0.50	0.65	0.35	0.40	0.10	0.50	1	0	1	0	0	1.00	0.00	1.00	0.00	0.00
Dorper	0.65	0.35	0.50	0.20	0.30	0.65	0.35	0.50	0.20	0.30	1	0	1	0	0	0.75	0.25	0.60	0.10	0.30
Dorset	0.60	0.40	0.30	0.10	0.60	0.60	0.40	0.30	0.10	0.60	1	0	1	0	0	0.85	0.15	0.70	0.00	0.30
Finn	0.85	0.15	0.70	0.00	0.30	0.85	0.15	0.70	0.00	0.30	1	0	1	0	0	1.00	0.00	1.00	0.00	0.00
Katahdin	0.95	0.05	0.90	0.00	0.10	0.90	0.10	0.80	0.10	0.10	1	0	1	0	0	1.00	0.00	1.00	0.00	0.00
Rambouillet	0.65	0.35	0.50	0.20	0.30	0.65	0.35	0.50	0.20	0.30	1	0	1	0	0	0.90	0.10	0.80	0.00	0.20
Romanov	0.25	0.75	0.10	0.60	0.30	0.25	0.75	0.10	0.60	0.30	1	0	1	0	0	1.00	0.00	1.00	0.00	0.00
Suffolk	1.00	0.00	1.00	0.00	0.00	1.00	0.00	1.00	0.00	0.00	1	0	1	0	0	1.00	0.00	1.00	0.00	0.00
Texel	0.80	0.20	0.60	0.00	0.40	0.80	0.20	0.60	0.00	0.40	1	0	1	0	0	1.00	0.00	1.00	0.00	0.00
Total	0.711	0.289	0.556	0.133	0.311	0.706	0.294	0.544	0.144	0.311	1	0	1	0	0	0.944	0.056	0.9	0.01	0.09
BHS	0	1	0	1	0	0	1	0	1	0	1	0	1	0	0	1	0	1	0	0
Bovine	0	1	0	1	0	0	1	0	1	0	1	0	1	0	0	1	0	1	0	0

Figure 3.1 Sheep γ-glutamyl carboxylase SNPs. (A) Individual SNPs and their locations within sheep γ-glutamyl carboxylase DNA. (B) Individual haplotypes of the most recent common ancestor (H1), haplotypes present within the sheep flock (H2-H5), and the haplotype associated with the heritable coagulopathy (H5). The diameter of each node reflects the frequency of that haplotype in the MARC sheep diversity panel 1.1. (C) SNP frequency in MARC sheep diversity panel 1.1. BHS=Bighorn sheep

Figure 3.2 Restriction Fragment Length Polymorphism analysis of R686Stop.



Lane 1, 25bp ladder; lane 2, DNA from heterozygous animal (GAYY); lane 3, DNA from homozygous animal (GATT); lane 4, DNA from normal control (TGCC).

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Chapter 4

Characterization of the Naturally Occurring Mutations R486H and R686Stop in Ovine γ-Glutamyl Carboxylase

Abstract

Neonatal lambs with the mutations R486H and R686Stop in the γ-glutamyl carboxylase have a severe coagulopathy because of decreased biological activity of γ-glutamyl carboxylase and procoagulant clotting factors. Vitamin K administration prolonged the required plasma transfusion interval required to maintain adequate hemostasis, but did not correct this deficiency. We have estimated the frequency of the observed mutation R486H in affected lambs and concluded it is not the cause of the fatal coagulopathy observed; however to determine the impact of R486H, along with the mutation that terminates the peptide to residue 686 (R686Stop) we prepared crude liver microsomes from affected lambs (GATT), carrier sheep (GAYY), and control lambs (TGCC). By kinetic studies, we analyzed the catalytic activity of the various genotypes and the respective binding to vitamin K_1H_2, the synthetic substrate Phe-Leu-Glu-Glu-Leu (FLEEL), and determined the rate of

FLEEL enhancement of carboxylation by various vitamin K-dependent propeptides. The K_m values for FLEEL were similar between the 3 different genotypes. The addition of various propeptides stimulated γ-glutamyl carboxylase activity supporting the notion that the glutamate and propeptide bindings sites are nearer the NH_2-terminus of the peptide than residue 686. Additionally, the mutation R486H, does not significantly impact the propeptide binding site or glutamate binding site of the γ-glutamyl carboxylase.

Introduction

Vitamin K is a required cofactor for the post-translational enzymatic reaction converting glutamic acid residues (Glu) to γ-carboxylated glutamic acid (Gla).[34] Proteins that share this processing before being secreted are referred to as vitamin K-dependent (VKD) proteins. Post-translational modification occurs for VKD proteins involved in hemostasis, bone metabolism, mineralization, growth control, signal transduction, and cell survival.[2,13,30] Carboxylation allows calcium binding and activation of VKD proteins II, VII, IX, X, protein C, protein S[15] and osteocalcin-hydroxyapatite binding in bone. This unique gamma carboxylation is catalyzed by γ-glutamyl carboxylase (*GGCX*).

