GLOMERULAR DEPOSITION OF HOMOTRIMERIC TYPE I COLLAGEN IN THE COL1A2 DEFICIENT MOUSE

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Doctor of Philosophy

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by

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GLOMERULAR DEPOSITION OF HOMOTRIMERIC TYPE I COLLAGEN IN THE COL1A2 DEFICIENT MOUSE

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ABSTRACT

A novel type I collagen glomerulopathy was identified in COL1A2 deficient mice, which are homozygous null for functional proα2(I) collagen chains and synthesize exclusively homotrimeric type I collagen. The COL1A2 deficient mice accumulate fibrillar type I collagen in their renal mesangium. Under normal physiologic conditions, type I collagen is not present in the renal mesangium. The accumulation of glomerular collagen is pathologic, and occurs as a component of many chronic renal diseases.

The aims of this study were to 1) determine the natural progression of the type I collagen glomerulopathy, 2) determine if the pathologic findings correlate with progression of the glomerulopathy, 3) identify the mechanism responsible for glomerular type I collagen deposition in COL1A2 deficient mice, and 4) determine if matrix metalloproteinases (MMPs) cleave heterotrimeric and homotrimeric type I collagen similarly.

Picrosirius red staining of formalin-fixed kidneys from COL1A2 deficient, heterozygous, and wildtype mice demonstrates that both COL1A2 deficient and, to a lesser degree, heterozygous animals develop glomerular collagen deposition, exhibiting a gene dose effect. Further, the glomerulopathy begins postnatally, within one week of life, following a pattern of glomerular maturation. The glomerulopathy appears

progressive, resulting in albuminuria in severely affected animals. *In situ* hybridization revealed no gross differences in steady-state proα1(I) and proα2(I) collagen mRNA levels in all genotypes. However, quantitative RT-PCR using whole kidney showed a two-fold increase in steady-state proα1(I) collagen mRNA in 1-month COL1A2 deficient relative to wildtype and heterozygote animals, suggesting that the glomerular collagen deposition seen in the type I collagen glomerulopathy is, in part, due to pretranslational mechanisms. Further studies using *in vitro* proteolytic cleavage analysis demonstrate differential cleavage of heterotrimeric and homotrimeric type I collagen by MMPs 1, 2, 9, and 13, indicating that differential substrate recognition may play a role in the glomerulopathy. Therefore, we postulate that the mechanism responsible for glomerular collagen deposition seen in the type I collagen glomerulopathy is a combined mechanism, involving a pretranslational mechanism and aberrant degradation of glomerular collagen by MMPs. Based on these findings, further investigation of MMPs and their role in the type I collagen glomerulopathy is warranted.

CHAPTER I

INTRODUCTION

Glomerular injury is a broad category that can encompass both primary and secondary kidney disease, and may be inflammatory or non-inflammatory in nature. The initiating event leading to glomerular disease is often known and immunologic, but the molecular mechanism of the secondary progressive glomerular decline has not yet been well-characterized. Progression of glomerular injury is defined by the histologic appearance of glomerulosclerosis, as well as tubulointerstitial inflammation and fibrosis. Glomerulosclerosis and glomerular fibrosis both result in the accumulation of extracellular matrix proteins in the renal mesangium, promoting the cascade of events that will inevitably develop into end-stage renal disease (Cotran, Kumar et al. 1999; Alexakis, Maxwell et al. 2006). A novel glomerulopathy, identified in the COL1A2 deficient mouse model, allows for investigation of the role of homotrimeric type I collagen in progressive glomerular injury. Lacking the α2 chain, COL1A2 deficient mice demonstrate deposition of homotrimeric type I collagen into and expansion of the renal mesangium (Phillips, Pfeiffer et al. 2002). This is a common component of glomerulosclerosis and fibrosis and implies that different regulatory mechanisms may be involved in homotrimeric type I collagen expression and may be an important window in understanding the pathogenesis of glomerulosclerosis and renal fibrosis. The primary objectives of this study are aimed at understanding the molecular role of the α2 chain in

extracellular matrix deposition common to secondary progression of renal damage seen in a variety of kidney diseases and systemic disorders.

The research that I have conducted towards my Ph.D. degree has focused on further histologic and pathologic characterization of the type I collagen glomerulopathy first identified in our laboratory. In addition, I have worked to gain an understanding of the role of homotrimeric type I collagen and the α2 chain in glomerulosclerosis. Through this work I have 1) further characterized the type I collagen glomerulopathy by identifying that heterozygous mice are affected in addition to the COL1A2 deficient mice, 2) shown that the glomerulopathy begins postnatally in the first week of life, demonstrates a gene dose effect, progresses with age, and follows a pattern of initiation consistent with glomerular maturation, 3) shown that the COL1A2 deficient mice exhibit microalbuminuria as a result of the glomerular type I collagen deposition characteristic of the glomerulopathy 4) demonstrated fibrillar collagen deposition in the mesangium and subendothelial space of the glomeruli of COL1A2 deficient mice with concomitant effacement of the podocyte foot processes in severely affected animals, 5) demonstrated evidence that the mechanism responsible for the type I collagen glomerulopathy is, in part, due to increased levels of steady-state proα1(I) collagen mRNA, suggesting pretranslation upregulation of type I collagen expression contributes to the glomerular collagen accumulation, and 6) provided evidence that differential matrix metalloproteinase cleavage properties exist for homotrimeric and heterotrimeric type I collagen suggesting that differential cleavage of homotrimeric type I collagen and/or differential expression/activity of matrix metalloproteinases may be involved in the overall mechanism responsible for the glomerulopathy.

Type I Collagen

Type I collagen, one of over 20 characterized forms of collagen, is the predominant structural protein in the body (Kielty, Hopkinson et al. 1993; Byers, Scriver et al. 2001). Table I.1 outlines those collagen molecules discussed here. The role of type I collagen in connective tissue contributes to the strength and integrity of a variety of tissues, including bone, skin, tendon, and vasculature. In addition, type I collagen, a fibrillar collagen, is one of the major components of the extracellular matrix (ECM). Collagen along with proteoglycans and noncollagenous glycoproteins, comprise the ECM which serves to provide structure and support to a variety of organs and tissues. Different tissues contain a different makeup of ECM components and amounts of the various types of collagen. The relative amount of ECM components determines the overall structural characterization and physiologic determinants of each individual tissue. Also of interest are the properties of type I collagen that relate to its involvement in a variety of human diseases including atherosclerosis, arthritis, a variety of genetic diseases including osteogenesis imperfecta, renal disease, and many others (Kielty, Hopkinson et al. 1993; Byers, Scriver et al. 2001).

Type I collagen generally exists as a heterotrimeric protein (Figure I-1A) composed of two related, yet genetically distinct, procollagen chains. The heterotrimer is formed by the incorporation of two proα1(I) collagen chains and one proα2(I) collagen chain [$\alpha1(I)_2\alpha2(I)$] (Phillips, Wenstrup et al. 1992; Byers, Scriver et al. 2001). In some tissues, a homotrimeric isotype (Figure I-1B) of type I collagen containing three proα1(I) chains [$\alpha1(I)_3$] has been identified and comprises less than 0.5% of collagen found in the

Table I.1: Comparison of Type I Collagen to other Collagen Types

Collagen	α -chains	Isoforms	Tissue localization
Type I	α1(I) α2(I)	$\alpha1(I)_2\alpha2(I)$ $\alpha1(I)_3$	Widespread; skin, bone, tendon, etc.
Type III	α1(III)	$\alpha1(III)_3$	Soft tissues, tendon, aorta, cornea, etc.
Type IV	α1(IV) α2(IV) α3(IV) α4(IV) α5(IV) α6(IV)	$\alpha1(IV)_2\alpha2(IV)$ α3(IV)α4(IV)α5(IV)	Basement membranes
Type V	α1(V) α2(V) α3(V)	$\alpha1(V)_2\alpha2(V)$ α1(V)α2(V)α3(V)	Widespread; skin, bone, aorta, etc.

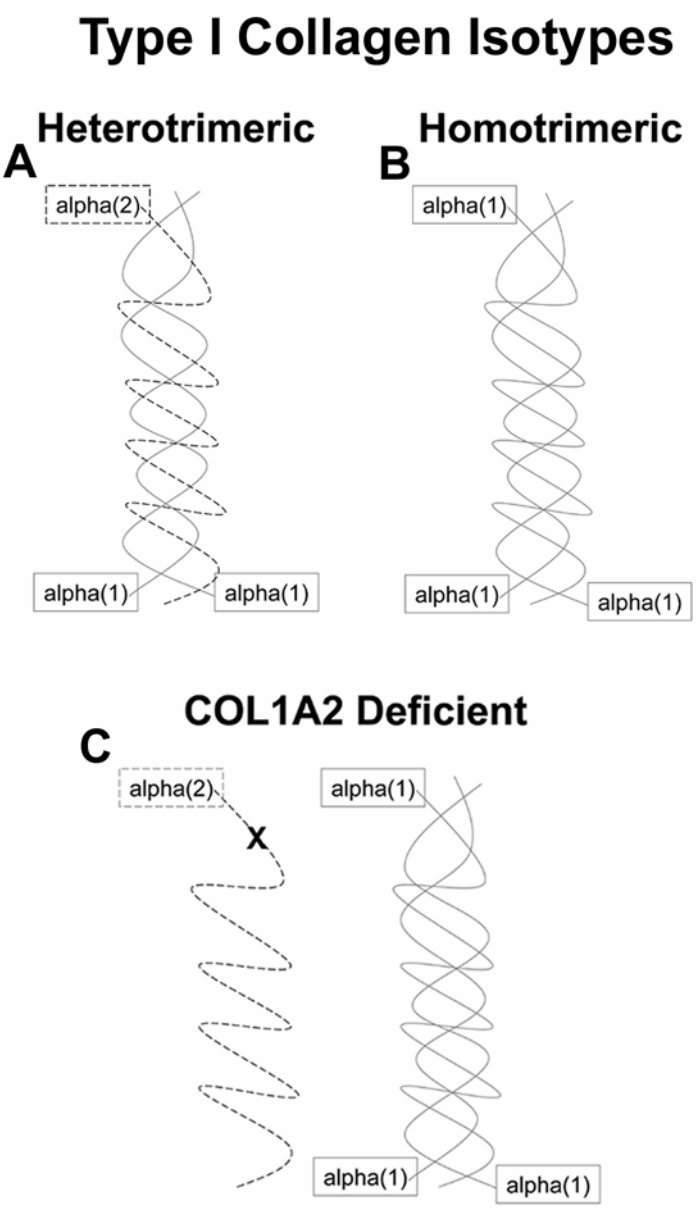

Figure I-1. Type I collagen exists as a heterotrimeric and a homotrimeric triple helical molecule. A) shows the mature heterotrimeric isotype of type I collagen [$\alpha1(I)_2\alpha2(I)$] consisting of two $\alpha1(I)$ chains and one $\alpha2(I)$ chain. B) demonstrates the less common homotrimeric type I collagen isotype [$\alpha1(I)_3$] consisting of three $\alpha1(I)$ chains. C) depicts formation of the homotrimer in COL1A2 deficient mice where, as a result of a nucleotide deletion (**X**) and frameshift, the $\alpha2(I)$ chain is unable to be incorporated into the triple helix.

body and less than 5% in human skin (Uitto 1979; Kielty, Hopkinson et al. 1993). Homotrimer has also been found early on embryologically, as well as occurring in some tumors and amniotic fluid (Jimenez, Bashey et al. 1977; Moro and Smith 1977; Rupard, Dimari et al. 1988; Ghersi, La Fiura et al. 1989). Further, cultured mesangial cells produce homotrimeric type I collagen, suggesting that homotrimer production may be related to injury and/or wound healing (Haralson, Jacobson et al. 1987). However, the role of the homotrimeric isotype is currently not well understood (Byers, Scriver et al. 2001).

Biosynthesis of type I collagen is a complex process which begins with transcription of the of the individual collagen genes. The proα1(I) chain is generated from an 18kb COL1A1 gene on chromosome 17, and the proα2(I) chain is coded for by the COL1A2 gene, which is 38kb and located on chromosome 7 (Kielty, Hopkinson et al. 1993). The COL1A1 and COL1A2 genes are transcribed at a ratio of 2:1 due to differences in transcriptional efficiency (Byers, Scriver et al. 2001). From the transcripts, the proα chains are synthesized, beginning with the N-terminus, on polyribosomes and transferred to the rough endoplasmic reticulum (RER) through the use of a 20 amino acid preproα signal (Phillips, Wenstrup et al. 1992). The type I collagen α chains are approximately 95kD in molecular weight and consist of a unique repeating triple amino acid sequence with a glycine in every third position, (Gly-X-Y)n. The X-position is often occupied by proline and Y is commonly hydroxyproline. Intracellular post- and co-translational modification are common in type I collagen biosynthesis and are responsible for the cleavage of the signal sequence as well as generation of the unique hydroxylation

and glycosylation of proline and lysine residues (Phillips, Wenstrup et al. 1992; Kielty, Hopkinson et al. 1993; Byers, Scriver et al. 2001).

The triple helical form of homotrimeric and heterotrimeric type I collagen is required for passage out of the RER. Type I collagen self-assembly begins with chain association stabilized by disulfide bonding at the C-terminus and nucleation proceeds in the direction of the N-terminus to form the triple helical region of the procollagen molecule. The procollagen molecule, consisting of the triple helical region, which contains approximately 1000 amino acids and the globular domains, is then transferred to the Golgi apparatus for packaging and secretion. Following secretion, the globular domains are cleaved by specific proteases and the individual helices are incorporated into greater order structure, or fibrils (Kielty, Hopkinson et al. 1993; Byers, Scriver et al. 2001). However, specific domains within the α1(I) and α2(I) chain have been shown to regulate collagen fibril assembly (Prockop and Fertala 1998; Malone, Alvares et al. 2005). Specifically, the α2(I) telopeptide contains nine amino acids which are sufficient to inhibit collagen fibril assembly, indicating a possible role for the α2(I) chain in fibril formation (Prockop and Fertala 1998).

Type I collagen, a fibrous collagen, has a crystalloid structure due to its symmetrical, insoluble structure of repeating subunits in which the collagen monomers associate through staggered lateral interactions in which the telopeptide region of one fiber interacts with the triple helix of an adjacent fiber. The fibers arrange to form long, unbranched, banded fibrils with a characteristic periodicity of 67nm. The helical region of type I collagen is a semirigid, right-handed helix with a 10nm repeat distance. The helix is formed by the coiling of the individual left-handed helices, the proα chains,

around a central axis. This conformation arises due to the steric repulsion that occurs between the X and Y position of the individual chains and the ability of the glycine residue to pack tightly into the center of the molecule. Based on the complex structural requirements of type I collagen, mutations in the amino acid sequence have the ability to profoundly impact the helical conformation and thus the physiology of the target tissue. Also unique to the triple helical structure is its sensitivity to proteolytic degradation. The globular domains and the individual proα chains are susceptible to degradation by a variety of proteases. However, the triple helical region is only susceptible to cleavage by specific proteases designed to recognize and cleave based on structural elements (Phillips, Wenstrup et al. 1992; Kielty, Hopkinson et al. 1993).

As mentioned above, the homotrimer is a triple helix which contains three α1 chains. Electron microscopy and meridional x-ray diffraction patterns demonstrate that homotrimeric type I collagen is similar in structure to heterotrimeric type I collagen. However, it has been suggested that there is a slight structural difference between homotrimer and heterotrimer based on comparative water binding studies, a differential micro-unfolding pattern, decreased rate of homotrimer assembly, decreased triple helix integrity in the homotrimer, and differential kinetics of thermal denaturation between the two molecules (McBride, Choe et al. 1997; Kuznetsova, McBride et al. 2001; Miles, Sims et al. 2002; Kuznetsova, McBride et al. 2003).

COL1A2 Deficient Mouse

The COL1A2 deficient mouse, also known as the *oim* mouse (osteogenesis imperfecta model), is a nonlethal, naturally occurring model that is homozygous for a

spontaneous nucleotide deletion at position 3983 in the COL1A2 gene (Chipman, Sweet et al. 1993; McBride and Shapiro 1994). The mutation results in an alteration of the last 48 amino acids of the carboxy-propeptide region of the proα2(I) chain generating a new translation stop site that incorporates an additional amino acid (Chipman, Sweet et al. 1993). The carboxy-propeptide region is not present in mature type I collagen but is responsible for coordination of the α2 chain with the two α1 chains during assembly of the triple helical heterotrimeric type I collagen molecule [$\alpha1(I)_2\alpha2(I)$] (McBride and Shapiro 1994). COL1A2 deficient mice synthesize α2 chains; however, they are postulated to be rapidly degraded intracellularly due to their inability to incorporate into the heterotrimeric type I collagen molecule (Figure I-1C), resulting in exclusive production of homotrimeric type I collagen [$\alpha1(I)_3$] (Deak, Nicholls et al. 1983).

Homozygous COL1A2 deficient mice are smaller than age-matched wildtype animals, are fertile, and have not been found to have premature mortality (Phillips, Bradley et al. 2000). The model has been shown to have an absence of α2(I) chains and secrete only homotrimeric type I collagen in skin, bone and fibroblast cultures (Chipman, Sweet et al. 1993). Additional work demonstrated an overall decrease in type I collagen content in the skin, bone, aorta, and left ventricle of COL1A2 deficient mice (McBride, Choe et al. 1997; Camacho, Hou et al. 1999; Weis, Emery et al. 2000; Pfeiffer, Franklin et al. 2005). This is in contrast with the aberrant accumulation of homotrimeric type I collagen in the kidney, more specifically the glomeurli (Phillips, Pfeiffer et al. 2002).

It is useful to study the glomerular accumulation of homotrimeric type I collagen in COL1A2 deficient mice due to the absence of the α2(I) collagen chain. There are numerous renal diseases that result in the accumulation of type I and other collagens in

the glomerular mesangium, resulting in renal impairment (Lenz, Elliot et al. 2000). However, it is unclear whether the type I collagen being deposited in these models is actually homotrimeric or heterotrimeric type I collagen and whether this represents an overcompensated wound healing response triggered by initial insult to the kidney. By studying the regulation of homotrimeric type I collagen deposition in the COL1A2 deficient mouse model, we may be able to gain insight into the regulation of collagen deposition in other models and diseases.

However, differentiation between homotrimeric and heterotrimeric type I collagen within tissue specimens cannot currently be carried out. There are several antibodies available that recognize the α1(I) chain of type I collagen, yet attempts to make antibodies to the triple helical region of the α2(I) chain have been largely unsuccessful. Antibodies to the propeptides of the α2(I) chain have been successful, but the propeptide regions are not present in the mature form of the molecule present in tissues (Fisher, Stubbs et al. 1995). In conjunction with another laboratory, our lab sought to generate an antibody to the triple helical region of the α2(I) chain. An antibody was generated that was capable of binding the α2(I) chain by Western blotting techniques, but was unable to bind the α2(I) chain within tissue specimens with sufficient specificity for use by immunohistochemical techniques.

Type I Collagen Glomerulopathy

As stated, a novel type I collagen glomerulopathy was discovered in the COL1A2 deficient mouse. The kidneys of the COL1A2 deficient mice demonstrate accumulation of an amorphous pink material within an expanded mesangial matrix when stained with

hematoxylin and eosin (Figure I-2. D & G). The accumulated material was determined to be type I collagen based on picrosirius red staining (stains for type I & type III collagen; Figure I-2. E-F & H-I), electron microscopy (demonstrates deposition of a fibrillar protein; Figure I-3), and immunohistochemistry comparing an α1(I) (Figure I-4) and α1(III) antibody. An α1(I) antibody demonstrated that the accumulated material was in fact type I collagen. There was no evidence of aberrant collagen accumulation in blood vessels, perivascular spaces, or the interstitium of COL1A2 deficient kidneys. Severely affected glomeruli demonstrate findings such as capillary wall thickening, crescent formation, periglomerular fibrosis and mononuclear cell inflammation. These findings were not present in mild to moderately affected animals (Phillips, Pfeiffer et al. 2002).

The primary event leading to the development of the novel glomerulopathy appears to be the deposition of type I collagen into the renal mesangium. While this is not consistent with initiation of primary glomerular injuries in other disease states, it does demonstrate similarity to progressive glomerular decline following initial injury. Secondary progressive decline is characterized by the development of glomerulosclerosis, as well as tubulointersitial inflammation and fibrosis (Cotran, Kumar et al. 1999). The glomerulopathy seen in the COL1A2 deficient model appears to have histologic findings similar to those of glomerular fibrosis, particularly accumulation of fibrillar ECM proteins. However, it remains to be determined if the glomerulopathy demonstrates all histologic features characteristic of renal fibrosis.