Gamma-glutamyl carboxylase catalyzes two chemical reactions at its active site.[24,39] The physiologically important reaction is the addition of carbon dioxide to Glu to form Gla, and the other reaction is the oxygenation of vitamin K hydroquinone to vitamin K 2,3 epoxide. Suttie et al. hypothesized that the epoxidation of vitamin K and carboxylation of Gla are carried out by the same enzyme in crude liver

microsomes,[35] and later confirmed in purified bovine liver carboxylase.[24] These reactions, while normally coupled, may become uncoupled in situations where CO_2 is reduced below saturating conditions, or when glutamyl substrate concentrations are low.[22,40]

Gamma carboxylation is the addition of a carboxyl group to the gamma carbon of glutamic acid [11] and occurs on the inner surface of the endoplasmic reticulum.[5] Energy to drive the first reaction is provided through the oxidation of vitamin K hydroquinone (K_1H_2) subsequent to binding the propeptide region of VKD proteins.[3,11] Calcium binding Gla residues are formed and K_1H_2 is converted to vitamin K_1 2,3 epoxide (KO). KO is recycled to K_1H_2, in a two-step process by vitamin K epoxide reductase, an enzyme sensitive to inhibition by warfarin. This complex process allows for coagulation proteins to fold, bind receptors, and participate in a sequential reaction process culminating in the formation of a stable fibrin clot.[10]

The substrate-GGCX recognition is thought to be mediated through the initial binding to an 18 amino acid propeptide sequence on the VKD protein.[14] Studies demonstrating disruption of this site in FIX, protein C, and prothrombin yields a mature protein either lacking or deficient in γ-carboxylation.[12,14,18] This observation and analysis of naturally occurring mutations in this region support the conclusion that the propeptide is required for γ-carboxylation.[8,32]

The γ-glutamyl carboxylase was purified to homogeneity from bovine liver in 1991,[42] and the bovine and human carboxylase cDNA's have been cloned.[41] The nucleotide sequence predicts a polypeptide 758 residues long with the

hydrophobic amino-terminal predicted to have three to seven trans-membrane domains, while the COOH-terminal half of the enzyme is relatively hydrophilic.[36,41] It has been demonstrated that the amino- and carboxy-termini are located on the cytoplasmic and luminal side of the endoplasmic reticulum (ER), respectively, and the carboxylase spans the ER membrane at least five times.[36] The propeptide binding site and the active site, where glutamate and vitamin K bind, are thought to face the lumen of the ER, where γ-carboxylation occurs.[5,7]

Little is known about the specific amino acids of the γ-glutamyl carboxylase that play a part in functional binding sites. Propeptide binding regions located via point mutation studies found important amino acids at 234/235, 406/408, and 513/515,[33] while others have proposed residues 495-513 function as the propeptide binding site.[23,28] Several studies have utilized peptide based affinity labels to identify portions of the carboxylase that form the functional binding sites. In one study the peptide labeled between residues 50 and 125[44], while a different group found the identical peptide to bind to residues between 438 and 507[43]. Site-directed mutagenesis studies have provided compelling evidence that residues 393-404 define important regions for interaction with the glutamate substrate.[25] Additionally, residue 394 may be involved in allosteric linkage between the propeptide and active binding sites.[26] Finally, mutagenesis studies support the notion that the vitamin K epoxidase activity domain may reside near the COOH terminus.[29] Together, these studies suggest that multiple non-contiguous regions of the carboxylase may form functionally important binding sites.

We report a coagulopathy in Rambouillet sheep similar to man.[4,9,17,27] A preliminary report eliminated vitamin K-antagonism as a cause for bleeding and demonstrated normal activity of non-VKD coagulation factors.[1] Breeding data suggests the coagulopathy is inherited as an autosomal recessive trait. Affected lambs were born alive but lacked the ability to achieve hemostasis of the umbilical artery and vein. Without intervention, newborn lambs continually bled from the umbilicus and had extensive subcutaneous and body cavity hemorrhage resulting in death. The mutation R686Stop, which prematurely terminates the peptide, has a strong association with selected bleeding phenotype, $p<0.001$.

The purpose of this study was to investigate the impact of the mutation R686Stop with regard to substrate (FLEEL), vitamin K_1H_2, and propeptide binding to determine the presence of functionally important binding sites within the COOH-terminal 72 amino acids. Additionally, enzyme kinetics were determined for the mutation R486H to determine if this site represents a critical amino acid for γ-glutamyl carboxylase function.

Materials and Methods

Animals

One animal representing each haplotype was used for determining individual kinetic parameters. All animals were euthanized at 40 days. Affected lambs were kept alive by periodic (every 4-7 days) plasma transfusions. Affected (GATT) and carrier (GAYY) lambs were from the Rambouillet flock described. The control lamb

was from an unrelated sheep flock. Kinetic parameters for each haplotype were determined from three separate reactions.

Haplotype and Genotype Determination of Samples

Prior to euthanasia, genotypes were determined based on sequencing individual animals as previously described.[1] Briefly, DNA was prepared from whole blood buffy coat preparations, using Qiagen QIAamp® DNA kit (Valencia, CA). Primers were designed to amplify two regions of the ovine GGCX DNA, which included all four polymorphisms associated with the fatal coagulopathy. Amplicons included part of exon 10, intron 10, part of exon 11 and part of exon 14, intron 14, part of exon 15, respectively. PCR products were extracted from 2% agarose, purified using Qiagen, and directly sequenced (see appendix I).

Preparation of Microsomes and Determination of Activity

Hepatocellular microsomes were prepared according to Kotkow *et al.*[20] using either fresh or snap frozen liver obtained from affected lambs (genotype GATT), heterozygous animals (genotype GAYY), and control lambs (genotype TGCC). Briefly, liver samples were divided into $\leq$1mm cubes, weighing approximately twenty-five grams, and combined with twenty-five mls of homogenization buffer (150mM NaCl, 50mM Tris-HCl, 5% (v/v) glycerol, 1mM EDTA, 1mM benzamidine, 1mM phenylmethysufonyl fluoride, pH 7.5). Twenty-five milligrams of liver/animal was prepared in a blender and homogenized using a 300A® homogenizer and 7mm saw-toothed generator (Pro Scientific Inc., Oxford, CT, USA). The homogenate was strained through gauze into a 25 X 89 mm centrifuge tube. Centrifuge tube weights were equilibrated with homogenization buffer and

centrifuged at 4°C for ten minutes at 10,000 g. The supernatant was filtered through gauze into a second centrifuge tube and weight equilibrated. Tubes were centrifuged at 4°C for one hour at 130,000 g. The supernatant was discarded and the pellet resuspended in 3 mls of homogenization buffer. Microsomal protein concentrations were determined according to the Lowry Method (Sigma Diagnostics), and all subsequent reactions were performed at 10mg/ml microsomal protein concentration unless otherwise stated.

Carboxylase activity and kinetic analysis

$^{14}C\,O_2$ incorporation was determined from standard reaction mixtures (125 μl) containing: 250 μg of microsomal protein, 0.8M $(NH_4)_2S\,O_4$, 28mM MOPS @ pH 7.5, 0.5M NaCl, 20μl 1% CHAPS, 3.6mM Phe-Leu-Glu-Glu-Leu (FLEEL), 8mM DTT, 10μCi $NaH^{14}C\,O_3$ (50mCi/mmol MP Biomedical), 220μM of vitamin K_1H_2, and 16μm proIX. Vitamin K_1 (10mg/ml) was reduced *in situ* with 2-β ME and sodium borohydride. Background counts/minute were subtracted from all data points. The determination of FLEEL kinetic parameters were performed by aliquoting the appropriate quantity of FLEEL into reaction tubes. Kinetic parameters of vitamin K_1H_2 were determined by aliquoting the vitamin K_1H_2 into tubes already containing DTT. When necessary vitamin K hydroquinone was diluted with PBS containing 0.5% CHAPS prior to use. Estimates of K_m and V_{max} were made using a lineweaver-burke plot (see appendix II). The propeptides were generated at Macromolecular Resources, Colorado State University, from residues –18 to –1 of the bovine proPT, human proIX, and bovine proX sequences.[31,32] Factor VII propeptide enhancement rates were not performed because of the presence of a

di-glutamate pair in the propeptide region. Under assay conditions this di-glutamate pair may become carboxylated, falsely elevating overall activity and estimated enhancement rates. The mixture was incubated at 25 °C for 30 minutes in sealed tubes. One milliter of 10% trichloroacetic acid was added to stop the reactions. Unbound $^{14}C\,O_2$ was removed by gently boiling the mixture for 10 minutes, or until the volume was reduced by approximately 90%. Total incorporation of $^{14}C\,O_2$ was determined by adding 6mls of Ecolyte scintillation cocktail, and counted for 5 minutes/sample on a Beckman LS 1801 scintillation counter. Data are expressed as dpm (degradation per minute) $^{14}C\,O_2$ incorporated per minute/ 250μg microsomal protein.

Results

Determination of K_m and V_{max} for Carboxylation of FLEEL

The control lamb carboxylase displayed non-Michaelis-Menten kinetics with concentrations of FLEEL above 17mM, thus FLEEL concentrations were used below 17mM to derive all kinetic parameters. The K_m of the ancestral haplotype enzyme for FLEEL was 0.891mM, a value similar to that reported for recombinant human carboxylase[29] and purified bovine liver derived enzyme.[24] The carrier haplotype enzyme exhibited similar K_m values to that of the control lamb, while the affected lamb had an apparent 2.2-fold increase in K_m (Table 1).

The V_{max} of each enzyme genotype for the substrate FLEEL was determined (Table 1). The V_{max} of the carrier genotype had an approximate 2-fold decrease compared to the control or ancestral genotype, while that of the affected

lamb genotype had an apparent 36-fold decrease. The K_m/V_{max} values were lower in the carrier and affected genotypes, demonstrating a decreased efficiency at catalyzing the carboxylation of the synthetic substrate compared to the ancestral genotype. The reduced efficiency of carboxylation in the carrier genotype is likely the result of an apparent half-dose affect of the enzyme as this genotype is heterozygous for R686Stop.

Table 4.1 Comparison of kinetic parameters for FLEEL with ancestral, carrier, and affected genotypes.

Genotype	K_m	V_{max}
	mM	*DPM/250μg protein*
TGCC[a]	0.879 ± 0.032	26,855 ± 2,217
GAYY[b]	0.859 ± 0.267	12,251 ± 730
GATT[c]	1.96 ± 0.369	739 ± 64

Reactions were performed with 250μg microsomes/125μl reaction.
The data represent the average of three independent determinations from one animal representing each individual genotype, and are presented as the mean ± S.D.
[a]Represents the ancestral or control genotype.
[b]Represents the carrier genotype.
[c]Represents the affected genotype.