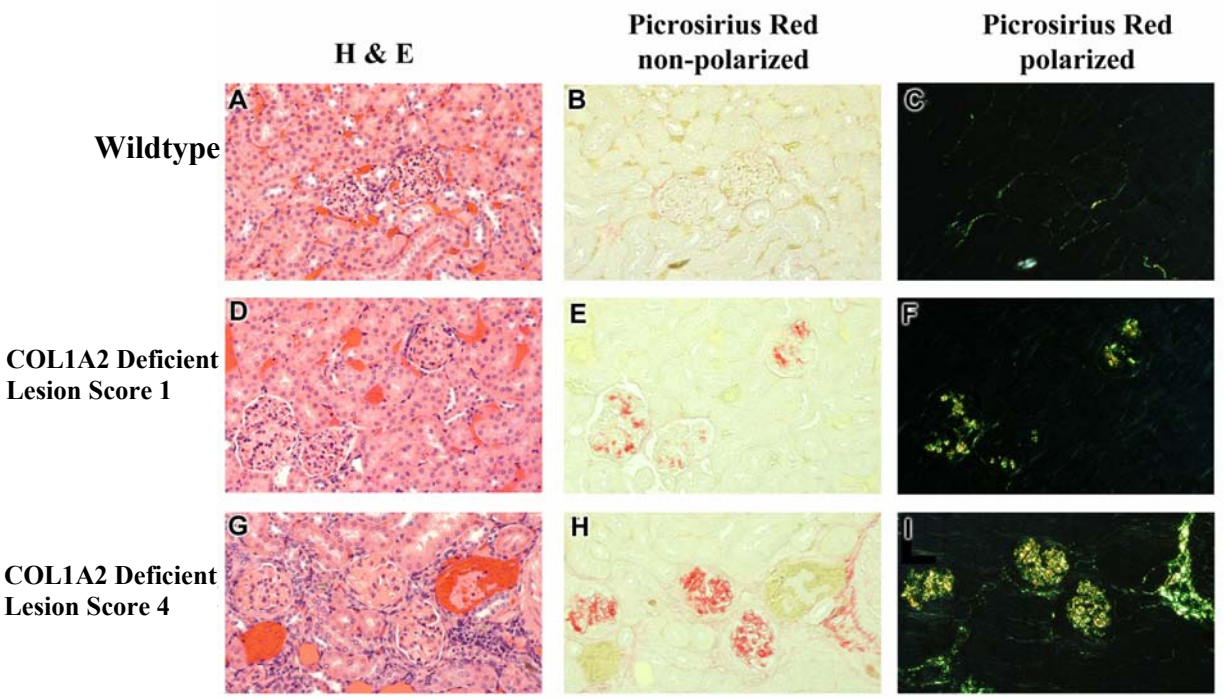

Figure I-2. COL1A2 deficient mice demonstrate glomerular accumulation of collagen. Serial sections at 200X magnification were evaluated by hematoxylin and eosin (H&E; A, D, and G) and picrosirius red (B, C, E, F, G, and H) staining. Wildtype (+/+) kidney (A-C) demonstrates normal glomerular architecture without collagen deposition. Serial sections from mild (lesion score 1, D-F) and severely (lesion score 4, G-I) affected kidneys demonstrate glomerular deposition of fibrillar collagen. Lesion score 1 indicates mild lesions; less than 50% of glomeruli affected. Lesion score 2 indicates moderate lesions; less than 50% of glomeruli affected. Lesion score 3 indicates moderate lesions; more than 50% glomeruli affected. Lesion score 4 indicates severe lesions; more than 50% of glomeruli affected. Adapted by permission from Macmillan Publishers Ltd: Kidney International 62(2): 383-91, copyright 2002.

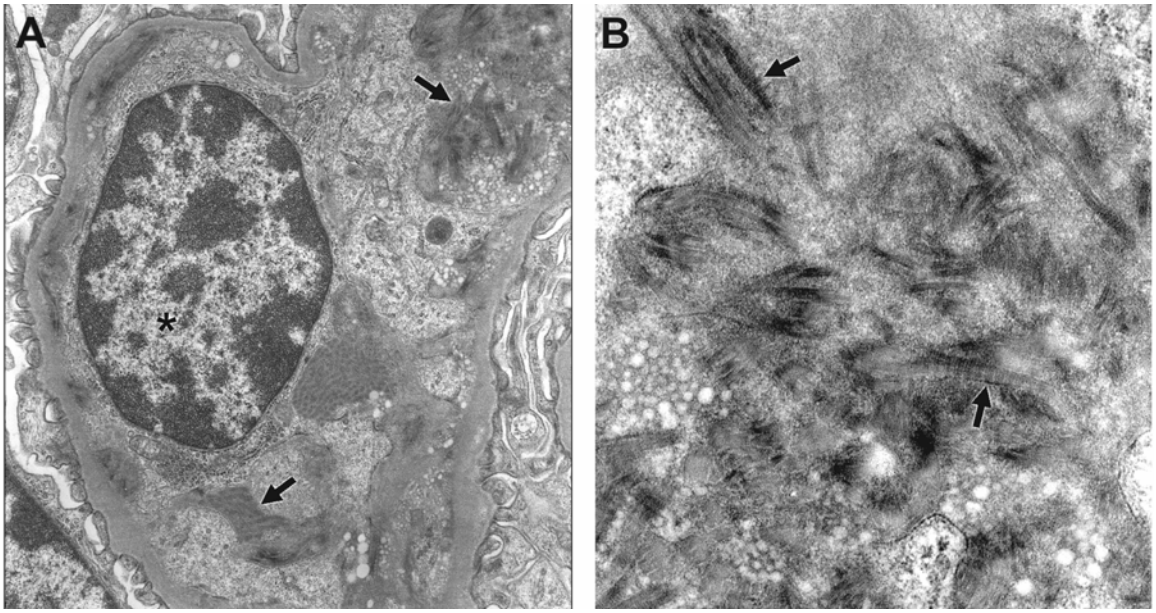

Figure I-3. Electron microscopy demonstrates fibrillar collagen deposition in the glomeruli of COL1A2 deficient animals. A) demonstrates mesangial extracellular deposits with fibrillar organization and bundles of fibrils (arrows; 9600X magnification). B) demonstrates typical cross-striation pattern of organized fibrillar collagen (arrows; 18,700X maginification). Reprinted by permission from Macmillan Publishers Ltd: Kidney International 62(2): 383-91, copyright 2002.

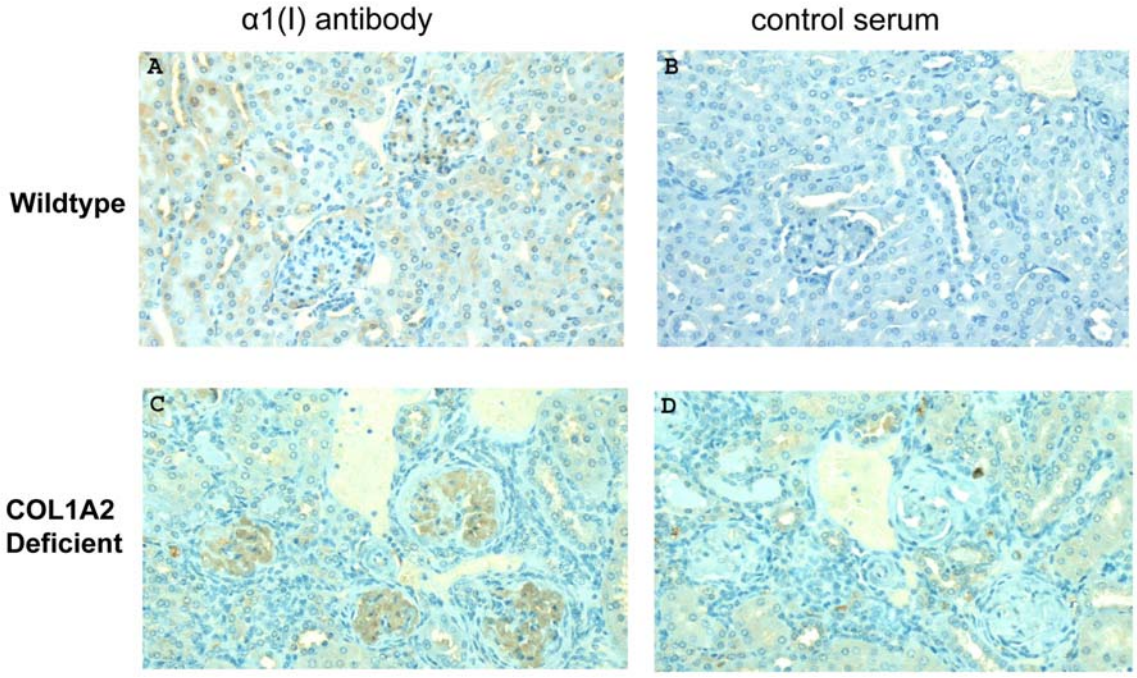

Figure I-4. Immunhistochemistry of COL1A2 deficient kidney demonstrates that the glomerular collagen is type I collagen. Serial kidney sections from wildtype (+/+, A and B) and COL1A2 deficient (-/-, C and D) kidneys treated with anti-proα1(I) collagen antibody (A and C) and with control serum (B and D). Adapted by permission from Macmillan Publishers Ltd: Kidney International 62(2): 383-91, copyright 2002.

Glomerular Structure and Function

A single glomerulus is a network of interlocking capillaries, which is surrounded by a filtration barrier and supported by mesangial cells (Stevens and Lowe 1997). The filtration barrier serves to remove unwanted solutes from circulating plasma by passage of the solutes through its three-layered structure. This structure (Figure 1-5A) is comprised of fenestrated endothelial cells, the glomerular basement membrane (GBM), and visceral epithelial cells called podocytes (Abrahamson 1987; Stevens and Lowe 1997).

The mesangial cells, 30-40% of the cell population, serve to create and integrate a structural scaffold for the capillary network and also aid in glomerular processing as well as filtration of macromolecules (Johnson, Floege et al. 1992). Mesangial cells secrete extracellular matrix proteins to provide a framework for the scaffold. Mesangial cells then enter the matrix to provide additional support (Mene, Simonson et al. 1989). Mesangial cells are mesenchymal in origin and have the ability to contract, phagocytize, and proliferate (Cotran, Kumar et al. 1999). Despite the ability of mesangial cells to produce ECM, there is very little type I collagen in the mesangium under normal physiologic conditions, and its accumulation is pathologic (Mene, Simonson et al. 1989; He, Hayashi et al. 2001). It is in the mesangium that ECM components are deposited in conditions such as glomerulosclerosis and chronic renal disease. Further evidence demonstrates that mesangial deposition of type I collagen decreases the filtering and

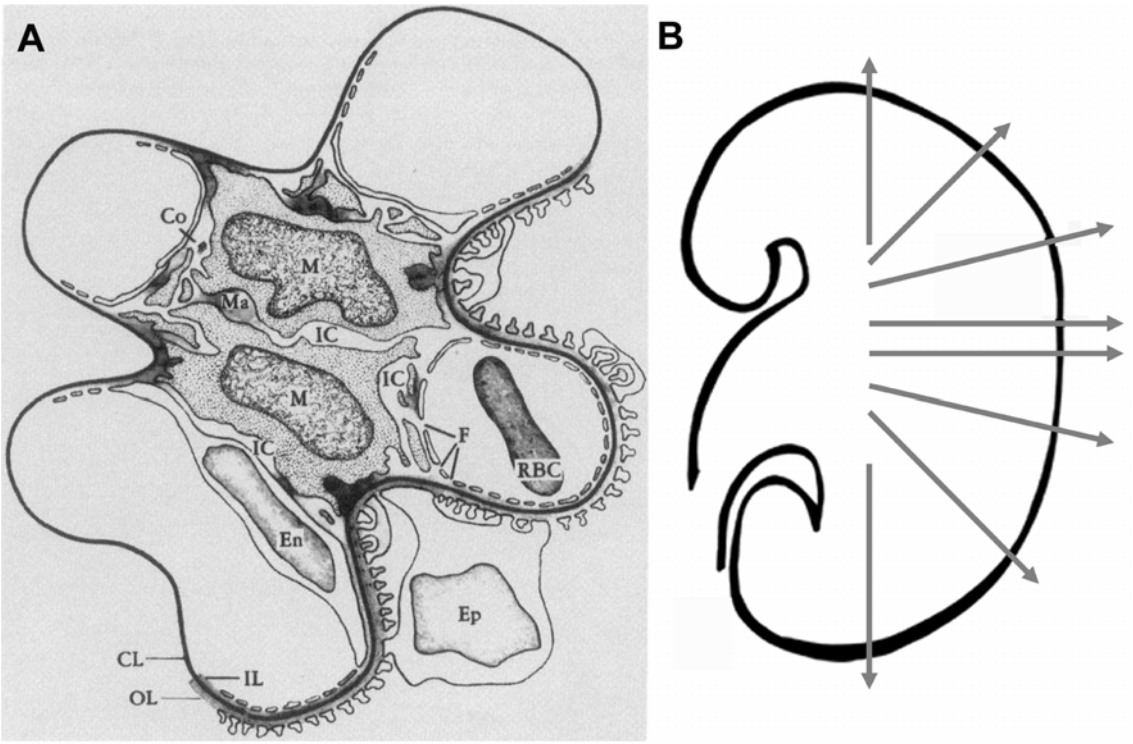

Figure I-5. Glomerular structure and maturation. A) is a diagram of a section of rat glomerulus. Mesangial cells (M) support the capillary network and secrete extracellular matrix proteins (Ma) that contribute to the structural scaffold. The fenestrated (F) endothelium (En) lines the inner layer (IL) of the glomerular basement membrane. The epithelial cell bodies (Ep) give rise to the epithelial foot processes that line the outer layer (OL) of the glomerular basement membrane. RBC, erythrocyte; CL, central layer of glomerular basement membrane; IC, intercellular channel. B) is a graphic representation of the direction of glomerular maturation. Arrows indicate a centrifugal pattern of glomerular maturation in which the earliest, embryologic functioning glomeruli are present in the juxtamedullary region near the base of the arrows and the latest glomeruli which gain function in the postnatal period are present in the outer cortex. Figure adapted with permission from Mene et. al. *Physiol Rev* 69(4): 1347-424, 1989.

processing functions of the cells that aid in removal of unwanted solutes (Liu 2006). Cultured mesangial cells produce type I collagen, approximately 50% of which is homotrimer, and prolonged culture results in accumulation of matrix components, including type I collagen, as seen in the mesangium of diseased glomeruli (Haralson, Jacobson et al. 1987). Histologically, mesangial cells are found between the capillaries. In addition, they have multiple extracellular processes, some of which make contact with the GBM, and a regular nucleus with occasional cytoplasmic granules. The mesangial cells are also in direct contact with endothelial cells within the glomerulus (Mene, Simonson et al. 1989).

Endothelial cells account for greater than 50% of the cells in the glomerulus. They are flat cells that line the lumen of glomerular capillaries and function as an integral part of the glomerular filtration barrier (Abrahamson 1987). Currently, there is no evidence that endothelial cells synthesize ECM components including type I collagen (Mene, Simonson et al. 1989). Structurally, the endothelial cells position their nucleus near the mesangium. The cytoplasm is interrupted by small circular fenestrations approximately 70nm in diameter, that are not closed by diaphragms. In addition, endothelial cells have cytoplasmic processes that extend into the capillary lumen in the region of mesangial cells. Endothelial cells are in direct contact with both the GBM and mesangial cells (Brenner and Rector 1981; Abrahamson 1987).

The glomerular basement membrane has three distinct layers. There is a central, dense layer termed the lamina densa, and two external layers that appear to be thinner, termed the lamina rara externa and the lamina rara interna. The glomerular basement membrane, the integral part of the filtration barrier, does not contain pores, it appears to

aid in filtration through a charge differential that exists along the membrane. There are several components that together makeup the GBM including type IV collagen, laminins, proteoglycans, and others (Brenner and Rector 1981; Abrahamson 1987).

Epithelial cells, or podocytes, the remaining cell type, have a variety of functions. They are thought to play a role in many glomerular functions, which include mediating perfusion of glomerular capillaries, establishing and maintaining the filtration properties of the glomerulus, and assisting in production of the components that comprise the GBM. Structurally, the podocytes have large cell bodies that give rise to foot processes which extend toward the glomerular capillaries and line the GBM. Foot processes from various podocytes interdigitate to form pores which are covered by a slit diaphragm, and all of these elements are involved in filtration. The podocyte cell body has a prominent nucleus, a well- developed Golgi apparatus, and prominent lysosomes. Interestingly, the cell body contains numerous organelles, while the foot processes contain very few. Of pathologic significance, podocytes are unable to regenerate or proliferate when they are destroyed or there is an increase in surface area of the GBM (Brenner and Rector 1981; Pavenstadt, Kriz et al. 2003).

Glomeruli are formed in a centrifugal pattern (Figure I-5B) surrounding the renal medulla. In mice at 16 days post-conception (pc), there is one large central glomerulus formed (Hogan, Costantini et al. 1986). At birth, there are numerous glomeruli present, yet only a fraction of them are functional. Between birth and 21 days of age, glomeruli begin to gain function in regions. The initial functioning glomeruli are found in the juxtamedullary region of the deep cortex (Kleinman and Reuter 1973; Spitzer and Brandis 1974). As the mouse develops, other regions of glomeruli gain functional

activity. Stepwise induction of new nephrons continues in mice until approximately 21 days of age; thus, a section of kidney from perinatal mice will contain nephrons at varying stages of development (Andrews, Betsuyaku et al. 2000). Once the kidney has reached its final state of maturation, the superficial glomeruli in the outer cortex are responsible for the majority of glomerular function (Kleinman and Reuter 1973; Spitzer and Brandis 1974). A similar progression of glomerular maturation has been demonstrated in other mammalian species, including humans (Potter 1965; Aperia and Herin 1975; He, Hayashi et al. 2001).

Matrix Metalloproteinases

Matrix metalloproteinases or (MMPs), of which 23 have been identified (Visse and Nagase 2003), function to regulate the composition and structure of the extracellular matrix (ECM). They do so by controlling signaling between the cellular components of the ECM through regulation of cell signals which affect cell proliferation, migration, differentiation, and death (Chegini, Kotseos et al. 2001). MMPs are involved in normal growth and development, thus regulation mediated by MMPs is tightly controlled (Massova, Kotra et al. 1998). MMPs are known to contribute widely to embryogenesis, wound healing, and inflammatory responses (Andrews, Betsuyaku et al. 2000). Yet MMPs are also involved in pathologic conditions, due to either decreased MMP activity leading to increased deposition of ECM components or increased MMP activity resulting in decreased ECM proteins. Decreased MMP activity is hypothesized to contribute to the development of cirrhosis, fibrotic lung disease, atherosclerosis, and multiple sclerosis, while increased MMP activity is thought to contribute to conditions such as rheumatoid

arthritis, osteoarthritis, metastatic cancer, and ulcers (Schnaper, Kopp et al. 1996; Parks and Mecham 1998). Therefore, the activity of this class of enzymes has wide-reaching effects on the body under many circumstances, and its control mechanisms may provide insight into modulating such activity.

MMPs are endopeptidases requiring Zn^{2+} at the catalytic site and the presence of Ca^{2+} for activity and stability (Massova, Kotra et al. 1998; Chegini, Kotseos et al. 2001). As a class of enzymes, MMPs have a wide range of substrates, and individually may cleave several ECM components in addition to non-matrix proteins. As a class, MMPs digest their substrates through cleavage of a peptide bond that is located before an amino acid with a hydrophobic side chain including Leu, Ile, Met, Phe, or Tyr. They rarely cleave peptide bonds occurring in front of charged residues (Visse and Nagase 2003). Further, they are secreted as active zymogens, which can be converted to their fully activated form via autocatalysis and/or other enzymes present in the ECM (Parks and Mecham 1998). Structurally, MMPs are similar and consist of a prodomain, a catalytic domain, a hinge region, and a hemopexin domain (Visse and Nagase 2003).

Matrix metalloproteinases are the primary physiologic regulators of glomerular extracellular matrix degradation. MMPs play a role in renal embryogenesis and serve to maintain glomerular ECM composition. An imbalance in ECM synthesis and degradation is known to occur in both inflammatory and non-inflammatory glomerular disease. However, the role of MMP regulation differs between the two types of glomerular disease. Non-inflammatory glomerular disease is characterized by a decrease in MMP activity responsible for glomerular ECM regulation, while inflammatory disease often demonstrates increased MMP activity (Lenz, Elliot et al. 2000).

Previous studies have identified MMP-1, MMP-2, MMP-3, MMP-9, MMP-13, and MT1-MMP (also known as MMP-14, a membrane-associated MMP) in the kidney (Lenz, Elliot et al. 2000). MMP-1, MMP-2, MMP-3, MMP-8, MMP-9, MMP-13, and MT1-MMP are known to cleave type I collagen and its molecular components (Barrett, Rawlings et al. 1998). Common features of these enzymes are provided in Table I-2.

MMP-1, MMP-8 and MMP-13 are collagenases that have the ability to cleave interstitial collagens (types I, II, and III), and though the mechanism is not well understood, they recognize a specific cleavage site approximately three-fourths of the length of the collagen molecule from the N-terminus (Visse and Nagase 2003). MMP-1 is expressed in humans and other vertebrates but not in mice (Massova, Kotra et al. 1998). MMP-8 is not found under normal circumstances in kidney, but is known to cleave type I collagen. MMP-13 is expressed in humans, mice and other vertebrates (Massova, Kotra et al. 1998).

MMP-2, or Gelatinase A, and MMP-9, or Gelatinase B, are capable of digesting denatured collagens, as well as interstitial collagens (Visse and Nagase 2003). MMP-2 is constitutively expressed in the kidney and is hypothesized to play a role in morphogenesis, as well as in abnormal deposition and/or cleavage of ECM components in a variety of kidney diseases (Lelongt, Legallicier et al. 2001). MMP-9 is known to be expressed in and has been localized to both healthy and diseased kidneys but has a restricted pattern of expression (Lelongt, Legallicier et al. 2001). It is hypothesized to play a role in morphogenesis, vascularization of a developing glomerulus, and in abnormal deposition and/or cleavage of ECM components in a variety of kidney diseases

TABLE I.2: Description of Matrix Metalloproteinases Known to Cleave Type I Collagen

Enzyme	Class	Name	Substrate	Pro	Active
MMP-1	Collagenase	Collagenase 1 & Human Interstitial Collagenase	Types I, II, III*, VII, and X collagen	52 kD	43 kD
MMP-2	Gelatinase	Gelatinase A	Denatured collagen and solubilized Type I collagen	72 kD	62 kD
MMP-3	Stromelysin	Stromelysin 1	Types I, III, IV, IX, X collagen and telopeptides	57 kD	45 kD
MMP-8	Collagenase	Neutrophil Collagenase & Collagenase 2	Types I*, II, and III collagen	85 kD	64 kD
MMP-9	Gelatinase	Gelatinase B	Types III, IV, V, denatured collagen, elastin and $\alpha 2$ chains	92 kD	84 kD
MMP-13	Collagenase	Collagenase 3 & Rodent Interstitial Collagenase	Type I, II*, monomeric, fibrillar, and denatured collagen	52 kD	42 kD
MMP-14	Membrane-Type	MT1-MMP	Types I, II, and III collagen, Pro-MMP-2, fibronectin	63kD	57kD

* preferred substrate, if known

(Andrews, Betsuyaku et al. 2000). MMP-2 and 9 are synthesized by podoctyes, while only MMP-2 is produced in the mesangial cells (Lelongt, Legallicier et al. 2001).

MMP-3, or Stromelysin-1, is capable of digesting several ECM components and is responsible for activating numerous proMMPs (Visse and Nagase 2003). Stromelysins, in general, are known for their broad substrate specificity. MMP-3 is expressed in humans, mice, and other vertebrates (Massova, Kotra et al. 1998) and is hypothesized to play a role in the activation of both MMP-2 and MMP-9 (Rao, Lees et al. 2005).

MT1-MMP (MMP-14) is a membrane-type MMP that functions to activate proMMP-2 and digest numerous ECM components, including the interstitial collagens. MT-MMPs are not secreted, but contain a transmembrane domain, attaching them to cell surfaces (Cao, Rehemtulla et al. 1996; Nelson, Fingleton et al. 2000; Visse and Nagase 2003).

MMPs are tightly regulated at several levels including transcription, activation of precursor enzymes, interaction with various ECM components, and inhibition by tissue inhibitors of metalloproteinases (TIMPs) (Visse and Nagase 2003). There are four TIMPs that have been isolated from vertebrates, TIMPs 1-4 (Massova, Kotra et al. 1998). Similar to MMPs, the TIMPS are expressed and tightly regulated as they play a role in normal development, tissue remodeling, and disease processes through direct regulation of MMP activity (Visse and Nagase 2003). All four TIMPs are capable of inhibiting any of the 23 MMPs by converting them to an inactive form that is unable to bind substrate; however, certain TIMPS have greater specificity for a given MMP based on molecular recognition and tissue expression (Nelson, Fingleton et al. 2000; Visse and Nagase 2003).

Renal Disease

Chronic kidney disease and renal fibrosis, which ultimately result in the loss of kidney function, is characterized by progressive accumulation of extracellular matrix components, or renal scarring, in which type I collagen may play a role (Alexakis, Maxwell et al. 2006). Chronic kidney disease has also been described as a failed wound-healing response following chronic sustained injury (Liu 2006). It involves several molecular and cellular pathways including mesangial cell and fibroblast activation, epithelial-to-mesenchymal transition in multiple cell lines, and disruption in the balance between extracellular matrix synthesis and degradation (Johnson, Floege et al. 1992; Alexakis, Maxwell et al. 2006). Renal disease is clinically categorized into the two broad categories of primary glomerular injury, nephritic and nephrotic syndromes. Nephritic syndrome is a type of renal disease in which patients present with macroscopic hematuria, hypertension, and progressive renal impairment. Typically, the patients are clinically ill and onset is often acute. In contrast, nephrotic renal impairments are defined by the triad of heavy proteinuria, hypoalbuminemia, and edema (Isbel 2005).

Proteinuria is the result of a disturbance in the glomerular filtration barrier. It can result from the disruption of the glomerular capillary wall, the glomerular basement membrane, the podocytes, or any combination of the three. Albumin is a protein that is commonly excreted in the early stages of renal disease. The clinically accepted measurement of proteinuria is a 24-hour urine collection and measurement of albumin excretion rate. However, a measurement of albumin:creatinine (a:c) ratio has been accepted as a sufficient substitution. An a:c ratio of $\geq$ 30mg/g qualifies as

microalbuminuria and ≥ 300mg/g indicates the presence of macroalbuminuria (Bennett, Haffner et al. 1995; Pavenstadt, Kriz et al. 2003).

Diabetic nephropathy is a form of chronic renal disease that has a profound impact on society and is characterized by impairment of glomerular basement membrane filtration capacity, as well as glomerular autoregulation of blood pressure. By definition, it is the presence of microalbuminuria in a patient with hyperglycemia due to insulin- or non-insulin dependent diabetes. The progression from mild to severe microalbuminuria and macroalbuminuria results in the development of nephrotic syndrome and eventually, end-stage renal disease. The progression in degree of microalbuminuria can be explained by alterations in kidney architecture which consist of progressive accumulation of ECM, including type I collagen at later stages, in both the mesangium and GBM of the kidney, as well as mesangial matrix expansion. As the matrix expands, it impinges on the capillaries and components of the filtration barrier, decreasing the surfaces available for filtration, resulting in declining glomerular function (Leon and Raij 2005; Schena and Gesualdo 2005).

While diabetic nephropathy is a common example of renal disease that progresses to glomerulosclerosis, a number of primary renal injuries also progress to develop a degree of glomerulosclerosis and collagen deposition. These include, but are not limited to, IgA nephropathy, mesangial proliferative glomerulonephritis, and systemic lupus erythematosis (SLE) nephropathy. IgA nephropathy, or Berger's disease, is a condition that results in macroscopic hematuria due primarily to the deposition of IgA into the renal mesangium. The deposition of IgA results in glomerular injury triggering mesangial enlargement and hypercellularity, and in the severely affected, glomerular mesangial

matrix deposition, including types I and III collagen. Mesangial proliferative glomerulonephritis is an example of nephrotic syndrome which initially presents with proteinuria. Initially, the glomeruli demonstrate mesangial hypercellularity with increased mesangial matrix. Again, as the disease progresses, the glomeruli become segmentally or totally sclerotic and demonstrate type III collagen deposition (Brenner and Rector 1981; Yoshioka, Tohda et al. 1990). Patients with SLE nephropathy have various clinical presentations and pathologic classifications. The most common renal manifestation is the presence of nephrotic syndrome with renal insufficiency due to immunoglobulin and complement component deposition in the glomeruli. In general, active SLE nephropathy glomeruli demonstrate a number of features that may include hypercellularity, leuckocyte infiltration, and subendothelial deposits. However, it is chronic SLE nephropathy that demonstrates fibrillar collagen deposition and the formation of glomerulosclerotic lesions (Brenner and Rector 1981; Hunter, Hurwitz et al. 2005). Therefore, examination of the regulation of type I collagen deposition, specifically homotrimeric type I collagen, may lead to a greater understanding of the regulation of ECM deposition in diabetic nephropathy and other renal diseases.

Potential Mechanisms for the Type I Collagen Glomerulopathy

The COL1A2 deficient mouse and its exclusive production of homotrimeric type I collagen allows for the further investigation of structure and function of homotrimer, or the absence of the α2 chain, in the regulation of extracellular matrix components within glomeruli. It also allows for the study of molecular and pathologic mechanisms that may be responsible for glomerular collagen deposition common to renal disease and

glomerulosclerosis. Such potential molecular and pathologic mechanisms have been described in other mouse models of glomerulosclerosis. In general the mechanisms responsible for glomerular collagen deposition can be grouped into the broad categories of increased synthesis of glomerular collagen, aberrant degradation mediated by matrix metalloproteinases, or a combination of the two processes. Select models which demonstrate these mechanisms are described here and in Table I-3.

Chatziantoniou et. al describe a model in which glomerular collagen deposition is due to an increase in synthesis. In their study, they treated mice, in which a luciferase gene was controlled by the murine proα2(I) collagen promoter, with the nitric oxide synthesis inhibitor L-NAME (N^G-nitro-L-arginine-methyl ester) and demonstrated that increased type I collagen synthesis and deposition into the glomeruli that occurs with L-NAME treatment is due to upregulation of the proα2(I) collagen gene (Chatziantoniou, Boffa et al. 1998). The L-NAME model produced by Chatziantoniou et. al is a model of nephroangio- and glomerulosclerosis due to type I collagen accumulation in renal vasculature and glomeruli as a result of systemic hypertension. They demonstrated early activation of the COL1A2 gene in both afferent arterioles and glomeruli in response to nitric oxide inhibition and endothelin administration. Interestingly, the activation of the COL1A2 gene preceded the increase in blood pressure. Together, this indicates that nitric oxide and its balance with endothelin plays a role in extracellular matrix synthesis and deposition. In addition, the inhibition of nitric oxide appears to induce glomerulosclerosis independent of blood pressure regulation (Chatziantoniou, Boffa et al. 1998).

TABLE I.3: Mechanisms Responsible for Glomerulosclerosis in Select Mouse Models

Author	Model	Renal Pathology	Deposition	Collagen expression	MMP expression	MMP activity
Chatziantoniou et. al (1998)	L-NAME Mice	Glomerulosclerosis	Type I collagen	mRNA, Increased	unknown	unknown
Uchio et. al (2000, 2004, 2005)	ICGN mice	Glomerulonephritis and glomerulosclerosis	Type I, III, and IV collagen	mRNA, proα1(I), proα1(III) increased	Protein, MMP-2, 9; MT1-MMP; TIMP-1,2 decreased	MMP-1, 2, 9 decreased
Tomita et. al (2004)	Double anti-Thy 1.1 antibody in rats	Mesangial proliferative glomerulonephritis and glomerulosclerosis	Type I and IV collagen	unchanged	RNA, MMP-9 decreased	unknown
Sanderson et. al (1995) **Kopp** et. al (1996) **Mozes** et. al (1999)	Increased TGF-β mice	Glomerulonephritis and glomerulosclerosis	Type I and III collagen	mRNA, proα1(I), proα1(III) increased	RNA, TIMP-1 increased	MMP-2 increased
Phillips et. al (2002)	COL1A2 deficient	Glomerulosclerosis	Type I collagen	mRNA, proα1(I) increased	unknown	unknown

Uchio et al. described the role of increased synthesis and aberrant collagen degradation in ICGN (Institute of Cancer Research (ICR)-derived glomerulonephritis) mice, an inbred strain of mice with idiopathic nephritic syndrome, in which the mice demonstrate proteinuria, hypoproteinemia, and hyperlipidemia. Histologically, the mice demonstrate glomerular types I, III and IV collagen deposition, glomerular basement membrane thickening, podocyte foot process effacement, and mesangial cell proliferation all of which increased with age (Uchio, Manabe et al. 2000; Uchio, Manabe et al. 2004; Uchio-Yamada, Manabe et al. 2005). Their work demonstrated that the glomerulosclerosis seen in the ICGN mice is a result of a combined mechanism of increased synthesis and aberrant degradation of the deposited ECM components. They demonstrate, by *in situ* hybridization, increased steady-state proα1(I) collagen and proα1(III) collagen mRNA levels as compared to control animals (Uchio, Manabe et al. 2004). They also show both a decrease in MMP activity and protein expression as mechanisms of aberrant degradation occurring in the ICGN mice. Using FITC-labeled substrate, they show a decrease in MMP-1, 2, and 9 activity through the use of a fluorogenic assay (Uchio, Manabe et al. 2000) and by Western blot analysis, they showed a decrease in MMP-2, MMP-9, MT1-MMP, TIMP-1, and TIMP-2 protein in ICGN mice (Uchio-Yamada, Manabe et al. 2005). However, they demonstrate the presence of MMP-2 in glomeruli of severely affected ICGN mice by immunohistochemistry, and interestingly, MMP-2 was not present by the same technique in the age-matched counterparts or in younger control and affected animals. This might indicate that the MMP-2 is present in response to injury, but is inactive and unable to degrade the deposited ECM components. Through the ICGN model, it has been shown that

deposition of ECM components in the ICGN mouse are the result of increased pretranslational synthesis in addition to a decrease in the degradative capacity of renal MMPs through decreased activity and expression (Uchio, Manabe et al. 2000; Uchio, Manabe et al. 2004; Uchio-Yamada, Manabe et al. 2005).

Similar findings have been demonstrated in two strains of a mouse model of glomerulosclerosis described by Sanderson et. al, in which a TGF-β1 transgene under the control of murine albumin promoter and its enhancers expressed high plasma TGF-β1 levels (Sanderson, Factor et al. 1995). Clinically these mice exhibited ascites and proteinuria consistent with glomerulonephritis. This progressed to chronic glomerulonephritis in which all glomeruli were affected. Histologically, severely affected mice demonstrated accumulation of ECM in their glomeruli, thickening of the glomerular basement membrane, capsular epithelial proliferation, and development of capsular crescents. Further examination by electron microscopy revealed that the mice demonstrated subendothelial extracellular matrix accumulation in their glomeruli, including deposition of fibrillar collagen in the severely affected animals (Kopp, Factor et al. 1996). Immunostaining revealed that the mice had glomerular types I and III collagen deposition. Northern blot analysis showed an associated increase in steady-state α1(I) and α1(III) collagen mRNA levels in young mice when compared with controls; however, the analysis showed a concomitant decrease in steady-state α1(I) mRNA with age in which the differential expression seen between affected and wildtype mice was no longer present at 5 weeks of age. In addition, they found increased activity of MMP-2 by gelatin zymography after 5 weeks of age when compared to wildtype (Northern blot analysis was not sensitive enough to detect changes in mRNA levels from total kidney

isolates for MMPs 2, 3, and 9. However, Northern blot analysis was able to detect a significant increase in expression of TIMP-1 mRNA in severely affected animals. This evidence indicates that in the presence of elevated TGF-β, glomerular collagen deposition is the result of an imbalance in pretranslational synthesis and degradative capacities mediated by MMP/TIMP interactions (Mozes, Bottinger et al. 1999). Further, this research provides a strong indication for the role of TGF-β as a modulator of renal and more specifically glomerular fibrosis.

Tomita et al., describe a rat model of mesangial proliferative glomerulonephritis in which the animals demonstrate mesangial cell proliferation and an increase in mesangial glomerular types I and IV collagen as a result of aberrant degradation alone. The rats develop proliferative glomerulonephritis and proteinuria as a result of injections of anti-Thy-1.1 monoclonal antibody 1-22-3. A single injection model results in reversible mesangial proliferative glomerulonephritis with a return to normal renal histology in 6-8 weeks. Double injection with anti-Thy-1.1 monoclonal antibody 1-22-3 results in a prolonged model of glomerulonephritis resulting in persistent mesangial cell proliferation, glomerular collagen deposition, and proteinuria (Jefferson and Johnson 1999). In this model, they described glomerular types I and IV collagen deposition that is not associated with increased steady-state type I or type IV collagen mRNA levels. However, they did observe a decrease in MMP-9 steady-state mRNA expression with no differences in expression of TIMP-1, MMP-2, MMP-13 and MT1-MMP steady-state mRNA (Tomita, Koike et al. 2004), suggesting that aberrant degradation is responsible for the glomerular collagen deposition.

CHAPTER II

TYPE I COLLAGEN GLOMERULOPATHY: DOSE DEPENDENT POSTNATAL INITIATION OF HOMOTRIMERIC COLLAGEN DEPOSITION FOLLOWS GLOMERULAR MATURATION IN THE PRESENCE OF A PRETRANSLATIONAL MECHANISM

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Type I Collagen Glomerulopathy: Dose Dependent Postnatal Initiation of Homotrimeric Collagen Deposition Follows Glomerular Maturation in the Presence of a Pretranslational Mechanism

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ABSTRACT

COL1A2 deficient (*oim*) mice, which lack functional proα2(I) collagen chains and synthesize exclusively homotrimeric type I collagen, exhibit a novel type I collagen glomerulopathy. Here we show that these mice accumulate fibrillar homotrimeric type I collagen in the mesangium that spreads to the subendothelial space in the peripheral capillary loops. Picrosirius red staining of formalin fixed kidneys demonstrates that in comparison to wildtype mice, COL1A2 deficient and heterozygous animals exhibit abnormal glomerular collagen deposition in a gene dosage dependent manner. Furthermore, the glomerulopathy initiates during the first postnatal week, following the pattern of glomerular maturation. The glomerulopathy appears progressive and results in albuminuria in severely affected animals. *In situ* hybridization revealed no gross differences in steady-state proα1(I) and proα2(I) collagen mRNA levels among the three genotypes. However, quantitative RT-PCR using whole kidney sections showed a 2-fold increase in steady-state proα1(I) collagen mRNA in 1-month COL1A2 deficient animals relative to wildtype and heterozygous animals, suggesting that glomerular collagen deposition seen in the type I collagen glomerulopathy is, in part, due to pretranslational mechanisms. Our results indicate that the mechanism responsible for the collagen deposition seen in the glomerulopathy is potentially a combination of increased synthesis and aberrant degradation of glomerular collagen.

INTRODUCTION

A novel type I collagen glomerulopathy was identified in the COL1A2 deficient mouse model; characterized by the deposition of homotrimeric type I collagen in the renal mesangium (Phillips, Pfeiffer et al. 2002). Under normal physiologic conditions, there is very little type I collagen in the glomerulus (Mene, Simonson et al. 1989; Yoshioka, Tohda et al. 1990). The primary event leading to the development of the novel glomerulopathy in the COL1A2 deficient mice appears to be the deposition of homotrimeric type I collagen into the glomerular mesangial matrix. In chronic renal disease, progressive accumulation of collagen and other extracellular matrix (ECM) components in the mesangial matrix results in fibrosis, glomerulosclerosis, and renal failure (Glick, Jacobson et al. 1992; Couser and Johnson 1994; McLennan, Death et al. 1999). The accumulation of ECM may be due to over compensation of glomerular wound healing responses (Haralson, Jacobson et al. 1987; Johnson, Floege et al. 1992). This collagen deposition is thus similar to progressive glomerular decline following an initial injury (Cotran, Kumar et al. 1999). In order to further characterize the type I collagen glomerulopathy and to elucidate the role of the α2 chain of type I collagen in kidney pathology, we determined the pattern of initiation, disease progression, and began investigating the mechanisms responsible for glomerular collagen deposition in COL1A2 deficient mice.

Type I collagen is the most abundant structural protein in the body. The predominant isotype is a heterotrimeric protein composed of two related, yet genetically distinct, procollagen chains (Byers, Scriver et al. 2001). The heterotrimer is formed by the incorporation of two proα1(I) collagen chains and one proα2(I) collagen chain

[$\alpha1(I)_2\alpha2(I)$] (Phillips, Wenstrup et al. 1992; Byers, Scriver et al. 2001). A homotrimeric isotype of type I collagen containing three pro$\alpha1$(I) collagen chains [$\alpha1(I)_3$] has also been identified (Byers, Scriver et al. 2001). However, the role of the homotrimeric isotype is currently not well understood. The homotrimer is found early embryologically in several tissues (Jimenez, Bashey et al. 1977; Rupard, Dimari et al. 1988), later in development in small amounts in skin (Uitto 1979), in some tumors and cultured cancer cell lines (Moro and Smith 1977; Rupard, Dimari et al. 1988; Ghersi, La Fiura et al. 1989), and during wound healing (Haralson, Jacobson et al. 1987). It has also been shown that cultured mesangial cells produce homotrimeric type I collagen, further suggesting that homotrimer production may be related to injury and/or wound healing (Haralson, Jacobson et al. 1987; Johnson, Floege et al. 1992).

The COL1A2 deficient mouse model, otherwise known as the *oim* mouse (osteogenesis imperfecta model) is homozygous for a spontaneous nucleotide deletion at position 3983 in the COL1A2 gene. The mutation results in an alteration of the carboxy-propeptide region of the pro$\alpha2$(I) collagen chain. The carboxy-propeptide region is not present in mature type I collagen but it is responsible for association of the $\alpha2$(I) chain with the two $\alpha1$(I) chains in assembly of the heterotrimeric type I collagen molecule [$\alpha1(I)_2\alpha2(I)$]. Because of this defect, COL1A2 deficient mice are postulated to synthesize nonfunctional $\alpha2$(I) chains which are rapidly degraded, thus resulting in exclusive production of homotrimeric type I collagen (Deak, Nicholls et al. 1983; Chipman, Sweet et al. 1993; McBride, Choe et al. 1997).

A glomerulus is a network of capillaries that is the filtration unit of the kidney serving to remove water and small solutes from circulating plasma. Mesangial cells, 30-

40% of the cell population of the glomerulus, create and integrate a structural scaffold for the capillary network (Mene, Simonson et al. 1989; Johnson, Floege et al. 1992). They also secrete extracellular matrix proteins to provide a framework for the scaffold. Mesangial cells then enter the matrix to provide additional support (Mene, Simonson et al. 1989). Glomeruli are formed in a centrifugal pattern surrounding the renal medulla. At birth, the mouse kidney has numerous glomeruli, yet only a fraction of them are functional. Between birth and 21 days of age, glomeruli gain function regionally (Andrews, Betsuyaku et al. 2000). The initial functioning glomeruli are found in the juxtamedullary region of the deep cortex(Kleinman and Reuter 1973; Spitzer and Brandis 1974). As the mouse develops, glomeruli in other regions gain functional activity. Stepwise induction of new nephrons continues until the kidney has reached its final state of maturation, and the superficial glomeruli in the outer cortex become responsible for the majority of glomerular function (Kleinman and Reuter 1973; Spitzer and Brandis 1974; Bard 1994). A similar progression of glomerular maturation has been demonstrated in other mammalian species, including humans (Potter 1965; Kleinman and Reuter 1973; Aperia and Herin 1975).