Propeptide Stimulation of Carboxylation of FLEEL

To determine if the V_{max} reduction observed in affected lambs was the result of loss of responsiveness to the propeptide we determined the rate of enhancement using 8μm proPT, proIX, and proX (Table 2). The rate of enhancement was determined by normalizing the carboxylase activity from a 30-minute assay including the various propeptides by the activity obtained under identical conditions without propeptide. While the rate of stimulation for each of the propeptides was similar between the carrier and ancestral genotypes, a 4-13-fold increase in enhancement was observed for the various propeptides and microsomes from the affected haplotype.

Table 4.2 Enhancement rate of FLEEL carboxylation by proPT, prolX, & proX for the ancestral, carrier, and affected genotypes.

Genotypes	FLEEL only	FLEEL + proPT	FLEEL + prolX	FLEEL + proX	Enhancement rates (Fold Increase compared to FLEEL Alone)		
	DPM/250μg protein	*DPM/250μg protein*	*DPM/250μg protein*	*DPM/250μg protein*	*8μm proPT*	*8μm prolX*	*8μm proX*
TGCC[a]	95,084 ± 1,942	141,259 ± 6,700	136,514 ± 14,030	109,075 ± 3,992	1.49 ± 0.042	1.43 ± 0.121	1.15 ± 0.024
GAYY[b]	42,179 ± 2,097	62,540 ± 4,767	89,989 ± 5,351	58,187 ± 1,283	1.48 ± 0.039	2.13 ± 0.023	1.38 ± 0.04
GATT[c]	561 ± 27	5,767 ± 188	3,823 ± 281	8,707 ± 153	10.3 ± 0.222	6.83 ± 0.692	15.5 ± 0.456

Reactions were performed with 250μg microsomes/125μl reaction.
The data represent the average of three independent determinations from one animal representing each individual genotype, and are presented as the mean ± S.D.
[a]Represents the ancestral or control genotype.
[b]Represents the carrier genotype.
[c]Represents the affected genotype.

Determination of K_m and V_{max} for Vitamin K hydroquinone

The ancestral genotype displayed non-Michaelis-Menten kinetics with concentrations above 440μm. Thus vitamin K_1H_2 concentrations below 440μm were used to determine enzyme kinetic parameters. The ancestral genotype had an apparent 6-fold increase in K_m for the vitamin K_1H_2 of 64.5μm (Table 4.3) compared to both the carrier (14.9μm) and affected (9.79μm) genotypes, a value 2-3 fold higher than previously reported for purified bovine liver enzyme and recombinant human enzyme,[24,29] but within the range reported for crude liver microsomes.[6,35]

Table 4.3 Comparison of kinetic parameters for vitamin K_1H_2 with ancestral, carrier, and affected genotypes.

Genotypes	K_m	V_{max}
	μM	*DPM/250μg protein*
TGCC	64.5 ± 11.6	17,668 ± 1,389
GAYY	14.9 ± 1.03	7,436 ± 1081
GATT	9.78 ± 1.69	647 ± 70

Reactions were performed with 250μg microsomes/125μl reaction.
The data represent the average of three independent determinations from one animal representing each individual genotype, and are presented as the mean ± S.D.
[a]Represents the ancestral or control genotype.
[b]Represents the carrier genotype.
[c]Represents the affected genotype.

Discussion

We have characterized an inbred Rambouillet sheep flock that has a heritable and fatal coagulopathy as a result of diminished GGCX activity. We have identified two distinct mutations, R486H & R686Stop, within the coding regions of the GGCX gene and have compared the frequency of these mutations with the U.S. sheep population. Based upon the frequency of homozygosity (54.5%) of R486H in the MARC 1.1 sheep diversity panel, we conclude it is not the cause of the bleeding phenotype. The current study explores the relationship of R486H with that of R686Stop while characterizing the impact of these mutations on the enzyme/substrate interactions.

The K_m values for FLEEL with the ancestral genotype and carrier genotype were similar, while that of the affected genotype was 2.2-fold higher. While the affected genotype has a somewhat higher K_m it is unlikely that this is the result of loss of a COOH-terminal mediated binding site, as higher concentration of FLEEL (16.4μm) did not result in higher activity of the enzyme. This apparent lack of effect of missing portions of the COOH-terminal on FLEEL recognition is consistent with previous studies.[21,29] The similarity between K_m values for the carrier and ancestral genotypes suggests that residue 486 is not important for FLEEL/enzyme interaction.

Conserved sequences within the propeptide region of vitamin K-dependent proteins serve as the recognition site of the carboxylase,[16,18,37] and have been shown to stimulate carboxylation of small peptide substrates such as FLEEL.[19,37] The synthetic peptides proPT, proIX, and proX, corresponding to residues –18 to

–1 of the bovine, human, and bovine propeptides, respectively, were used to stimulate FLEEL carboxylation . The rate of enhancement was approximately 4-13-fold higher in the affected genotype compared with similar enhancement rates observed in the ancestral and carrier genotypes depending upon the propeptide used. These results support the conclusion that neither truncation of the peptide to residue 686 nor the presence of R486H negatively impacts propeptide binding, and are consistent with reports localizing propeptide binding site to the NH_2-terminal third of the enzyme.[23,44]

The higher rate of enhancement observed is interesting. Reportedly, the enhancement by the propeptide is the result of an approximately 5-10-fold reduction in the K_m for FLEEL.[19] The increased enhancement rate is likely the result of this K_m reduction for FLEEL on the slighter higher K_m observed in the affected genotype. It should be noted that Uotila et al. reported the presence of $(NH_4)_2SO_4$ in bovine microsomes blocked the K_m lowering effect of the propeptide substrate.[38] In contrast, we found that including $(NH_4)_2SO_4$ along with the various propeptide substrates resulted in maximal stimulation of GGCX activity, suggesting that this reported substrate inhibition is not a property of sheep microsomes.

The K_m values for vitamin K_1H_2 were similar between the affected and carrier genotypes, while that of the ancestral genotype was approximately 6-fold higher. The K_m values for all three genotypes evaluated were within the normal range reported for crude bovine microsomes.[6,35] In contrast to our observations, Roth et al. reported that truncation of the carboxylase to 676 residues resulted in a 23-fold increase in the K_1H_2 K_m.[29] One explanation may be that an important site for

mediating vitamin K_1H_2 binding lies between residue 676 and 686, or an alternative explanation may be that the mutation R486H, which is present in the homozygous state in both the affected and carrier genotype, significantly lowers the enzyme affinity for K_1H_2, overcoming any impact of premature peptide termination. Site-directed mutagenesis studies will clarify this discrepancy and experiments are currently underway.

We conclude that neither mutation R486H nor R686Stop significantly impacts the interaction of the enzyme for the substrate FLEEL, nor the various VKD-propeptides and therefore these reactions occur nearer the NH_2-terminus than residue 686. We cannot rule out the possibility that the mutation R486H impacts the affinity of the enzyme for K_1H_2 by lowering the K_m, or whether residues 676-686 may represent an important site for mediating K_1H_2 binding.

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CHAPTER 5

CONCLUSIONS

The major accomplishments of this investigative work are as follows:

1. A flock of inbred Rambouillet sheep has been established to study the effects of impaired vitamin K metabolism due to a heritable decrease in the activity of the enzyme γ-glutamyl carboxylase.
2. Affected homozygous lambs have a fatal coagulopathy characterized by extensive subcutaneous and body cavity hemorrhage with both male and female animals affected, supporting an autosomal inheritance pattern.
3. Affected lambs have markedly prolonged prothrombin times, activated partial thromboplastin times, with variably decreased activity of the vitamin K-dependent coagulation factors, II, VII, IX, and X supporting abnormal vitamin K metabolism.
4. This fatal coagulopathy can be compensated for by periodic plasma transfusions, and required plasma transfusions are prolonged by the administration of supraphysiologic vitamin K_1; however, vitamin K alone does not correct the heritable coagulopathy.

5. Affected lambs have two mutations in the open-reading-frame of the γ-glutamyl carboxylase gene, R486H and R686Stop.
6. Based on the high frequency of the homozygous state in the U.S. sheep population (54.5%) and our flock (38%) R486H is not the cause of the fatal coagulopathy.
7. The mutation, R686Stop was only detected in our inbred flock and homozygosity was strongly associated with the fatal coagulopathy, $p<0.001$.
8. The mutations R486H and R686Stop do not significantly impact the interaction of the enzyme for the substrate FLEEL or the various VKD-propeptides and therefore these reactions occur NH_2-terminus to residue 686.

This investigation focused on characterizing a spontaneous and fatal coagulopathy in an inbred flock of Rambouillet sheep. In completing this investigation we have a clearer understanding of the underlying cause of the observed coagulopathy from a molecular and biochemical standpoint.

We have demonstrated that aberrant vitamin K metabolism is the result of markedly decreased activity of the enzyme γ-glutamyl carboxylase. Furthermore, based upon the findings of this study, and the support of similar work in other laboratories, we have strong evidence that the mutation R686Stop is the cause of the observed bleeding phenotype.

Additionally, we have demonstrated that the glutamate and propeptide binding sites for the ovine γ-glutamyl carboxylase are not within the COOH-terminus of the peptide, but instead are NH_2-terminus of residue 686.

There are many questions that still remain regarding this spontaneous defect and future investigations may resolve some of these important questions.

1) What are the long-term impacts of this defect involving cellular signaling, apoptosis, neoplasia, bone metabolism and mineralization?
2) What events are occurring within the COOH-terminus of the enzyme that results in significant loss of function in affected lambs?
3) Why are some of the VKD proteins carboxylated in this system, specifically, coagulation factor II, and VII?
4) Recent evidence supports an anti-oxidant effect in cultured oligodendrocytes and neutrons. What protective effects does vitamin K_1H_2 have in other systems of the body?

APPENDIX I

HAPLOTYPES OF ANIMALS WITHIN THE RAMBOUILLET SHEEP FLOCK.