Studies using transgenic models have demonstrated that glomerular collagen deposition (Chatziantoniou, Boffa et al. 1998; Francki, Bradshaw et al. 1999; Mozes, Bottinger et al. 1999; Uchio, Manabe et al. 2000; Tomita, Koike et al. 2004; Uchio, Manabe et al. 2004; Uchio-Yamada, Manabe et al. 2005) can be the result of either an increase in pretranslational expression of type I collagen, a decrease in enzymes such as matrix metalloproteinases (MMPs) that are responsible for degradation of the collagen, or a combination of the two mechanisms (Lenz, Elliot et al. 2000). For example,

Chatziantoniou et. al, treated mice, in which a luciferase gene was controlled by the murine proα2(I) collagen promoter, with the nitric oxide synthesis inhibitor L-NAME (N^G-nitro-L-arginine-methyl ester) and demonstrated that increased type I collagen synthesis and deposition into the glomeruli occurs via upregulation of the proα2(I) collagen gene (Chatziantoniou, Boffa et al. 1998). Uchio et. al demonstrated that both an upregulation of mRNA expression and a decrease in degradation through decreased activity and expression of matrix metalloproteinases results in glomerulosclerosis in ICGN mice (Uchio, Manabe et al. 2000; Uchio, Manabe et al. 2004; Uchio-Yamada, Manabe et al. 2005). Further, Tomita et al, demonstrated that a decrease in MMP-9 mRNA expression results in mesangial matrix expansion in a rat model of mesangial proliferative glomerulonephritis (Tomita, Koike et al. 2004).

We sought to determine whether or not the glomerulopathy in the COL1A2 deficient model was progressive with pre- or postnatal initiation and whether there were any functional consequences of collagen deposition. Further, we sought to determine whether pretranslational upregulation of type I collagen was involved in the deposition of collagen. Through histologic examination and RT-PCR, we determined that the type I collagen glomerulopathy in COL1A2 deficient mice initiates postnatally, progresses with age and glomerular maturation, is associated with a gene dose effect, and is, in part at least, due to increased pretranslational synthesis.

METHODS

Animals

Homozygous B6C3Fe *a/a-Col1a2*$^{oim/J}$ (COL1A2 deficient, -/-); heterozygous (-/+); and wildtype (+/+) mice were purchased from the Jackson Laboratory (Bar Harbor, ME, USA). Animals were housed in an AAALAC accredited animal facility, provided with water and food (Purina 5008 Formulab Diet; Purina Mills Inc., Richmond, IN, USA) ad libitum, and cared for in accordance with an approved University of Missouri Animal Care and Use protocol. +/+, +/-, and -/- genotypes were determined by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) analysis (Phillips, Bradley et al. 2000).

Experimental Design

Animals were divided into one of four age groups 1-month (n=52 animals), 2-weeks (n=56 animals), 1-week (n=55 animals), and 1-day (n=26 animals) of age. Animals were sacrificed and kidneys were harvested. The right kidney was snap frozen immediately in liquid nitrogen, and stored at -80ºC until further use. The left kidney was fixed in 10% neutral-buffered formalin for 48 hours.

Microscopy

Formalin-fixed kidneys were embedded in paraffin, longitudinally sectioned at 5µm, and stained with Picrosirius red (PSR) stain. Glomeruli within individual sections were examined and a lesion score for each individual kidney was recorded. Glomerular lesion scores were determined using the following scale: 1- mild lesions; less than 50% of

glomeruli affected; 2- moderate lesions; less than 50% of glomeruli affected; 3 - moderate lesions; more than 50% glomeruli affected; 4 - severe lesions; more than 50% of glomeruli affected. A positive correlation was demonstrated between the amount of collagen deposited in the renal glomeruli and the lesion score assigned to the mouse (Phillips, Pfeiffer et al. 2002).

For electron microscopy, kidneys were harvested and fixed in 4% paraformaldehyde, 4% glutaraldehyde, and 0.1M cacodylate buffer for thin sectioning. Tissue processing was carried out as described previously (Noakes, Miner et al. 1995). A diamond knife was used to cut thin sections which were stained with lead citrate plus uranyl acetate for transmission electron microscopy (Kikkawa, Virtanen et al. 2003). Reagents were purchased from Polysciences Inc (Warrington, PA).

Morphometry Mapping

PSR-stained sections were also used to assess individual glomerular lesion distribution and severity within longitudinal sections as depicted in Figure II-4. Formalin fixed kidneys from 1-month [n=10 -/- (lesion score 2-4), n=10 +/+ (lesion score 0), and n=10 +/- (lesion score 1-4)] and 2-week [n=10 -/- (lesion score 1-3), n=10 +/+ (lesion score 0), and n=10 +/- (lesion score 1)] old mice were used. Longitudinal sections were assessed using four 200X fields that bracketed the renal cortex, from the corticomedullary junction to the renal capsule. Each field was divided into corticomedullary and cortical halves (zones), using the midcortical interlobular arteries as landmarks. Within these two zones, the following were determined: number of glomeruli, number of glomeruli without collagen deposition (assigned a morphometry

score of 0), number of glomeruli in which <25% of glomerular area is occupied with collagen (assigned a score of 1), number of glomeruli in which 25-50% of glomerular area is occupied with collagen (assigned a score of 2), number of glomeruli in which 50-75% of glomerular area is occupied with collagen (assigned a score of 3), and number of glomeruli in which 75-100% of glomerular area is occupied with collagen (assigned a score of 4). A total score for each animal was determined by multiplying the number of glomeruli in each category by their respective glomerular morphometry score, adding the scores from each category, and dividing by the total number of glomeruli. For example, if 40 glomeruli were found in the four cortical fields examined and 20 had no collagen deposition, 10 had morphometry scores of two, 8 had morphometry scores of three and 2 had morphometry scores of four, the total score was [(20x0)+(10x2)+(8x3)+(2x4)]/40 = 1.3. Mean corticomedullary and cortical glomerular morphometry scores, mean corticomedullary and cortical glomerular numbers, mean number of affected glomeruli and the percentage of affected glomeruli were determined for each genotype at 2-weeks and 1-month of age.

Albumin to Creatinine Ratio Measurement

Urine was collected from mice prior to sacrifice and stored at -20ºC. To determine whether the COL1A2 deficient mice exhibit microalbuminuria, urine albumin concentration was measured using the Albuwell M kit (Exocell, Philadelphia, PA) following a 1:13 dilution of the urine sample (Animal Models of Diabetic Complications Consortium, http://www.amdcc.org/shared/showFile.asp?docTypeID=3&docID=22,

March 9, 2006) (Burke June 25, 2003). Creatinine concentration was analyzed using an automated Jaffe alkaline picrate assay on a urine sample diluted 1:2.

In Situ Hybridization (ISH)

Formalin fixed kidneys from 1-month [n=7 -/- (lesion score 2-4), n=5 +/+ (lesion score 0), and n=5 +/- (lesion score 1-4)], 1 to 2-week [n=7-/- (lesion score 1-2), n=5 +/+ (lesion score 0), and n=5 +/- (lesion score 1-2)] and 1 to 3-day [n=3 -/- (lesion score 0-1), n=4 +/+ (lesion score 0), and n=4 +/- (lesion score 0)] old mice were used. Paraffin sections of formalin-fixed kidneys were deparaffinized in xylene and rehydrated in serial ethanol washes. Sections were then treated with proteinase K for 7 minutes at room temperature to increase the tissue permeability for hybridization and then post-fixed in 10% neutral-buffered formalin for 20 minutes. The treated sections underwent prehybridization for 1 hour followed by hybridization with ^{35}S-UTP labeled riboprobes overnight at 50ºC. Following hybridization, sections were washed in FSM (50% formamide, 2x SSC, and 20 mM mercaptoethanol) at 65°C for 1 hour. Sections were digested with 6μg/ml RNase A at 37°C for 30 min and again washed in FSM at 65°C for 1.5 hours. Tissue sections were then dehydrated and coated with Kodak NTB-2 emulsion for autoradiography. After one-week exposure at 4°C, the slides were developed and counter-stained with hematoxylin (Reneker, Silversides et al. 1995; Xu, Overbeek et al. 2002).

RNA probe templates were synthesized from cDNA fragments of murine α1(I) and α2(I) collagen chains. Templates were generated by PCR amplification using T3 engineered forms of the primers in Table II-1 followed by gel purification and extraction

Table II-1: Primer sequences used for *in situ* hybridization and quantitative reverse transcriptase-polymerase chain reaction analysis

Primer	gi Number	Sequence	Nucleotide Segment	Amplicon Size
ISH[a] & RT-PCR[b]				
COL1A1 Forward	470673	5'- AGC CTG AGT CAG CAG ATT GAG A -3'	3676-3698	404 bp
COL1A1 Reverse	470673	5'- CTT GCA GTG ATA GGT GAT GTT CT -3'	4058-4080	
ISH[a]				
COL1A2 Forward	50488	5'- CTT CTT GGT GCT CCC GGT ATT CT -3'	2693-2715	1365 bp
COL1A2 Reverse	50488	5'- TTT TGG AGC AGC CAT CGA CTA -3'	4038-4058	
RT-PCR[b]				
COL1A2 Forward	50488	5'- TGA AGT GGG TCT TCC AGG TCT TTC -3'	946-969	235 bp
COL1A2 Reverse	50488	5'- CAC CCT TGT TAC CGG ATT CTC CTT -3'	1158-1181	

[a] *in situ* hybridization

[b] quantitative reverse transcriptase-polymerase chain reaction

(Qiagen, Valencia, CA) (Schulman and Setzer 2003). Anti-sense and sense probes were synthesized using T3 RNA polymerase *in vitro* transcription kit (Ambion, Austin, TX) in the presence of ^{35}S-UTP. Within each experiment sense and anti-sense probes were generated, measured for activity, and used at a concentration to normalize for specific activity. The sense probe served as a reference to detect non-specific binding.

Quantitative Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

Snap frozen kidneys from 1-month [n=6 -/- (lesion score 3-4), n=6 +/+ (lesion score 0), and n=6 +/- (lesion score 1-4)], 2-week [n=7 -/- (lesion score 1-4), n=6 +/+ (lesion score 0), and n=6 +/- (lesion score 1)] and 1-week [n=6 -/- (lesion score 1-2), n=6 +/+ (lesion score 0), and n=7 +/- (lesion score 1)] old mice were used. Snap frozen kidneys were thawed in a solution of phenol and guanidine isothiocyanite (TRIzol Reagent, Invitrogen Corporation, Carlsbad, CA). The tissue was lysed using a tissue homogenizer (TissueLyser, QIAGEN, Valencia, CA). Total RNA was isolated according to manufacturer's protocol (TRIzol Reagent, Invitrogen Corporation, Carlsbad, CA). The extracted RNA was dissolved in 100µl of water treated with diethyl pyrocarbonate (DEPC, Sigma-Aldrich Co., St. Louis, MO). The quantity of RNA was assessed by measuring the absorbance at 260 nm. Five micrograms of total RNA was reverse transcribed with oligo(dT) primers according to manufacturer's protocol (Superscript, Invitrogen Corporation, Carlsbad, CA). The cDNA was diluted with diethyl pyrocarbonate (DEPC, Sigma-Aldrich Co., St. Louis, MO) treated water to a concentration of 20 ng/µl. The primer sequences for COL1A1 and COL1A2 are found in Table II-1. Primer sequences for hypoxanthine-guanine phosphoribosyltransferase

(HPRT) have been previously reported (O'Garra, Chang et al. 1992). Quantification was done by comparing fluorescence of kidney isolates to that of plasmid standards. Standards were generated from linearized plasmids containing desired, cloned amplicons using the Zero Blunt Topo PCR-cloning kit (Invitrogen Corporation, Carlsbad, CA). COL1A1 and COL1A2 gene expression was quantified using real-time RT-PCR (LightCycler®, Roche Diagnostic Corporation). PCR reactions were performed in a 20 µl volume in glass capillaries that contained isolated cDNA, 0.5 µM of forward and reverse primers, 3mM $MgCl_2$, SYBR© green, and QuantiTect™ SYBR© Green PCR Master Mix which contains dNTP mix, HotStart Taq DNA Polymerase, reaction buffer I, and SYBR© green (QIAGEN, Valencia, CA) (Morrison, Weis et al. 1998; Bustin 2000). Copy number of the desired genes was determined through generation of a standard curve using known concentrations, 10^1 to 10^6 copies, of the pCR-Blunt II-TOPO (Invitrogen Corporation, Carlsbad, CA) plasmid which contains the amplicon of interest. The PCR reactions for COL1A1 and COL1A2 were incubated at 95°C for 15 minutes to activate the polymerase. Forty cycles consisting of a 15 second denaturation at 94°C, a 30 second annealing at 60°C, and a 30 second extension at 72°C were used to amplify COL1A1 and COL1A2. Fluorescence was monitored at the end of each extension phase at 80°C for 5 seconds. HPRT levels were used to normalize COL1A2 deficient, heterozygous, and wildtype samples.

Statistics

All statistical analyses were performed using SAS (SAS Institute Inc., Cary, NC). Morphometry data was analyzed as a split plot in space. The main plot contains effects

of gene, age, and the interactions between genotype and age. The subplot contains effects of side (cortical versus corticomedullary) and all possible interactions between side and the main plot effects. Polynomial orthogonal contrasts were performed to test for linear and/or quadratic gene dose effect.

The urine data was analyzed as a 1-way completely randomized design analysis of variance. Due to heterogeneous variances among albumin to creatinine ratios, a log transformation was used to stabilize the variation. Data presented are the actual mean and standard error but the differences in albumin to creatinine ratio within genotype and lesion score were analyzed using the transformed data.

The RT-PCR data was analyzed as a completely randomized design in which genotype and age were arranged as a 3 by 3 factorial (3 genotypes, 3 ages). Due to heterogeneous variances among copy numbers, a log transformation was again used to stabilize the variation. The means presented are the actual mean and standard error but the differences in copy number with age and genotype were determined using the transformed data.

Mean differences were ascertained using Fisher's Least Significant Difference (LSD). All results are presented as mean ± standard error. Differences were considered to be statistically significant at p-value < 0.05.

RESULTS

Heterozygous mice demonstrate collagen glomerulopathy

Picrosirius Red (PSR)-stained sections of COL1A2 deficient, heterozygous, and wildtype mice were examined by light microscopy for the purpose of lesion scoring.

Heterozygous mice were found to have collagen deposition in their glomeruli characteristic of the type I collagen glomerulopathy identified in the COL1A2 deficient mice. However, lesions found in the heterozygous animals were found to be less severe than those visualized in COL1A2 deficient mice. Figure II-1 demonstrates the presence of the type I collagen glomerulopathy in heterozygous mice as well as the difference in lesion severity seen between COL1A2 deficient and heterozygous age-matched animals.

Glomerulopathy Initiation

To determine whether initiation of glomerular collagen deposition occurred pre- or postnatally, we examined lesion scores of kidney sections from mice at various ages (1-day, 1-week, 2-week, and 1-month of age). Figure II-2 highlights the postnatal initiation of collagen deposition within renal glomeruli. Glomeruli examined at 1-day of age lack collagen deposition across all genotypes. However, at 1-week of age, deposition had initiated in COL1A2 deficient and heterozygous mice as demonstrated by the positive PSR staining and the development of mild to moderate glomerular lesions. At 1-month of age, COL1A2 deficient mice exhibit lesions that range from mild to severe, whereas heterozygous mice have mild to moderate lesions. Further, in younger COL1A2 deficient mice, affected glomeruli were near the cortico-medullary junction where initiation of glomerular function occurs. In contrast, in older mice, lesions were also found in glomeruli of the outer cortex. This lesion development coincides with glomerular maturation which progresses from the cortico-medullary region outward

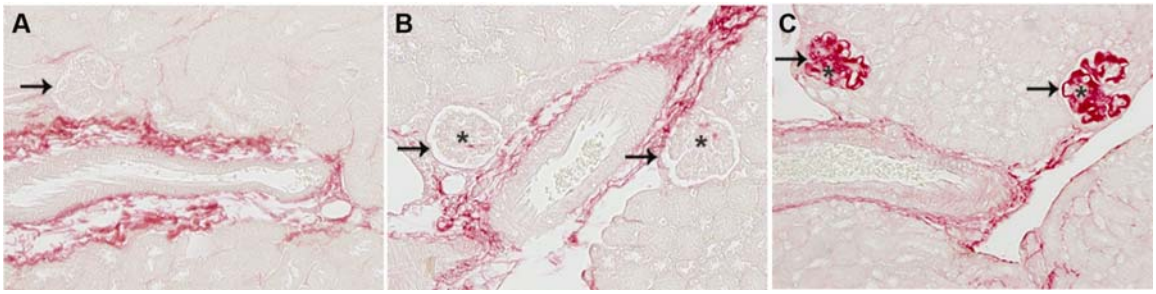


Figure II-1. Deposition of type I collagen in heterozygous and COL1A2 deficient glomeruli. PSR stained sections of A) wildtype (lesion score 0), B) heterozygous (lesion score 1), and C) COL1A2 deficient (lesion score 4) kidneys from 1-month old mice. Arrows indicate glomeruli. Asterisks denote affected (PSR+) glomeruli.

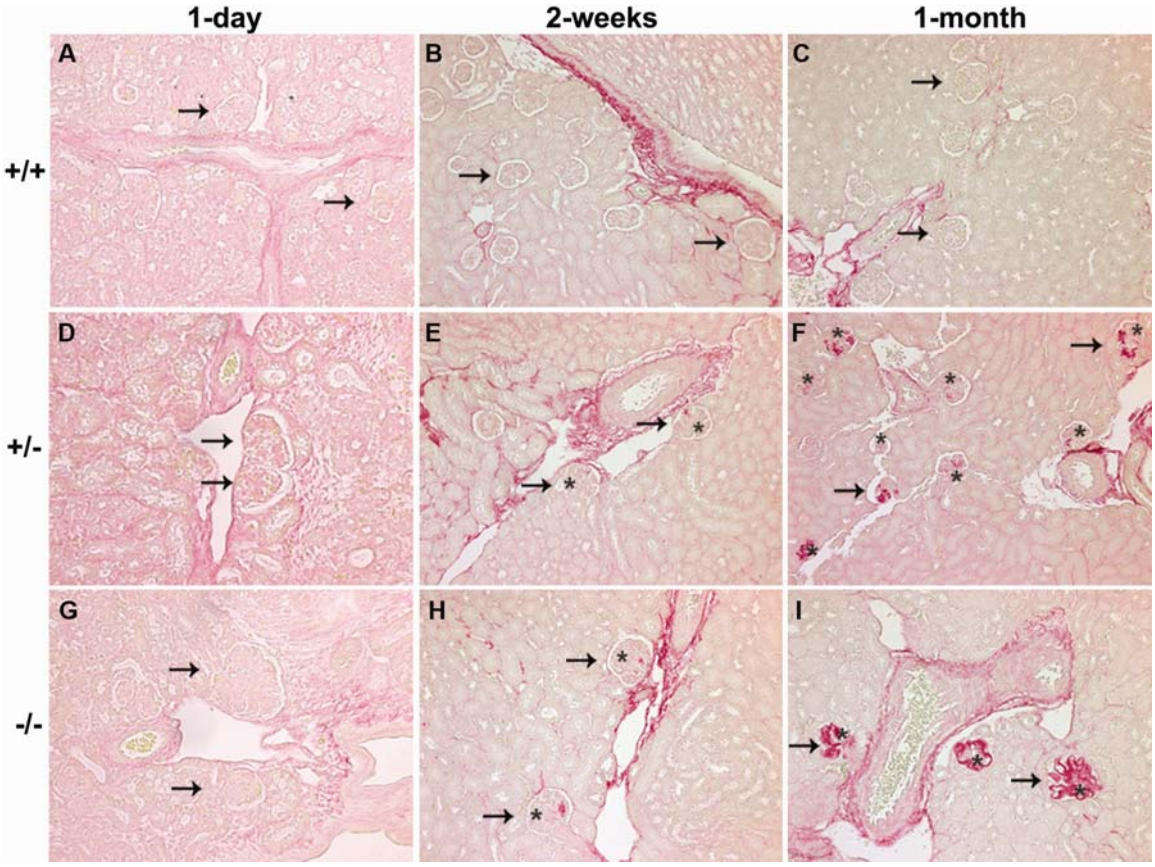


Figure II-2. Initiation of type I collagen deposition in glomeruli occurs postnatally. PSR stain of 1-day old (A, D, G), 2-week old (B, E, H), and 1-month old (C, F, I) mice. +/+ (wildtype, A-C) mice do not demonstrate collagen deposition and have lesion scores of 0. +/- (heterozygous, D-F) mice show evidence of disease at 2 weeks, demonstrating lesion scores of 0 at 1-day, 1 at 2-weeks, and 1 at 1-month. -/- (COL1A2 deficient, G-I) mice also show evidence of deposition at 2-weeks with a lesion score of 0 at 1-day, 1 at 2-weeks, and 4 at 1-month. Arrows indicate glomeruli. Asterisks denote affected (PSR+) glomeruli.

toward the cortex (Kleinman and Reuter 1973; Spitzer and Brandis 1974; Andrews, Betsuyaku et al. 2000). These data confirm that initiation of the type I collagen glomerulopathy occurs in the postnatal period at some point prior to 1-week of age and suggests that initiation of disease coincides with the maturation of renal glomeruli into functional filtration units (Bard 1994).

Type I Collagen Glomerulopathy is Progressive

To determine whether the type I collagen glomerulopathy progresses with age, we examined PSR-stained sections to determine lesion scores of COL1A2 deficient kidneys from mice 1-day, 1-week, 2-week and 1-month of age (timepoints were within 48 hours of specification). Figure II-3 demonstrates progression from 1-day to 1-month of age in COL1A2 deficient mice. 1-day old mice had a lesion score of 0 and the majority of mice 1-week old demonstrated a lesion score of 1. Once mice reached 2-weeks of age, they exhibited a range of lesions scores, 1-3, with a lesion score of 2 occurring most commonly. By 1-month of age, COL1A2 deficient mice demonstrated lesion scores from 2-4, and a majority of the mice demonstrated the most severe lesion scores of 3 and 4. Therefore, increased accumulation of collagen as monitored by lesion scoring, was associated with increasing age in COL1A2 deficient mice. This indicates that there is a pattern of disease progression in the type I collagen glomerulopathy.

Morphometry

In order to refine our previously described lesion scoring system and to further investigate lesion distribution and progression, we developed a morphometry mapping

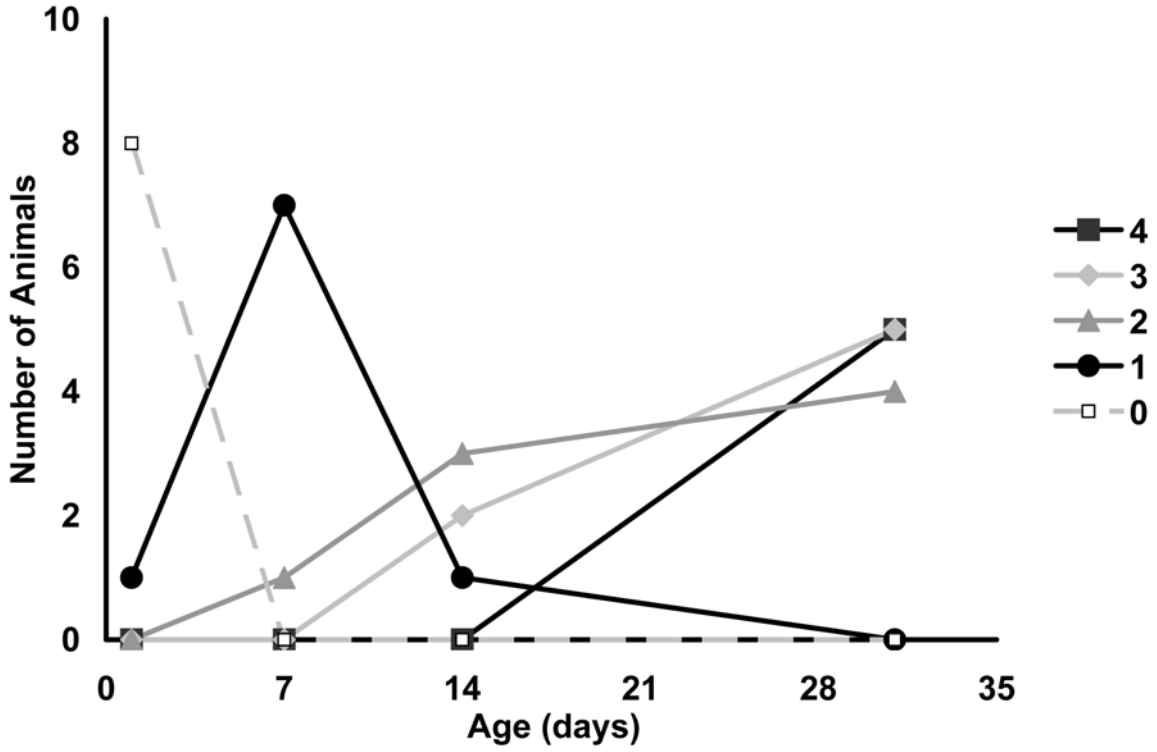


Figure II-3. Increased accumulation of type I collagen, monitored by lesion scoring, with increasing age in COL1A2 deficient mice. Mice 7-days and younger demonstrate lesion scores from 0-2. Mice at approximately 14 days of age demonstrate lesion scores from 1-3, and mice older than 14-days have lesion scores greater than 2.

system, as depicted in Figure II-4, which allowed us to examine the severity and localization of individual lesions. As seen in Figure II-5, the morphometric analysis confirmed our previous histologic findings that the type I collagen glomerulopathy is progressive and exhibits a gene dose effect. At 2-weeks of age, 55% of COL1A2 deficient glomeruli were affected, this increased to 95% being affected at 1-month of age. Based on these data and those shown in Figures 2 and 3, it was concluded that this type I collagen glomerulopathy is progressive and that by 1-month of age a majority of COL1A2 deficient glomeruli may be severely affected. In comparison, 8% of heterozygous glomeruli were affected at 2-weeks of age compared to 55% of age-matched COL1A2 deficient mice. Further, at 1-month of age 53% of heterozygous glomeruli were affected, which is increased from 8% at the 2-week time point; however, when compared to 1-month COL1A2 deficient mice in which 95% of glomeruli are affected an approximate 2-fold difference is seen, providing further support for the presence of the gene dose effect. Polynomial orthogonal contrast analysis of the morphometric data confirmed that the linear gene dose effect exists.

Further analysis using the morphometric scoring procedure demonstrated a difference in lesion severity and percent of glomeruli affected between the cortical and corticomedullary regions of individual kidney sections. As seen in Figure II-6A, the morphometry score in the corticomedullary region was greater and significantly different from the cortical region in COL1A2 deficient mice at 2-weeks and 1-month of age. The mean corticomedullary morphometry score in 2-week COL1A2 deficient animals was 0.94, whereas the 1-month corticomedullary morphometry score was 2.1, again demonstrating disease progression. Similar findings were true of percent glomeruli

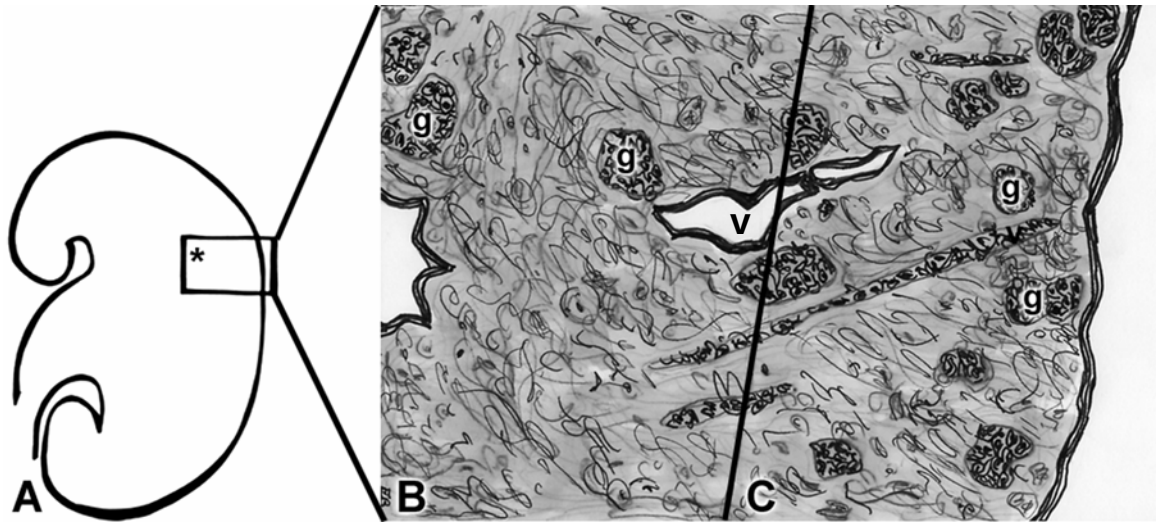


Figure II-4. Morphometry mapping diagram. To determine the morphometry score and glomerular number for a given longitudinal section, outlined in A, individual glomeruli (g) and the percent collagen deposition within each glomeruli within the section were recorded. Data was recorded and analyzed using four microscopic sections (*1 of 4) at 200 magnification and by dividing the renal cortex into corticomedullary (B) and cortical regions (C). The cortical and corticomedullary regions were differentiated using the midcortical interlobular vessels (v) as visual landmarks.

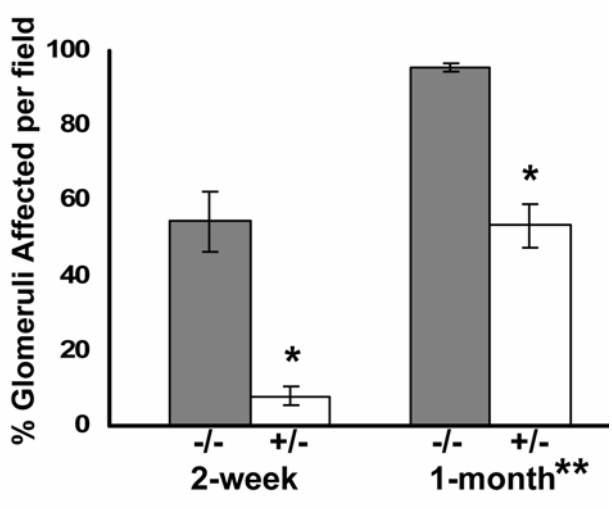


Figure II-5. The type I collagen glomerulopathy in heterozygous mice demonstrates a gene dose effect (*$p<0.0001$) that is progressive with age (**$p<0.0001$). The percent of affected glomeruli per field is significantly greater in COL1A2 deficient (-/-) than heterozygous (+/-) kidneys at both 2-weeks and 1-month of age. Significant differences in the percent of glomeruli affected between kidneys examined at 2-weeks and 1-month of age is also shown irregardless of genotype.

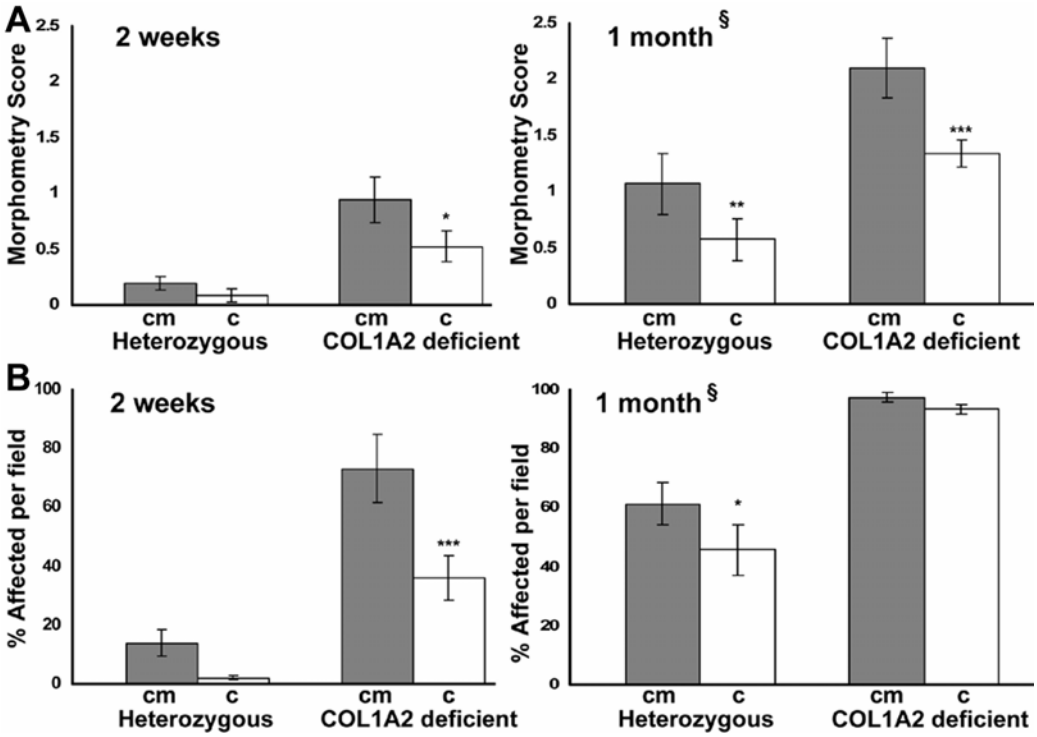


Figure II-6. The type I collagen glomerulopathy initiates postnatally and glomeruli sequentially become affected in a centrifugal pattern from the corticomedullary (cm) to the cortical (c) region in a distribution consistent with glomerular maturation and initiation of function. A) demonstrates that there is a significant difference between the morphometry score in cm and c glomeruli at 2-weeks and 1-month of age in COL1A2 deficient mice with more severely affected glomeruli located in the cm region where glomerular function initiates. B) demonstrates a significant difference between percent of glomeruli affected per field between cm and c glomeruli in COL1A2 deficient mice at 2-weeks of age while greater than 90% of cm and c glomeruli per field are affected by 1-month of age. Conversely, the heterozygous animals lack a significant difference at the earlier time point but demonstrate a significant difference in both lesion severity and percent glomeruli affected per field between cm and c glomeruli at 1-month of age. Further the lesion severity and percent glomeruli affected is significantly different between the 2-week and 1-month ages (§), confirming the progressive nature of the glomerulopathy. (*$p<0.05$, **$p<0.005$, ***$p<0.0001$).

affected as seen in Figure II-6B. At 2-weeks 73% of corticomedullary glomeruli were affected in COL1A2 deficient mice, while 36% of cortical glomeruli were affected. In contrast, at 1-month of age 97% of corticomedullary and 93% of cortical glomeruli were affected. A similar pattern was seen in the heterozygous mice, though the shift in affected glomeruli from predominantly corticomedullary to both corticomedullary and cortical was not as dramatic. These data suggest a centrifugal pattern of glomerular collagen deposition as the animal ages, with disease initiation occurring primarily in the corticomedullary region and progressing to involve both the corticomedullary and cortical regions of the kidney.

Subendothelial Type I Collagen Accumulation

In an effort to further characterize glomerular collagen deposition ultrastructurally, we examined tissue sections from wildtype and severely affected COL1A2 deficient (lesion score 4) mice using electron microscopy. Figure II-7 illustrates that the type I collagen was extracellular, accumulating in the mesangial matrix, as well as between the fenestrated endothelial cells and the glomerular basement membrane within the glomeruli. In addition, there was podocyte foot process effacement in areas demonstrating severe type I collagen deposition along with marked separation of endothelial cells and basement membrane. However, in all cases, the glomerular basement membrane was intact and no lesions were seen.

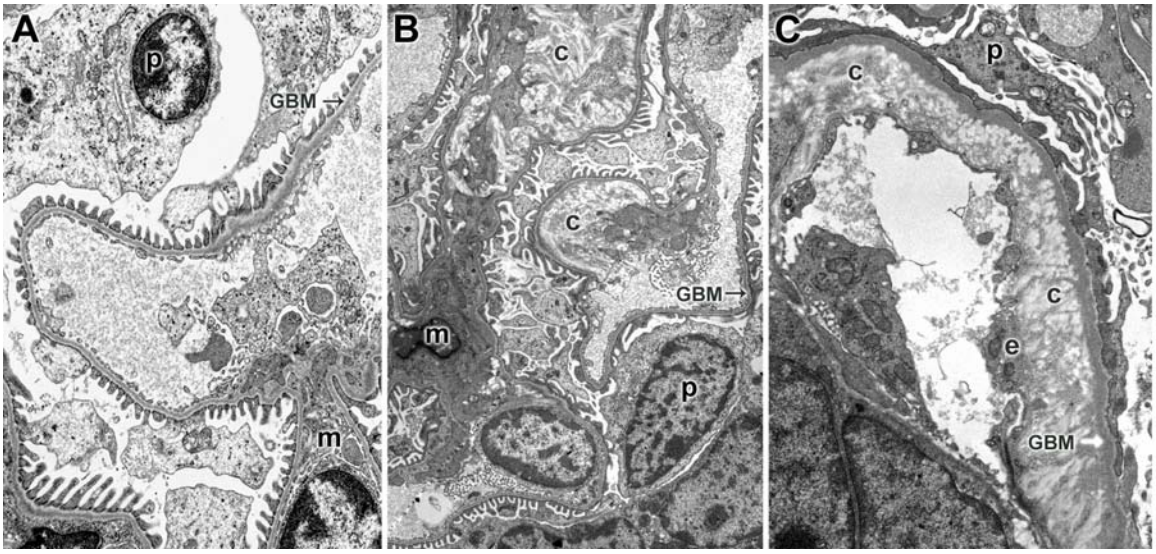


Figure II-7. Electron microscopy of glomeruli from wildtype (A) and COL1A2 deficient mice (lesion score 4; B,C). The COL1A2 deficient sections demonstrate deposition of extracellular fibrillar type I collagen (c) into the subendothelial space displacing the fenestrated endothelium (e) from the underlying basement membrane (B). In areas of severe collagen deposition and expansion of the subendothelial space, podocyte (p) foot process effacement is seen (C). It is hypothesized that the type I collagen is being produced by the mesangial (m) cell followed by deposition in the subendothelial space. In all mice examined, the glomerular basement membrane was intact. Wildtype provided for comparison (A). Magnification 4000X (A&B) and 8000X (C).

COL1A2 Deficient Mice and Albuminuria

To better characterize the COL1A2 deficient mouse as a model of glomerular injury, we examined the level of albumin excreted in the urine of wildtype and COL1A2 deficient mice as compared to the amount of creatinine excreted by each mouse. A significant increase in albumin excretion is evident in COL1A2 deficient mice when compared to the wildtype animals as presented in Table II-2. The COL1A2 deficient mice demonstrated a mean albumin to creatinine ratio of 38.2 μg albumin/mg creatinine, while the wildtype demonstrate a mean ratio of 0.5 μg albumin/mg creatinine. It was also determined that mice with higher lesion scores had a higher degree of albuminuria than those with less severe lesion scores. These data suggest that increasing deposition of homotrimeric collagen into the renal glomeruli results in a decrease in renal function resulting in increased albumin excreted in the urine.

Steady-state Proα1(I) and Proα2(I) Collagen mRNA Levels

To determine whether increasing type I collagen deposition in the glomerulus of COL1A2 deficient mice is the result of altered mRNA expression, steady-state levels of proα1(I) and proα2(I) collagen mRNA transcripts were evaluated using *in situ* hybridization and quantitative RT-PCR. *In situ* hybridization studies were performed on kidneys from 1-day, 1-week, 2-week, and 1-month old mice from all three genotypes, with varying degrees of collagen deposition in COL1A2 deficient and heterozygous animals as determined by lesion score. Figure II-8 demonstrates a similar pattern of mRNA steady-state expression of proα1(I) and proα2(I) collagen in both the wildtype and COL1A2 deficient animals with mild and severe lesions. Proα1(I) and proα2(I)

Genotype	N	µg Albumin/mg Creatinine (range)
Wildtype	12	**0.5 ± 0.1** (0.1-1.4)
COL1A2 deficient	14	**38.2 ± 12.3 *** (0.4-128.6)
Lesion Score		
0	12	0.5 ± 0.1
1	1	0.4
2	4	8.9 ± 6.5 **
3	6	42.3 ± 20.6 *
4	3	81.7 ± 25.2 *

Table II-2: Comparison of Urinary Albumin/Creatinine Ratios in Wildtype and COL1A2 Deficient Animals.