Animal ID	Genotype	Haplotype H1	Haplotype H2	Animal ID	Genotype	Haplotype H1	Haplotype H2
[redacted]				o135	KRYY	1	5
				o136	KRYY	1	5
				o137	KRYY	1	5
				o138	GAYY	3	5
				y23	GACY	3	4
b100	KRYY	1	5	y39	KRYY	1	5
b133	GAYY	3	5	b133M04	GAYY	3	5
b134	KRYY	1	5	o132M04	KRYY	1	5
b136	KRYY	1	5	b145M04	GAYY	3	5
b137	KRYY	1	5	Harley	GAYY	3	5
b138	GAYY	3	5	cr102L02	KRCC	1	3
b139	GAYY	3	5	cr108L02	TGCC	1	1
b143	KRYY	1	5	b145F04	GACC	3	3
b145	GAYY	3	5	b131	KRCC	1	3
b148	KRYY	1	5	b141	TGCC	1	1
b150	KRYY	1	5	b144	KRCC	1	3
g158	KRYY	1	5	b139M04	KRCC	1	3
g164	KRYY	1	5	b146	KRCC	1	3
g165	KRYY	1	5	b147	TGCC	1	1
g3	GAYY	3	5	r101	KRCC	1	3
r102	KRYY	1	5	r115	GACY	3	4
r104	GAYY	3	5	g161	TGCC	1	1
r110	GAYY	3	5	g168	TGCC	1	1
g169	KRYY	1	5	o126	TGCC	1	1
w8	KRYY	1	5	o127	KRCC	1	3
o128	KRYY	1	5	o129	KRCC	1	3
o131	KRYY	1	5	o130	TGCC	1	1
o132	GAYY	3	5	o133	KRCC	1	3
o134	KRYY	1	5				

HAPLOTYPE	GENOTYPE	LOCATION/SNP
H1	TGCC	
H2	GGCC	INTRON 10
H3	GACC	EXON 11/R486H
H4	GACT	INTRON 14
H5	GATT	EXON 14/R686STOP

COLOR KEY

[redacted]

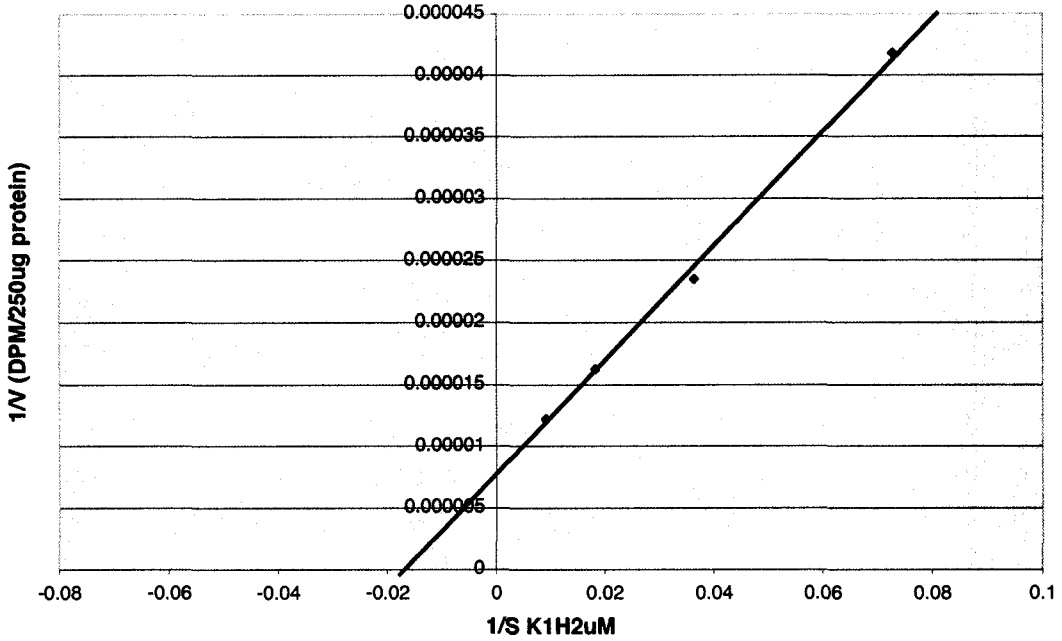
YELLOW=CARRIER

WHITE=NON CARRIER

APPENDIX II

LINEWEAVER-BURK PLOTS FOR HAPLOTYPES TGCC, GAYY, GATT TO DETERMINE K_m/V_{max} VALUES USING K_1H_2 & FLEEL.

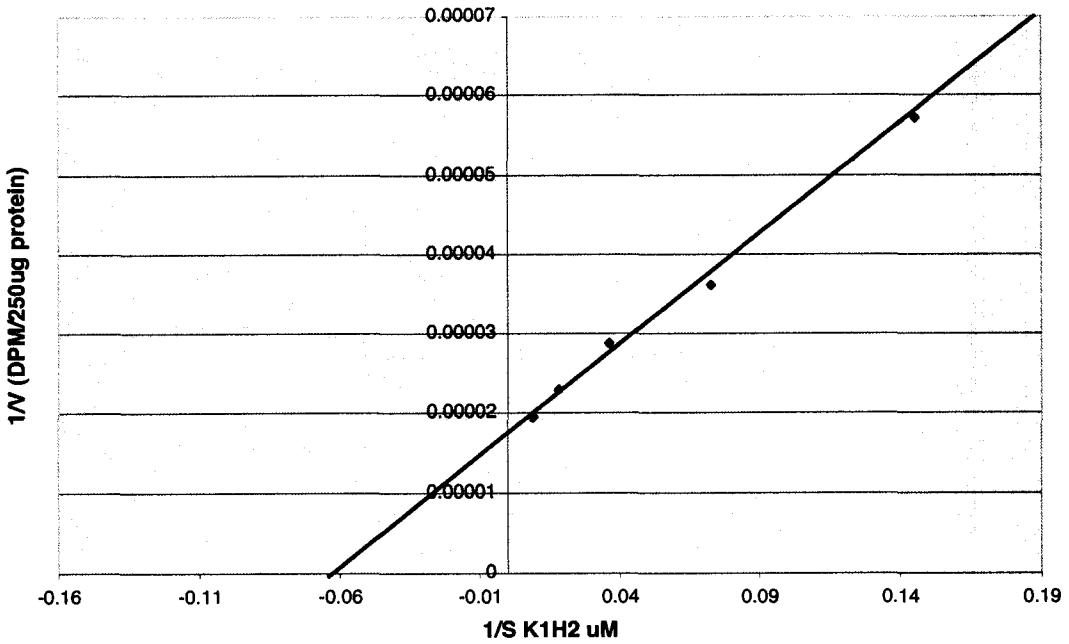
TGCC



$y=4.65X10^{-4}x + 7.63X10^{-6}$, $r^2=0.997$

K_m =60.98 μm V_{max} =17,475 DPM/μg protein

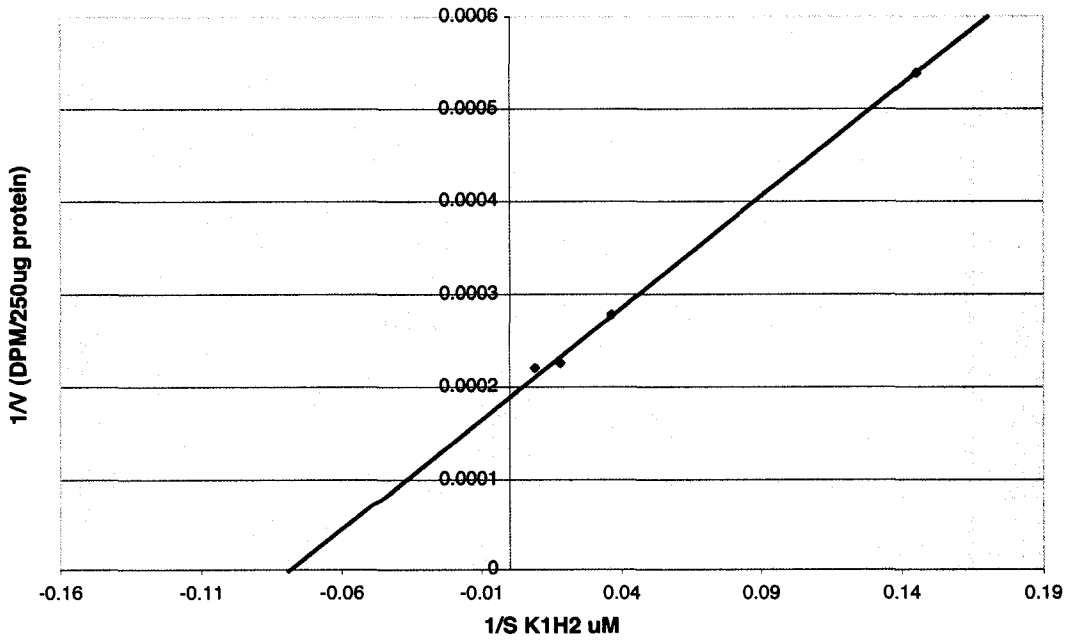
GAYY



$y=2.70X10^{-4}x + 1.77X10^{-6}$, $r^2=0.996$

K_m =15.27 μm V_{max} =7,532 DPM/μg protein

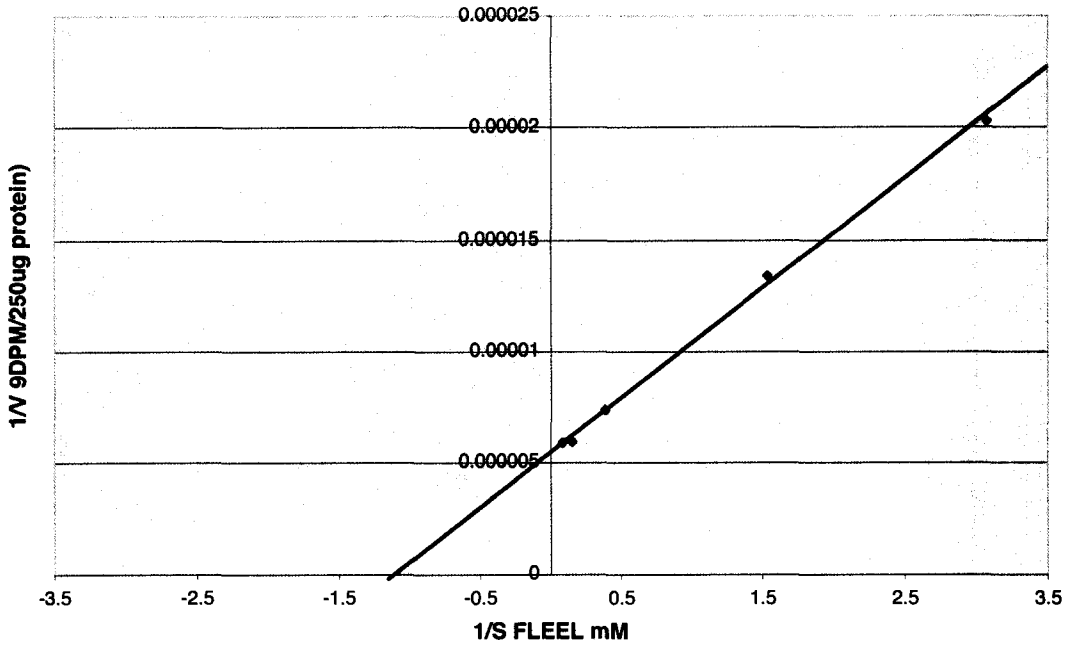
GATT



$y=2.38X10^{-3}x + 1.92X10^{-4}$, $r^2=0.998$