Urinary albumin/creatinine ratios (mean ± SE) from mice with varying lesion scores demonstrate a significant difference in albumin excretion in the urine. COL1A2 deficient mice demonstrate albuminuria as compared to wildtype animals. COL1A2 deficient mice with lesion scores 2, 3 and 4 had significantly greater albuminuria than wildtype animals. * indicates $p<0.0001$. ** indicates $p<.005$

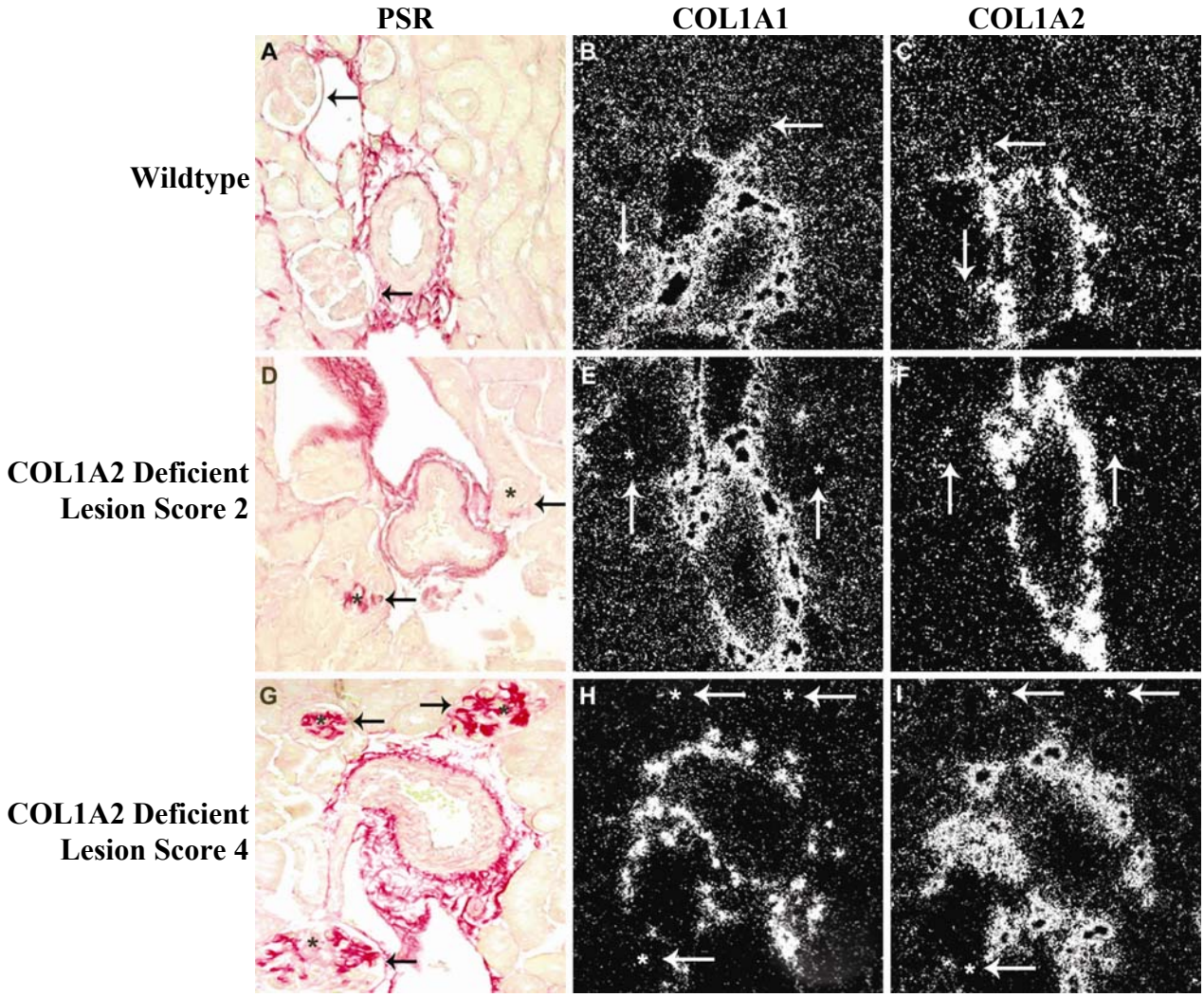


Figure II-8. *In situ* hybridization demonstrates similar temporal proα1(I) and proα2(I) collagen mRNA expression in COL1A2 deficient, heterozygous, and wildtype mice ages 1-week to 1-month. Wildtype (+/+) mouse kidney at 2-weeks of age with a lesion score of 0 (A-C) demonstrates proα1(I) (B) and proα2(I) (C) collagen mRNA expression predominantly associated with the vasculature. COL1A2 deficient (-/-) mouse kidney at 2 weeks of age lesion score 2 (D-F) and 1 month of age lesion score 4 (G-I) demonstrate similar proα1(I) (E &H) and proα2(I) (F&I) collagen mRNA expression associated with the vasculature and demonstrating no difference in signal intensity within affected glomeruli as wildtype. Arrows indicate glomeruli. Asterisks denote affected (PSR+) glomeruli.

collagen mRNA expression is seen in the wall of the arteries, as expected. To further assess collagen gene expression, quantitative RT-PCR analyses were performed to quantify steady-state proα1(I) and proα2(I) collagen mRNA levels in COL1A2 deficient, heterozygous, and wildtype mice at 1- week, 2-weeks, and 1-month of age. However, as seen in Figure II-9, there is a significant increase in proα1(I) collagen mRNA in 1-month COL1A2 deficient animals, as compared to age-matched heterozygous and wildtype animals. In contrast, a significant difference in proα2(I) collagen mRNA is not seen. Figure 9 also demonstrates a significant decrease in proα1(I) collagen mRNA with increasing age across all three genotypes. Examination of proα2(I) collagen mRNA levels with age, shows that there is a similar difference between animals 1- and 2-weeks of age when compared with 1-month animals. This suggests that the glomerular type I collagen deposition can be accounted for in part by an alteration in pretranslational regulation specifically an increase in proα1(I) collagen mRNA synthesis or stability.

DISCUSSION

In the original characterization of the type I collagen glomerulopathy in COL1A2 deficient mice, it was thought that heterozygous mice did not demonstrate glomerular collagen deposition (Phillips, Pfeiffer et al. 2002). However, revision of the lesion scoring system to include the examination of kidney sections stained with picrosirius red staining in addition to hematoxylin and eosin stained sections revealed that heterozygous mice do indeed demonstrate glomerular deposition of collagen. Through this method of histological examination it became evident that the type I collagen glomerulopathy is present to a lesser degree in the heterozygous mice. Heterozygous mice demonstrate a

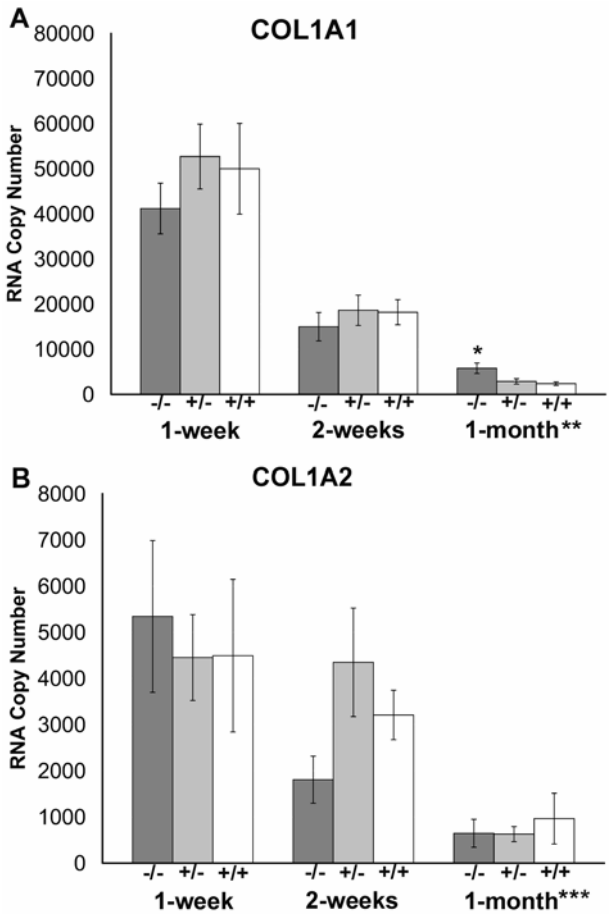


Figure II-9. Quantitative RT-PCR demonstrates a significant increase in proα1(I) collagen mRNA expression in 1-month COL1A2 deficient (-/-) animals when compared with age-matched heterozygous (+/-) and wildtype (+/+) animals (*p <0.05). It also demonstrates a decrease in proα1(I) collagen mRNA expression with age independent of genotype (**p <0.001) and a significant difference in proα2(I) collagen mRNA expression when comparing 1-month and 1-week animals for all three genotypes (*** p<0.01).

range of lesion scores at various ages, as do COL1A2 deficient mice; however the age dependent severity is less in the heterozygous animals, suggesting that the loss of proα2(I) collagen or the presence of homotrimeric type I collagen has a gene dose effect. It is not an uncommon phenomenon for heterozygous animals to be less severely affected than their homozygous counterparts as demonstrated in several disorders including achondroplasia, Waardenburg syndrome, Machado-Joseph disease, and others (Rousseau, Bonaventure et al. 1994; St George-Hyslop, Rogaeva et al. 1994; Zlotogora, Lerer et al. 1995; Zlotogora 1997).

Through these studies, we have also determined that the deposition of homotrimeric type I collagen initiates postnatally, prior to 1-week of age. Homotrimeric type I collagen is normally seen embryonically (Jimenez, Bashey et al. 1977; Rupard, Dimari et al. 1988); however, its postnatal production in the kidneys of wildtype animals is present only in wound healing or disease states (Haralson, Jacobson et al. 1987; Rupard, Dimari et al. 1988).

We have also shown that disease initiation and progression follow a pattern similar to that of glomerular maturation. Initially, within 1- week of life, affected glomeruli were confined to the corticomedullary region, where glomerular function initiates prenatally. However, by 1-month of age, affected glomeruli were evident throughout the corticomedullary and cortical regions in COL1A2 deficient mice, thus following the pattern for functional maturation of glomeruli, which is complete by 21 days of age (Andrews, Betsuyaku et al. 2000). From these findings, we conclude that the glomerulopathy progresses in a centrifugal pattern, coinciding with initiation of glomerular function or maturation (Kleinman and Reuter 1973; Spitzer and Brandis

1974). We postulate that deposition of type I collagen in COL1A2 deficient mice may be in response to increased glomerular filtration pressures and/or initial exposure to circulating solutes that occurs with glomerular maturation (functional activation).

Further, we have shown that as COL1A2 deficient mice age, increasing amounts of homotrimeric collagen may be deposited resulting in glomerular injury. This deposition has clinical significance, in that COL1A2 deficient mice exhibit microalbuminuria when compared to wildtype mice, and those mice with increasing lesion scores demonstrate a higher degree of albuminuria than those with less severe collagen deposition. Therefore, with age, the mice demonstrate increasing severity in glomerular lesions and functional decline of the affected kidneys, consistent with glomerular injury.

Through further investigation, it was determined that fibrillar collagen was being deposited into the subendothelial space between the fenestrated endothelium and the basement membrane. In areas of severe deposition, this results in podocyte foot process effacement. Podocytes have been proposed to be the cell type involved in ultrafiltration and establishment of the glomerular barrier to protein (Pavenstadt, Kriz et al. 2003). Further, foot process effacement and overall decrease in podocyte number has been shown to result in increased proteinuria, a hallmark of glomerular injury (Laurens, Battaglia et al. 1995; Pavenstadt, Kriz et al. 2003). Electron microscopic examination demonstrated that the basement membrane was normal in appearance, without deposition of pathologic material. It can be hypothesized that the deposition of the type I collagen into the mesangial matrix and in the subendothelial space results in impaired podocyte/endothelial cell interactions, podocyte foot process effacement, increasing

proteinuria, and is responsible for the progressive glomerular functional decline (Remuzzi and Bertani 1998). Similar findings have been demonstrated in two strains of a mouse model of glomerulosclerosis described by Sanderson et. al, in which a TGF-β1 transgene under the control of murine albumin promoter and its enhancers expressed high plasma TGF-β1 levels (Sanderson, Factor et al. 1995). Further examination revealed that they demonstrated subendothelial extracellular matrix accumulation in the glomeruli of these mice, including deposition of fibrillar collagen in addition to proteinuria in the severely affected animals (Kopp, Factor et al. 1996).

In situ hybridization (ISH) analyses of COL1A2 deficient, heterozygous, and wildtype kidneys demonstrated no gross differences in proα1(I) and proα2(I) collagen steady-state mRNA localization and levels between genotypes. Subsequently, quantitative RT-PCR was performed due to inherent insensitivities of ISH to subtle changes in steady-state mRNA. Quantitative RT-PCR demonstrated a significant increase in proα1(I) collagen mRNA in 1-month COL1A2 deficient animals, as compared to age-matched heterozygous and wildtype animals. Analysis of the RT-PCR data, suggests that proα1(I) collagen mRNA expression is not coordinately regulated with proα2(I) collagen mRNA in 1-month old COL1A2 deficient kidneys. This would be consistent with the concept that the glomerular collagen deposition seen in the COL1A2 deficient mice may be an overcompensation of the glomerular wound healing response in glomerular diseases and activation of mesangial cell collagen production. Haralson et. al, demonstrated that cultured rat mesangial cells produced type I collagen, greater than 50% of which was homotrimer, in response to culture, this along with the data presented here indicates that homotrimeric type I collagen deposition may play a

role in glomerulosclerosis common to several renal diseases (Haralson, Jacobson et al. 1987).

Evaluation of the RT-PCR data also revealed that there was a significant decrease in proα1(I) collagen and proα2(I) collagen mRNA in animals 1-month of age when compared to animals less than 1-month, suggesting that there is a high degree of synthesis of type I collagen molecules at early ages, at which time the glomeruli are undergoing development (Kleinman and Reuter 1973; Spitzer and Brandis 1974). The presence of an increased quantity of proα1(I) and proα2(I) collagen mRNA at early ages may be due to developmental changes and thus mask any differences that might be occurring due to initiation of the glomerulopathy. Further, the quantitative RT-PCR analysis was performed on whole kidney sections, and to clarify whether proα1(I) and proα2(I) collagen steady-state mRNA increases occur with initiation of disease, quantitative RT-PCR analyses needs to be performed on glomerular isolates.

Under normal physiologic conditions, a balance exists between the synthesis and degradation of type I collagen, and more broadly, in extracellular matrix components. In order to maintain homeostasis, synthesis and degradation must remain balanced. However, in disease states, if the balance is disrupted resulting in increased synthesis and/or decreased degradation, accumulation can result (Lenz, Elliot et al. 2000). Our quantitative RT-PCR findings demonstrated that steady-state levels of proα1(I) collagen mRNA were increased by 2-fold in 1-month in COL1A2 deficient animals when compared to age-matched heterozygous and wildtype animals, suggesting that the aberrant accumulation is due, at least in part, to a pretranslational increase in type I collagen gene expression. However, we cannot rule out that in addition to an increase in

proα1(I) collagen expression, a decrease in degradative mechanisms may also be involved similar to mechanisms seen in other glomerulosclerotic or disease models. Particularly since 1-month heterozygous animals also demonstrate glomerular deposition in a gene dosage dependent manner, yet there is no significant increase in collagen gene expression in heterozygous animals as measured by quantitative RT-PCR.

Type I collagen is degradatively regulated by matrix metalloproteinases (MMPs) and their associated tissue inhibitor of metalloproteinases (TIMPs) as are other extracellular matrix components. Tomita et al., describe a model of proliferative glomerulonephritis in rats as a result of injections of anti-Thy-1.1 monoclonal antibody 1-22-3 in which glomerular type I and IV collagen deposition occurs but is not associated with increased steady-state type I or type IV collagen mRNA levels. However, they did observe a decrease in MMP-9 steady-state mRNA expression with no differences in expression of TIMP-1, MMP-2, MMP-13 and MT1-MMP steady-state mRNA (Tomita, Koike et al. 2004). This contrasts what was seen by Uchio et al. in the ICGN (Institute of Cancer Research (ICR)-derived glomerulonephritis) mice, a model of idiopathic nephritic syndrome, in which the mice demonstrate proteinuria, hypoproteinemia, and hyperlipidemia, as well as type I collagen deposition in their glomeruli (Uchio, Manabe et al. 2000; Uchio, Manabe et al. 2004; Uchio-Yamada, Manabe et al. 2005). Uchio et al. demonstrated a decrease in MMP-1, 2, and 9 activity in the ICGN mice (Uchio, Manabe et al. 2000). In subsequent studies, these authors, using Western blot analysis, showed a decrease in MMP-2, MMP-9, MT1-MMP, TIMP-1, and TIMP-2 protein in ICGN mice as well (Uchio-Yamada, Manabe et al. 2005). In their later work, they demonstrate, by *in situ* hybridization, increased steady-state proα1(I) collagen and proα1(III) collagen

mRNA levels as compared to control animals (Uchio, Manabe et al. 2004). Therefore, deposition of extracellular matrix components in the ICGN mouse are the result of increased pretranslational synthesis in addition to a decrease in the degradative capacity of renal MMPs through decreased activity and expression. This is similar to what was described by Mozes et. al in the TGF-β transgenic mouse discussed above. The mice were found to have glomerular type I collagen deposition associated with an increase in steady-state α1(I) mRNA levels in young mice. In addition, they found increased activity of MMP-2 and increased expression of TIMP-1 in severely affected animals indicating that in the presence of elevated TGF-β glomerular collagen deposition is the result of an imbalance in pretranslational synthesis and degradative capacities mediated by MMP/TIMP interactions(Mozes, Bottinger et al. 1999).

Thus, in addition to an increase in proα1(I) collagen mRNA, the mechanism responsible for type I collagen deposition may involve decreased MMP enzyme expression and/or activity associated with glomerular matrix accumulation or related to the specific cleavage properties of certain MMPs when presented with homotrimeric versus heterotrimeric type I collagen as a substrate.

In summary, it is evident that type I collagen deposition and regulation of glomerular extracellular matrix components are controlled by a delicate balance between increased pretranslational synthesis and matrix metalloproteinase/tissue inhibitor of metalloproteinase proteolytic degradation. In this study, we demonstrate that the type I collagen glomerulopathy affects heterozygous animals demonstrating a dose response effect, initiates postnatally following a pattern of glomerular maturation, and is a progressive disease resulting in functional renal decline. We also suggest that the

glomerulopathy is, in part, due mechanistically to pretranslational upregulation of proα1(I) collagen mRNA expression, but is most likely due to a combined mechanism that includes failure of regulatory degradative enzymes, matrix metalloproteinases, in removal of homotrimeric type I collagen. Further investigation must be done to provide insight into the cleavage properties of MMPs in the presence of homotrimeric and heterotrimeric type I collagen, as well as investigate *in vivo* expression and activity of MMPs in COL1A2 deficient mice. As seen in our model and those described here, it is evident that glomerular extracellular matrix deposition may be the result of a number of mechanisms and many stages of control from transcription to enzymatic degradation. It is important to understand the mechanisms of deposition, the various levels of regulation, and their respective implication to disease in order to devise effective therapy and clinical approaches to glomerular injury common to so many renal diseases.

CHAPTER III

DIFFERENTIAL CLEAVAGE OF HETEROTRIMERIC AND HOMOTRIMERIC TYPE I COLLAGEN BY MATRIX METALLOPROTEINASES

In Preparation

ABSTRACT

Type I collagen exists, most commonly, as a heterotrimeric triple helical molecule [$\alpha1(I)_2\alpha2(I)$] and less commonly, as a homotrimeric triple helix [$\alpha1(I)_3$]. COL1A2 deficient (*oim*) mice, lack functional proα2(I) collagen chains and exclusively synthesize homotrimeric type I collagen. The mice have also been found to exhibit a novel type I collagen glomerulopathy, or renal injury due to glomerular type I collagen deposition. Previous studies indicated that the glomerular deposition was, at least in part, due to an increase in synthesis of the homotrimer. Evidence from previous studies and presented here indicates that the mechanism responsible for glomerular collagen deposition may be two-fold involving both synthesis of glomerular collagen and aberrant degradation by matrix metalloproteinases (MMPs). Degradation of type I collagen within the glomerulus is primarily regulated by MMPs. Previous studies have identified the presence of MMPs 1, 2, 3, 9, 13, and 14 in the kidney, and MMPs 1, 2, 3, 8, 9, and 13 are known to cleave type I collagen. *In vitro* proteolytic cleavage analysis demonstrates differential cleavage of heterotrimeric and homotrimeric type I collagen by MMPs 1, 2, 9, and 13. Further, this work presents optimized type I collagen isolation parameters for assessment of recombinant MMP proteolytic activity, and potential methods to further characterize the differential proteolytic cleavage of heterotrimeric and homotrimeric type I collagen.

INTRODUCTION

A novel type I collagen glomerulopathy was identified in COL1A2 deficient mice (Phillips, Pfeiffer et al. 2002). COL1A2 deficient mice exclusively synthesize homotrimeric [$\alpha1(I)_3$] type I collagen (Chipman, Sweet et al. 1993), a form of collagen that normally comprises <0.5% of the type I collagen found in the body (Uitto 1979). Further, it has been shown that cultured mesangial cells produce homotrimeric type I collagen, suggesting that homotrimer production may be related to injury and/or wound healing (Haralson, Jacobson et al. 1987). The glomerulopathy seen in the COL1A2 deficient mice is characterized by the deposition of glomerular collagen in the renal mesangium and subendothelial space of their glomeruli (Phillips, Pfeiffer et al. 2002). Under normal physiologic conditions, there is very little type I collagen in the glomerulus and the primary event leading to the development of the novel glomerulopathy in the COL1A2 deficient mice appears to be the deposition of homotrimeric type I collagen into the glomerular mesangial matrix (Mene, Simonson et al. 1989; Yoshioka, Tohda et al. 1990; Brodeur submitted 3/10/06) The mechanism responsible for glomerular collagen deposition is of interest due to the histologic presence of type I collagen and other matrix proteins in the glomeruli of other mouse models as well as humans with chronic renal disease. Studies of mouse models that have glomerular collagen deposition demonstrate that the mechanisms of deposition can be one of increased matrix synthesis, aberrant proteolytic degradation, or a combination of the two (Sanderson, Factor et al. 1995; Chatziantoniou, Boffa et al. 1998; Uchio, Manabe et al. 2000; Tomita, Koike et al. 2004; Uchio, Manabe et al. 2004; Uchio-Yamada, Manabe et al. 2005).

Previous studies by this lab demonstrate that the glomerular collagen deposition is, at least in part, due to pretranslational upregulation and demonstrate a two-fold increase in steady-state proα1(I) collagen mRNA in COL1A2 deficient mice in comparison to wildtype and heterozygous age-matched animals. However, studies also demonstrate the occurrence of the glomerulopathy in heterozygous animals at 1-month of age without a corresponding increase in collagen gene expression. Thus, the mechanism responsible for the type I collagen glomerulopathy was postulated to be one of increased synthesis combined with aberrant proteolytic degradation of the glomerular type I collagen.

Proteolytic pathways responsible for degradation of type I collagen and other matrix proteins produced in the glomeruli are composed primarily of matrix metalloproteinases, or MMPs. MMPs are the primary regulators of glomerular matrix composition (Lenz, Elliot et al. 2000). The glomerular accumulation of homotrimeric type I collagen could be due to the absence of a specific MMP, a decrease in MMP activity, or an altered interaction between MMPs and their regulators, tissue inhibitors of metalloproteinases (TIMPs) unique to COL1A2 deficient mice. Or, the glomerular accumulation of homotrimer could result from the inability of an MMP or class of MMPs to cleave the homotrimeric substrate.