K_m=12.41 μm V_{max}=694 DPM/μg protein

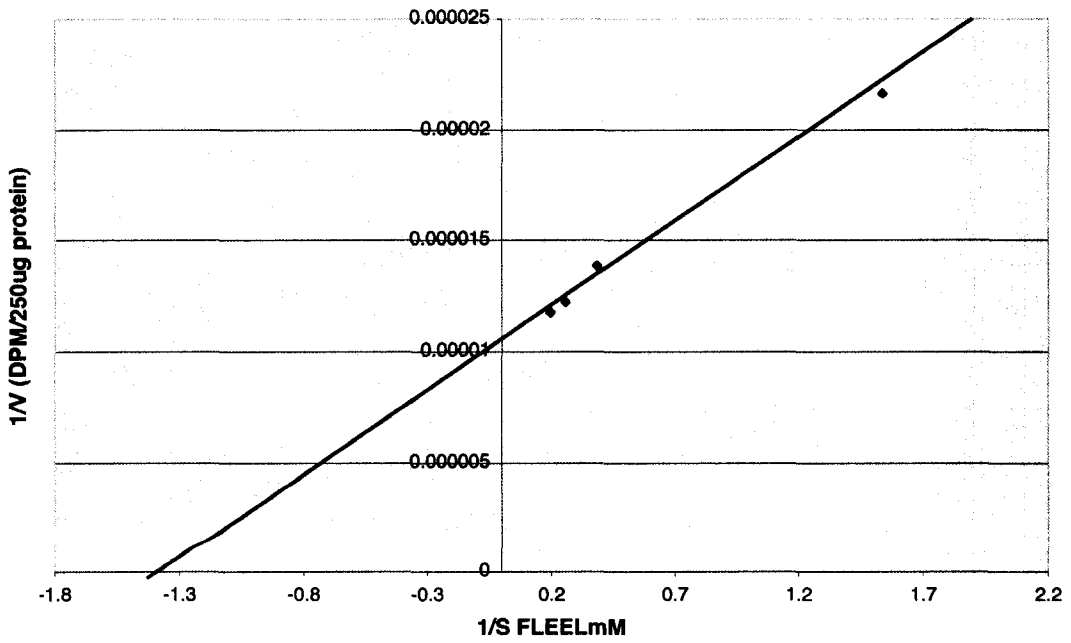
TGCC



$y=4.89X10^{-6}x + 5.48X10^{-6}$, $r^2=0.998$

K_m=0.89 mM V_{max}=24,331 DPM/μg protein

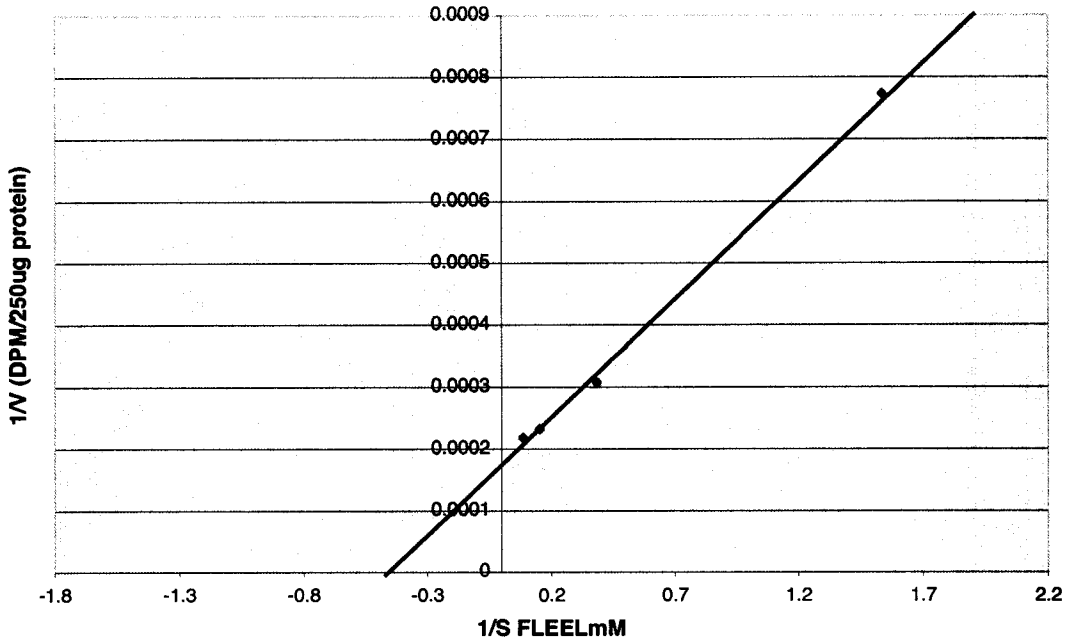
GAYY



$y=7.24X10^{-6}x + 1.06X10^{-5}$, $r^2=0.994$

K_m=0.68 mM V_{max}=12,578 DPM/μg protein

GATT



$y=3.88X10^{-4}x + 1.74X10^{-4}$, $r^2=0.998$

K_m=2.23 mM V_{max}=766 DPM/μg protein