To explore the latter possibility, *in vitro* proteolytic assays were developed to examine cleavage properties of MMPs known to cleave the heterotrimeric [$\alpha1(I)_2\alpha2(I)$] type I collagen, MMPs 1, 2, 3, 8, 9, and 13. Currently, the function of the homotrimeric [$\alpha1(I)_3$] isotype is under investigation, and the ability of MMPs to cleave the homotrimeric substrate is unknown. However, structural studies comparing

heterotrimeric and homotrimeric type I collagen have been conducted. Homotrimeric type I collagen has been shown by electron microscopy and meridional x-ray diffraction patterns to be similar to heterotrimeric type I collagen. However, there is growing evidence that the homotrimer may be structurally different from the heterotrimer based on comparative water binding studies, a differential micro-unfolding pattern, decreased triple helix integrity, and differential kinetics of thermal denaturation (McBride, Choe et al. 1997; Kuznetsova, McBride et al. 2001; Miles, Sims et al. 2002; Kuznetsova, McBride et al. 2003). The results of *in vitro* proteolytic analysis demonstrate differential cleavage of heterotrimeric and homotrimeric type I collagen by MMPs 1, 2, 9, and 13 in support of the hypothesis that structural differences may exist between heterotrimeric and homotrimeric type I collagen. Techniques for collagen isolation, MMP activation, and various *in vitro* proteolytic analyses are also presented.

METHODS

Animals

Homozygous B6C3Fe a/a-Col1a2$^{oim/J}$ (COL1A2 deficient, -/-); heterozygous (+/-); and wildtype (+/+) mice were purchased from the Jackson Laboratory (Bar Harbor, ME, USA). Animals were housed in an AAALAC accredited animal facility, provided with water and food (Purina 5008 Formulab Diet; Purina Mills Inc., Richmond, IN, USA) ad libitum, and cared for in accordance with an approved University of Missouri Animal Care and Use protocol. +/+, +/-, and -/- genotypes were determined by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) analysis (Phillips, Bradley et al. 2000).

Collagen Isolation

Mouse tail tendons and whole tails and were harvested from wildtype and COL1A2 deficient animals. Tail tendons were isolated and agitated in 0.5N acetic acid overnight at 4ºC. Samples were then separated by centrifugation for 45 min at 4500 rpm at 4ºC. The supernatants were isolated and the collagen precipitated in a 30% NaCl/0.5N acetic acid salt solution. The NaCl was added incrementally over 60 min and then agitated at 4ºC for 48 hr. The collagen was pelleted by centrifugation at 4500 rpm for 20 min at 4ºC and reconstituted in 0.5N acetic acid.

Whole tails were skinned and homogenized in 3mL of 0.1M Na_2HPO_4/20% EDTA buffer with a mortar and pestle. For decalcification, 2mL of 0.1M Na_2HPO_4/20% EDTA buffer were then added to the homogenates and the homogenates were incubated for a minimum of 45 min at 4ºC. The sample was separated by centrifugation at 4500 rpm for 20 min at 4ºC, the supernatant was decanted off, and the collagen-containing pellet was collected. The pellet was resuspended in 5mL 0.1M Na_2HPO_4 buffer and recentrifuged at 4500 rpm for 20 min at 4ºC, the supernatant was discarded. The collagen-containing pellet was resuspended in 0.5N acetic acid and agitated at 4ºC for 24 hr and followed by centrifugation at 4500 rpm for 20 min at 4ºC. The supernatants were isolated, subjected to salt precipitation in a 30% NaCl/0.5N acetic acid salt solution, centrifuged, and reconstituted as described above. Collagen concentrations were determined using the Sircol Collagen Assay (Biocolor, Westbury, NY), a colorimetric-based assay. Concentration and purification quality were confirmed using 8% acrylamide/2M urea SDS-PAGE.

Fluorogenic Assay

In order to evaluate activation and the enzyme activity of the individual MMPs, a fluorogenic assay was used. Recombinant MMPs 1, 2, 8, 9, and 13 (R&D Systems, Minneapolis, MN) were incubated in the presence of TCNB (50mM Tris, 5mM $CaCl_2$, 200mM NaCl, 0.02% Brij-35, pH 7.5; BioRad, Hercules, CA) and 1mM 4-aminophenylmercuric acetate (APMA, Sigma, St. Louis, MO) for 2 hours at 37ºC to allow for complete enzymatic activation. The activated reaction was then added to a fluorogenic peptide substrate, 2mM ES001 (R&D Systems, Minneapolis, MN) that contains a fluorescent 7-methoxycoumarin that is quenched by resonance energy transfer to 2,4-dinitrophenyl following cleavage of amide bonds, and allowed to incubate for 1 hour. Excitation and emission were read at 320 and 405nm respectively on a Cary Eclipse spectrophotometer (Varian, Palo Alto, CA). The spectrophotometer was blanked with a substrate, TCNB, and APMA solution (no enzyme). Enzymatic reactions in the absence of APMA were also set up in order to examine the activity of the pro-MMP form. An increase in fluorescence above that generated by the pro-form was considered sufficient activation. Fluorescence was also examined over a 1-hour time course and increased with increasing time of substrate and enzyme incubation. MMP-3 does not cleave fluorogenic substrate ES001, therefore the same assay was performed using a fluorogenic peptide substrate with a different peptide sequence, ES003 (R&D Systems, Minneapolis, MN) to verify enzymatic activation.

Proteolysis

Individual recombinant MMP 1, 2, 3, 8, 9, and 13 were activated in the suggested concentrations of TCNB and 1mM APMA for 2 hours. Following activation, the enzymatic reaction was added to 7µg of the desired substrate (homotrimer or heterotrimer murine type I collagen) and incubated for 24 hr at 37ºC. Heterotrimeric and homotrimeric type I collagen was purified as described above and lyophilized and reconstituted in TCNB to bring the substrate to a more physiologic pH. Reactions were terminated by addition of denaturing buffer (20% Glycerol, 0.1M Tris pH 6.8, 2% SDS, 1M Urea, and Bromophenol Blue) and boiled for 3 min. Proteolytic cleavage was evaluated by 8% acrylamide/2M urea SDS-PAGE followed by staining with GelCode Blue (Pierce, Rockford, IL).

Ninhydrin

In order to quantitate proteolytic cleavage of heterotrimeric and homotrimeric type I collagen substrates, an assay was devised using the colorimetric change exhibited by reaction of ninhydrin with free α-amino acids when heated. The assay was based on the principle that the absorbance which occurs as a result of the ninhydrin color change would increase with the generation of free α-amino acids following proteolytic cleavage of heterotrimeric and homotrimeric type I collagen by individual MMPs. To quantitate the increase in absorbance, four general reactions were prepared: (1) 20 and 30µg of purified and lyophilized heterotrimeric type I collagen isolated as described above with 24µg of MMP-2 (R&D Systems, Minneapolis, MN) following 2hr activation in 1mM APMA and TCNB and its control, (2) 24µg of MMP-2 following activation in 1 mM

APMA and TCNB in the absence of substrate (3) 20 and 30µg of purified and lyophilized heterotrimeric type I collagen hydrated in TCNB and its control, (4) TCNB alone. In addition, a standard curve was generated using increasing concentrations of heterotrimeric type I collagen. The above samples were incubated at 37ºC for 3 hours, mixed with 500µL freshly prepared ninhydrin reagent (100mg ninhydrin (Sigma, St. Louis, MO), 10mg Tn(II)Cl (Sigma, St. Louis, MO) in 5mL ethylene glycol mixed with 5mL Buffer B (0.5M acetic acid and 0.1M citric acid, pH 5.0), and boiled for 20 min. The solutions were immediately cooled in an ice water bath for 4 min and absorbance was measured at 570nm using a Genesys 5 spectrophotometer (Spectronic Instruments, Rochester, NY). The change in absorbance was calculated using the following equation ((reaction 1 - control 2) - (reaction 3 – control 4)) and compared to the standard curve to generate the concentration of amino-termini generated by proteolytic cleavage (Doi, Shibata et al. 1981; Yin, Tomycz et al. 2002).

Statistics

All statistical analyses are one-way analyses of variance. All results are presented as mean ± standard deviation. Differences were considered to be statistically significant at p-value < 0.05.

RESULTS

Collagen Isolation Yield

Heterotrimeric and homotrimeric type I collagen were isolated from harvested mouse tails using the tail tendon isolation procedure. The age of the mouse at sacrifice was found to have an impact on the milligrams of acid-extractable collagen isolated from the tail tendons. Figure III-1A demonstrates that collagen extraction from wildtype mouse tail tendon is inversely proportional to age and is significantly different in animals 1-month of age compared to those 18-months of age. As the mice age, the amount of collagen that was isolated from the tendons decreased. At 1-month of age, a mean of 5.8mg of collagen was isolated, while only 1.2mg was isolated from 18-month animals. In addition, the amount of collagen isolated decreased inversely proportional to the amount of tail tendon tissue harvested as seen in wildtype animals in Figure III-1B. It was also determined that collagen isolation yields were consistently lower for isolation of homotrimeric type I collagen from COL1A2 deficient mice at all ages (data not shown).

MMP Activation

Recombinant MMPs are provided in their pro-form and require activation (Lenz, Elliot et al. 2000). In order to confirm that equivalent activation is occurring with each lot of enzyme a fluorogenic assay was developed. Reactions were run in duplicate, one containing the activating compound 4-aminophenylmercuric acetate, or APMA, and one without an activating compound, or a proform. Figure III-2 is a graphical representation of the activation of MMPs 1, 2, 3, 8, 9, and 13 at 10, 20, 30, and 60 minutes as compared

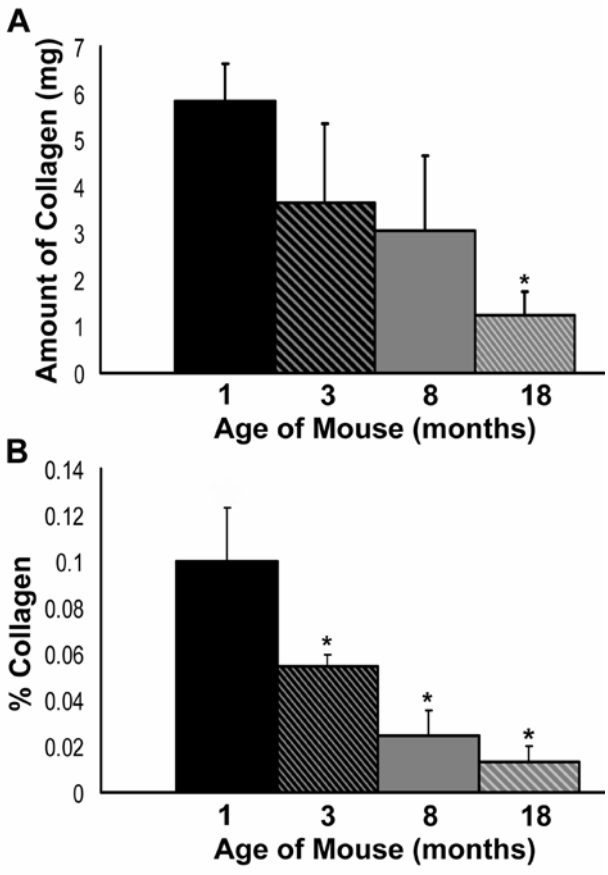


Figure III-1. Type I collagen isolation is dependent upon age of the animal. A) demonstrates a decrease in mg of collagen isolated with age in wildtype animals (+/+), demonstrating a significant difference between 1- and 18-month animals ($p<0.05$). B) demonstrates decreasing collagen yields despite increasing tail tendon weight with age in wildtype animals (+/+). The percent collagen yield is greatest in 1-month animals and is significantly different from the yield seen in all other age groups ($p<0.05$). % collagen is mg of collagen isolated per mg of tail tendon harvested.

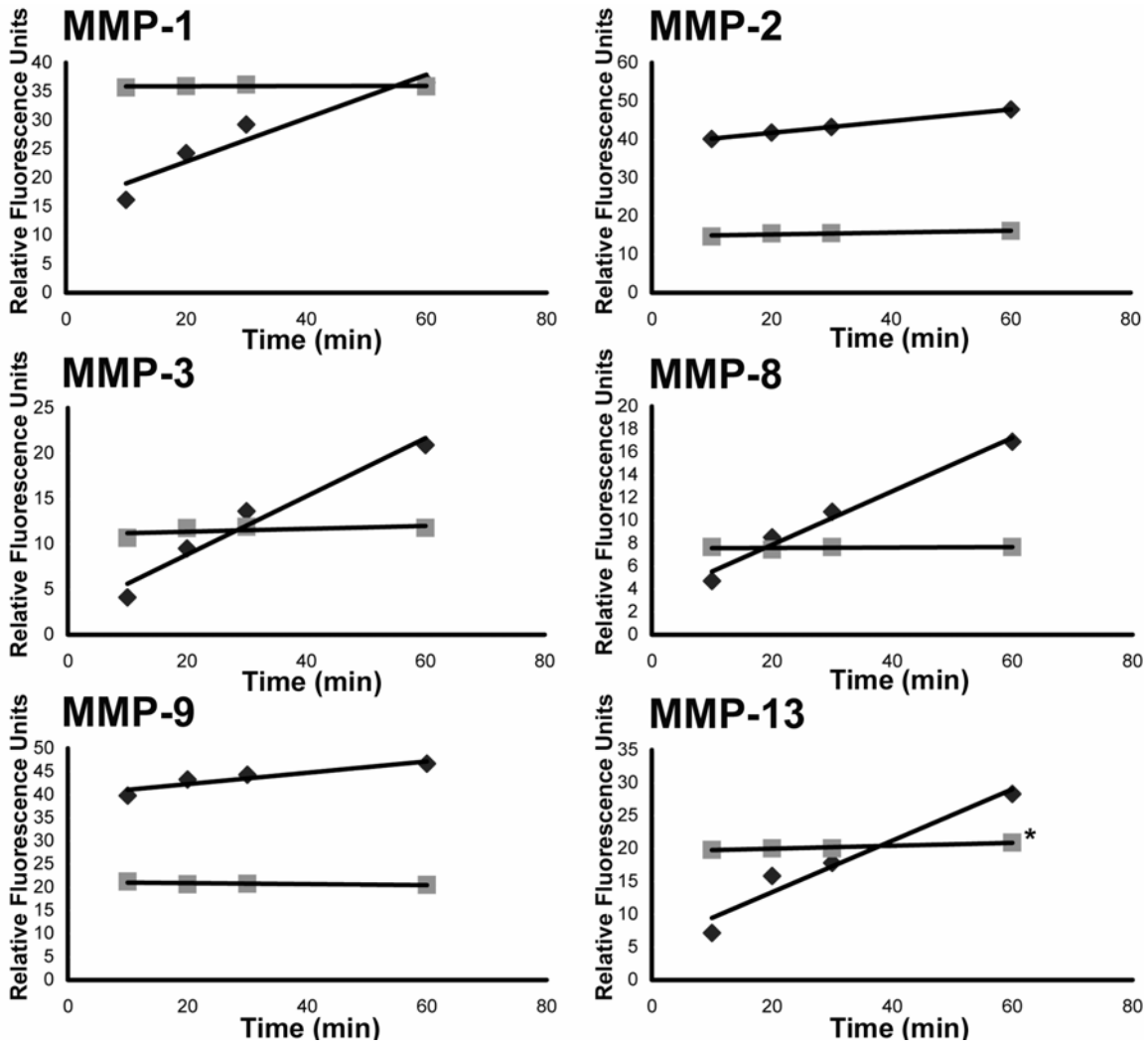


Figure III-2. Fluorogenic substrate cleavage demonstrates activation of individual MMPs. As seen in this representative assay, activation (♦) of recombinant MMPs 1, 2, 3, 8, 9, and 13 results in increasing cleavage, or increasing slope, of their respective fluorogenic substrates above the activity of the proenzyme (■) for each individual MMP. Proenzyme (■) reactions, lacking APMA, show no change in cleavage of the fluorogenic substrate as expected. It is unclear why the proform of MMPs 1, 3, 8, and 13 demonstrate baseline cleavage above that of the activated reaction. (*) indicates a discrepancy in control and activated enzyme incubation. The control for MMP-13 was incubated at 37ºC for 1hr rather than 2hr as in all other experiments.

to the activity of the respective proenzyme. The activated state of the respective MMPs is demonstrated by an increase in proteolytic cleavage of the fluorogenic substrate, as indicated by an increasing slope. The activation reactions for each MMP also demonstrate continuous cleavage of the fluorogenic substrate with time as compared to the proform of the enzyme, which is linear, or unchanging for each respective proform. It is unclear why the proform of MMPs 1, 3, 8, and 13 demonstrate baseline cleavage above that of portions of the activated reaction. Therefore, each MMP reaches its activated state within 1 hour and repeat experiments can be compared to determine the degree of activation achieved by individual lots of enzyme.

Differential Cleavage of Heterotrimeric and Homotrimeric Type I Collagen

Preliminary *in vitro* proteolytic cleavage analyses of heterotrimeric [$\alpha1(I)_2\alpha2(I)$] and homotrimeric [$\alpha1(I)_3$] type I collagen by collagenases MMP-1, MMP-8, and MMP-13 (Figure III-3), stromelysin MMP-3 (Figure III-4), and gelatinases MMP-2 and MMP-9 (Figure III-4) suggest that all of these MMPs are capable of cleaving both substrates to some degree. Further as seen in Figure III-3, MMPs 1, 2, 9, and 13 differentially cleave heterotrimeric and homotrimeric type I collagen. MMP-1 and MMP-13 demonstrate more efficient cleavage of the homotrimeric molecule, while MMP-2 and MMP-9 appear to preferentially cleave the heterotrimeric molecule. MMP-3 and MMP-8 appear to demonstrate similar cleavage of both molecules. Based on this and other preliminary data, it appears that MMPs do, in fact, cleave heterotrimeric and homotrimeric type I collagen differentially.

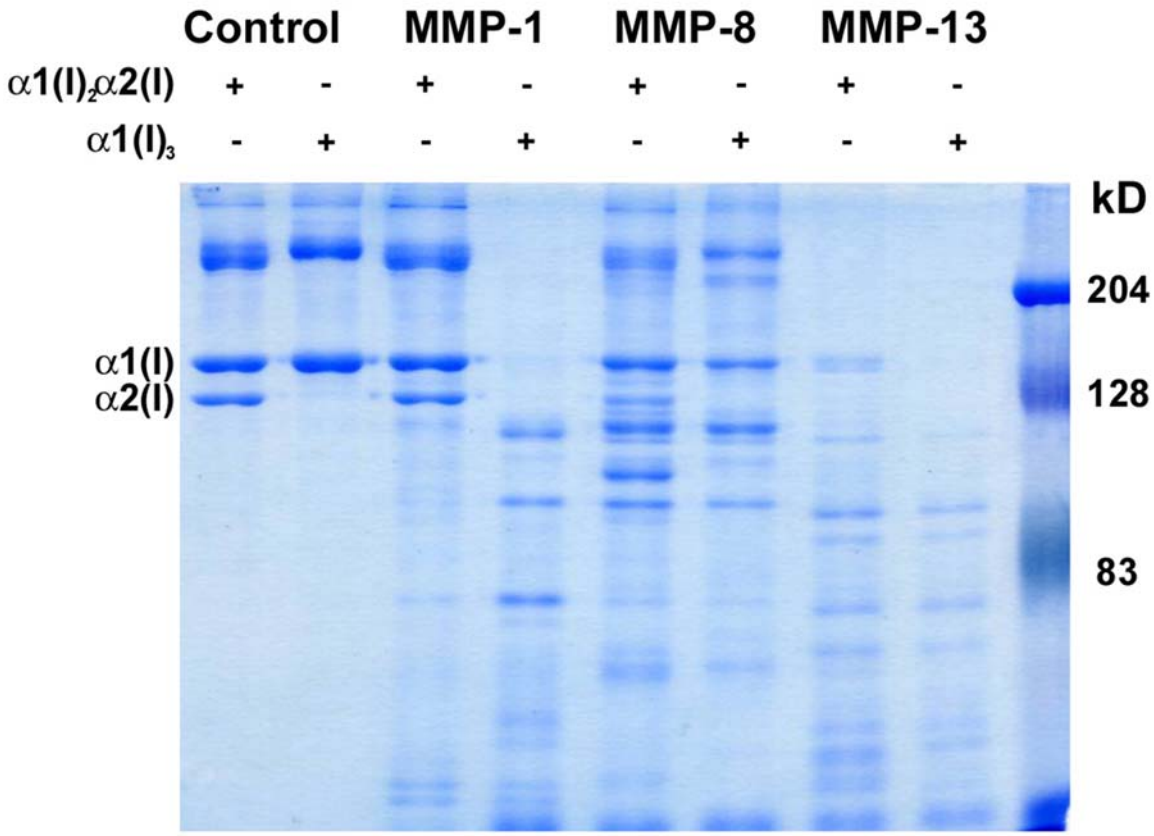


Figure III-3. *In vitro* proteolytic cleavage of heterotrimeric and homotrimeric type I collagen by MMP-1, MMP-8, and MMP-13. Proteolysis experiments suggest that MMP-1 and MMP-13 differentially cleave heterotrimeric [$\alpha1(I)_2\alpha2(I)$] and homotrimeric [$\alpha1(I)_3$] type I collagen. MMP-1 and MMP-13 appear to more efficiently cleave homotrimeric type I collagen. Uncleaved α1(I) and α2(I) collagen chains and molecular weight markers are indicated.

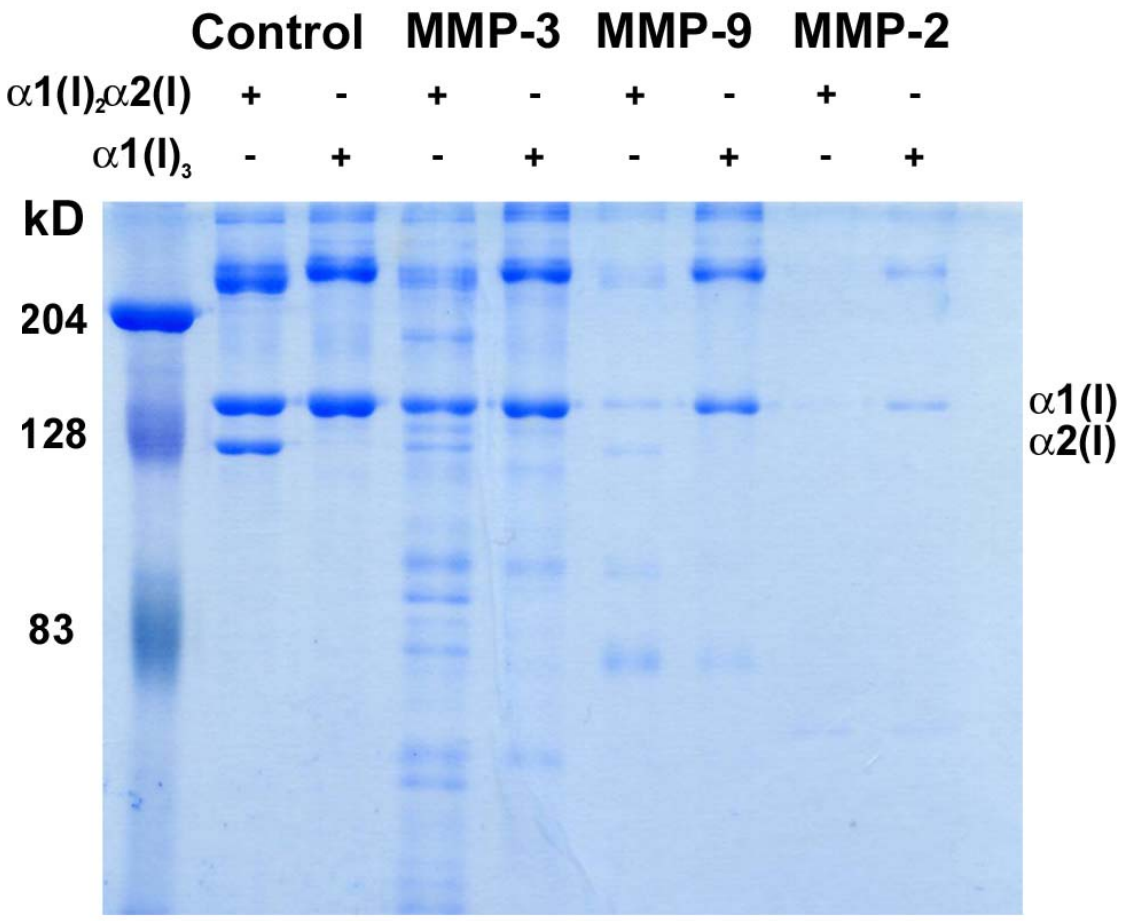


Figure III-4. *In vitro* proteolytic cleavage of heterotrimeric and homotrimeric type I collagen by MMP-3, MMP-9, and MMP-2. Proteolysis experiments suggest that MMP-9 and MMP-2 differentially cleave heterotrimeric [$\alpha1(I)_2\alpha2(I)$] and homotrimeric [$\alpha1(I)_3$] type I collagen. MMP-9 and MMP-2 demonstrate more efficient cleavage of heterotrimeric type I collagen. Uncleaved α1(I) and α2(I) collagen chains and molecular weight markers are indicated.

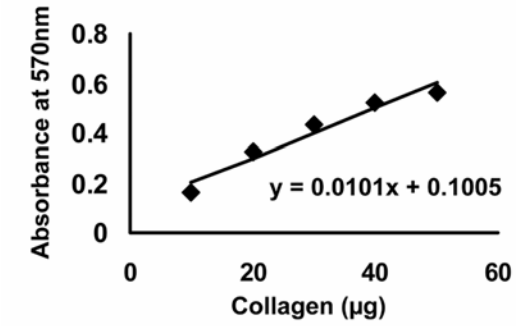
Alternative Method of Demonstrating Proteolytic Cleavage

As an alternative method of assessing proteolytic degradation of type I collagen, ninhydrin was used to detect the creation of free α-amino acids following MMP proteolytic cleavage of heterotrimeric and homotrimeric type I collagen. Figure III-5A shows the generation of a standard curve and equation relating absorbance to the amount of protein, using wildtype (+/+) or heterotrimeric type I collagen. Figure III-5B demonstrates that ninhydrin is capable of detecting proteolytic cleavage of heterotrimeric type I collagen by MMP-2. Activation of MMP-2 and incubation with 20 and 30 μg of heterotrimeric type I collagen resulted in 31 and 48μg of calculated protein, an approximately 60% increase in binding of ninhydrin as detected by color change and absorbance at 570nm. It has also been demonstrated that the ninhydrin assay can be used to generate a similar standard curve for both the homotrimeric and heterotrimeric type I collagen substrates (data not shown). These data suggest that the above-described ninhydrin assay is a sufficient alternative in determining and/or confirming differential cleavage of heterotrimeric and homotrimeric type I collagen by MMPs.

DISCUSSION

The combination of homotrimeric type I collagen deposition in the glomeruli of COL1A2 deficient mice and the structural differences seen in heterotrimeric and homotrimeric type I collagen led us to examine the potential roles of matrix metalloproteinases (MMPs) in development of the type I collagen glomerulopathy.

A



B

Initial Substrate (µg)	Product Absorbance (nm)	Product (µg)	Percent Increase
20	0.422	31.8	59%
30	0.587	48.2	61%

Figure III-5. Ninhydrin is capable of detecting an increase in free amino-termini generated by proteolytic cleavage of heterotrimeric type I collagen by MMP-2. A) shows generation of a standard curve using heterotrimeric type I collagen isolated from wildtype animals (+/+). B) demonstrates increased absorbance following MMP-2 proteolytic cleavage of 20 and 30 µg of heterotrimeric type I collagen.

Tail tendon isolation of heterotrimeric type I collagen demonstrates greater collagen yield from tails harvested from younger animals relative to older animals. It can be postulated that the decrease in yield with age may be due to differences in intermolecular crosslinking within the tissue as the animal ages. It has been shown that there is an increase in overall crosslinking with age along with a conversion to a greater number of mature, or nonreducible, crosslinks with age, either of which could lead to decreased collagen yield (Reiser, Hennessy et al. 1987; Reiser 1994; Bailey, Paul et al. 1998). In order to examine MMP cleavage of heterotrimeric and homotrimeric type I collagen *in vitro*, purified substrates were required. Previously in our laboratory, collagen was isolated from mouse tail tendon, but the need for greater yields spurred development of the whole tail method also described in the Methods section. Development of the whole tail extraction method allowed purification of sufficient amounts of heterotrimeric and homotrimeric type I collagen for the proposed *in vitro* studies.

In vitro activation of recombinant MMPs was crucial to the examination of heterotrimeric and homotrimeric type I collagen as independent substrates of MMPs 1, 2, 3, 8, 9, and 13. Through the use of the fluorogenic assay it was determined that 4-aminophenylmercuric acetate, or APMA, was sufficient for *in vitro* activation and allowed for comparison of enzyme activity between enzyme lots purchased from R&D Systems (Minneapolis, MN). APMA is a thiol-modifying agent that serves to convert pro-MMPs into active enzymes and differs from the activation of MMPs *in vivo*. *In vivo*, MMPs are partially activated by cleavage of a fragment of the propeptide sequence termed the bait region. This is followed by intermolecular processing which results in

cleavage of the remainder of the propeptide and full activation of the enzyme. Chemical activation with compounds such as APMA is initiated by modification of a specific sulfhydryl group which promotes intramolecular cleavage of a segment of the propeptide resulting in partial activation. Full enzymatic activation is then achieved following removal of the remaining propeptide by intermolecular processing (Visse and Nagase 2003).

With sufficient substrate and activation conditions, we proceeded to examine MMP cleavage of heterotrimeric and homotrimeric type I collagen. Preliminary *in vitro* proteolytic analyses demonstrate differential cleavage of the two substrates. Based on SDS-PAGE visualization, MMP-1 and MMP-13 demonstrate more efficient cleavage of the homotrimeric collagen. However, MMP-2 and MMP- 9 preferentially cleave the heterotrimeric substrate. It can be postulated that these differences may be due to the structural differences in the heterotrimeric and homotrimeric type I collagen substrates.

The synthesis of homotrimeric type I collagen in COL1A2 deficient mice has been accepted for sometime; however, the functional significance of the homotrimeric isotype and the α2(I) chain remains unknown. Examination of the α chains independently reveals that they are nearly homologous, yet the α2(I) chain has been shown to be more hydrophobic and have decreased imino acid content when compared with the α1(I) chain. It has been postulated that the increased concentration of hyprophobic residues found in the α2(I) chain may play a role in the formation of associations between the α chains, the triple helix, and other noncollagenous proteins. Further, a forty-fold increase in critical concentration for fibril formation has been

demonstrated in the homotrimer, which suggests that the α2(I) chain assists in the self-assembly process (McBride, Choe et al. 1997).

Studies of the triple helix have further highlighted differences in heterotrimeric and homotrimeric type I collagen molecules. Electron microscopy and x-ray diffraction patterns demonstrate that homotrimeric type I collagen is similar in structure to heterotrimeric type I collagen, in that both molecules demonstrate diffraction patterns consistent with a meridional repeat of approximately 67nm. However, x-ray diffraction studies show that the absence of the α2(I) chain results in a decreased order of axial packing and a loss of lateral packing in the homotrimeric molecule when compared to heterotrimeric type I collagen, and the homotrimer demonstrated an overall decrease in meridional order (McBride, Choe et al. 1997). It has also been suggested that structural differences exist between homotrimer and heterotrimer based on studies that show the presence of a differential microunfolding pattern, decreased rate of homotrimer assembly, decreased triple helix integrity in the homotrimer, and differential kinetics of thermal denaturation between the two molecules (McBride, Choe et al. 1997; Kuznetsova, McBride et al. 2001; Kuznetsova, McBride et al. 2003). For example, the homotrimeric and heterotrimeric isotypes of type I collagen share two microunfolding sites, or short regions in which the triple helix unwinds for a brief time. However, the heterotrimeric isotype has an additional microunfolding site approximately 150 residues from the N-terminus. Yet, the homotrimer microunfolding segments, regions in which the individual α-chain are exposed, are more thermally stable and resistant to further proteolytic cleavage than those in the heterotrimer. In addition, it has been shown that homotrimeric type I collagen denatures approximately 100 times more slowly than heterotrimer, which

is postulated to be of significance in tissue growth and wound healing (Kuznetsova, McBride et al. 2003).

MMP cleavage of triple helical type I collagen is dependent upon the primary and supersecondary structure of the collagen molecule. The region of the type I collagen molecule recognized by the active site of the enzyme, or the cleavage site, is described as hydrophobic with 24 residues, <10% of which are charged. It is flanked by two regions with specific amino acid compositions. The region preceding the cleavage site is known to have a relatively high percentage of imino acids, resulting in a tight triple helix. The region following the cleavage site is noted to have a relatively low percentage of imino acids, resulting in a looser helical structure, lending greater flexibility to the molecule. MMPs recognize a specific amino acid sequence, based on which interstitial collagen molecule is present, in combination with the pattern of supersecondary structure consisting of tight helical structure in front of the primary sequence and a loose helical structure following (Lauer-Fields, Juska et al. 2002).

Based on the structural differences described, it can be postulated that homotrimeric type I collagen may have inherent qualities that make proteolytic degradation by MMPs more difficult. Slower denaturation may prohibit cleavage in some way, and once unwound, as seen in microunfolding studies, the homotrimer is more thermally stable than the heterotrimeric isotype. Furthermore, decreased integrity of the triple helical structure of homotrimeric type I collagen may result in an altered supersecondary structure and thus, altered orientation upon attempted cleavage by specific MMPs.

These findings confirm the need to further characterize properties of MMP cleavage of heterotrimeric and homotrimeric type I collagen. Preliminary results presented indicate that the ninhydrin assay is capable of measuring quantitative differences in MMP cleavage of the two substrates. The ninhydrin assay, as compared to the *in vitro* proteolytic analysis, allows greater freedom in varying the substrate concentration and examining the time-course in a given reaction. This will allow for determination of the kinetics of individual cleavage reactions for both heteotrimeric and homotrimeric type I collagen.

Previous studies from this laboratory demonstrate that the glomerular collagen deposition is, at least in part, due to pretranslational upregulation and demonstrate a 2-fold increase in steady-state proα1(I) collagen mRNA in COL1A2 deficient mice compared to wildtype and heterozygous age-matched animals. However, studies also demonstrate the occurrence of the glomerulopathy in heterozygous animals at 1-month of age without a coordinate increase in collagen gene expression. Preliminary results presented here indicate that MMP-2 and MMP-9 demonstrate preferential cleavage of the heterotrimeric type I collagen molecule. Further, studies outlined previously demonstrate glomerular collagen deposition as a result of pretranslational upregulation and the current study suggests decreased proteolytic degradation may also play a role. Therefore, we postulate that the type I collagen glomerulopathy is due to a mechanism in which both pretranslational upregulation and aberrant MMP degradation play a role. Thus, further examination into the role of MMPs in the mechanism responsible for the type I collagen glomerulopathy in COL1A2 deficient and heterozygous animals is warranted. Such

examination should include quantitation and localization of the individual MMP proteins within the glomeruli.

CHAPTER IV

PERSPECTIVES AND FUTURE DIRECTIONS

The overall aims of the research presented in this dissertation were 1) to determine the natural progression of the type I collagen glomerulopathy and determine whether or not pathologic findings correlate with the progression of the glomerulopathy in COL1A2 deficient mice, 2) to determine whether the mechanism of glomerular type I collagen deposition in COL1A2 deficient mice is due to increased synthesis, aberrant matrix metalloproteinase degradation, or a combination of the two, and 3) to determine if MMPs cleave heterotrimeric and homotrimeric type I collagen similarly. Towards these goals, the results presented in this dissertation have established that 1) COL1A2 deficient and heterozygous mice both develop the type I collagen glomerulopathy, 2) the glomerulopathy begins postnatally in the first week of life, demonstrates a gene dose effect, progresses with age, and follows a pattern of initiation consistent with glomerular maturation, 3) COL1A2 deficient mice exhibit pathologic findings of microalbuminuria, which correlate with progression of the glomerulopathy, 4) the mechanism responsible for the type I collagen glomerulopathy is most likely an imbalance that exists between type I collagen synthesis and aberrant MMP degradation, and 5) MMPs differentially cleave heterotrimeric and homotrimeric type I collagen.

During the progression of any research project, the questions and answers generated often spark additional questions. In the following section, three objectives are outlined for future studies and include development of glomerular isolation techniques, determination of *in vitro* and *in vivo* MMP activity and its relationship to the type I

collagen glomerulopathy, and examination of mesangial proteins and factors involved in the development of glomerulosclerosis and chronic kidney disease.

Objective 1. To optimize glomerular isolation techniques in order to examine the mechanism of the type I collagen glomerulopathy.

Rationale. The studies presented in this dissertation including *in situ* hybridization and quantitative RT-PCR were carried out on whole kidney sections rather than mesangial cells or glomeruli alone, which comprise approximately 10% of kidney tissue. Therefore, the steady-state mRNA levels presented are those of the kidney section rather than glomeruli alone and include mRNA that was located in the tubules, interstitium, and blood vessels. In order to gain a greater understanding of the glomerulopathy and glomerular collagen deposition, it is imperative that mechanistic studies be carried out on glomerular isolates in addition to whole kidney sections.

Previous attempts to isolate glomeruli and mesangial cells by our lab have been largely unsuccessful. The use of cellular sieving techniques, described by Francki et. al, resulted in isolation of murine mesangial cells (Francki, Bradshaw et al. 1999). However, upon culturing these cells, cells from both wildtype and COL1A2 deficient mice secreted homotrimeric type I collagen, indicating that the technique had spurred a wound healing response in the cells and that *in vitro* mesangial cell cultures were not representative of the *in vivo* condition. In addition, laser capture microscopy was also attempted in an effort to isolate individual glomeruli from renal tissue. However, the hypercellularity of a single glomerulus prevented sufficient glomerular isolation.

Approach 1. To optimize glomerular isolation from whole kidney tissue using magnetic bead technology described by Takemoto et. al.

Anesthetized mice undergo trans-cardiac perfusion with the magnetic beads, or Dynabeads. The spherical Dynabeads are sized such that they selectively accumulate in the capillaries of the glomeruli. The kidneys are then removed and gently pressed through a 100 μm sieve twice. The homogenate is centrifuged and the pellet containing the Dynabead-laiden glomeruli are selectively captured using the magnetic particle concentrator. It is hoped that this technique will result in isolation of a sufficient amount of glomeruli at 97% purity, as documented, in order to further characterize the mechanism responsible for the type I collagen glomerulopathy (Takemoto, Asker et al. 2002).

Anticipated Result: Based on recent attempts at isolation in our lab, we do not anticipate difficutly in isolating individual glomeruli from COL1A2 deficient, heterozygous and wildtype animals. It is yet to be determined if isolation yield will be sufficient to carry out desired experiments, in which case it may be necessary to consolidate glomeruli. It is hoped that quantitative RT-PCR analysis of COL1A1, COL1A2, and potentially individual MMP genes will help further elucidate the mechanism of the type I collagen glomerulopathy.

Objective 2. To 1) complete *in vitro* analysis of the kinetics of heterotrimeric and homotrimeric type I collagen by MMPs 1, 2, 3, 8, 9, and 13; 2) examine *in vivo* activity of MMPs 1, 2, 3, 8, 9, and 13 in wildtype, heterozygous, and COL1A2 deficient animals;

and 3) determine steady-state mRNA levels of MMPs, TIMPs, and the α-chains of type I collagen mRNA in glomerular isolates if differences in protein activity exist.

Rationale. As demonstrated in Chapter II, increased type I collagen synthesis plays a role in the mechanism responsible for the type I collagen glomerulopathy. However, the glomerulopathy was also identified in heterozygous animals without a coordinate increase in steady-state collagen mRNA expression, also seen in Chapter II. Further, as presented in Chapter III, preliminary results indicate that MMPs 1, 2, 9, and 13 differentially cleave heterotrimeric and homotrimeric type I collagen. Based on these findings, it can be postulated that the mechanism responsible for the type I collagen glomerulopathy involves both increased matrix synthesis and aberrant degradation controlled by MMPs and their relationships with TIMPs. Therefore, further studies are indicated to examine protein activity, protein localization, and mRNA expression of type I collagen, MMPs, and TIMPs in an effort to fully characterize the mechanism responsible for glomerular collagen deposition.

Approach 1. The current work must be repeated and expanded upon to confirm the cleavage properties of MMPs 1, 2, 3, 8, 9, and 13 and further characterize the differential cleavage seen by MMPs 1, 2, 9 and 13 by *in vitro* proteolytic analysis as described in Chapter III.

In order to further characterize cleavage of heterotrimeric and homotrimeric type I collagen, we sought to examine the kinetics of cleavage of each substrate with individual recombinant MMPs. We are in the process of refining a method of examining the

kinetics of cleavage in which the two substrates are subject to *in vitro* proteolytic cleavage by individual MMPs over a series of time points and visualized using SDS-PAGE. From this, we hope to determine a relative rate of MMP cleavage for each substrate and enzyme. We intend to confirm the potential findings of the *in vitro* time-course studies and expand upon them using the ninhydrin assay described in Chapter III. Through the ninhydrin assay, by varying the substrate concentration, determination of Vmax and Km for each individual cleavage reaction (MMPs 1, 2, 3, 8, 9, and 13) using heterotrimer and then homotrimer as a substrate should be possible.

Approach 2. MMP extracts obtained from wildtype, heterozygous, and COL1A2 deficient kidneys will be run on both heterotrimeric and homotrimeric type I collagen zymogen gels to determine relative extract MMP activity and to identify the specific MMPs by size present in each genotype. *In situ* zymography may also be performed to localize and further characterize *in vivo* MMP activity. Finally, quantitation of the individual MMPs and confirmation of the identity of the specific MMPs within each genotype will be obtained using immunohistologic and/or Western blot analyses.

Approach 3**.** To determine steady-state mRNA levels of MMPs, TIMPs, and the α-chains of type I collagen within glomeruli, glomerular isolation must be optimized and isolates from wildtype, heterozygous, and COL1A2 deficient animals analyzed by quantitative RT-PCR, as described in Chapter II.

Anticipated Results: Based on preliminary results, differential cleavage of the homotrimeric and heterotrimeric substrate does, in fact, occur. We postulate that further studies using proteolytic cleavage and colorimetric based assays will demonstrate differential kinetics of cleavage. At this time, it is unclear whether the mechanism responsible for the glomerulopathy will also include differences in MMP activity and mRNA expression in COL1A2 deficient, heterozygous, or wildtype animals. However, there is the potential for the mechanism to be multifactorial incorporating an increase in collagen pretranslational regulation, differential kinetics of cleavage, and differential MMP activity and/or expression, as partially demonstrated here and seen in other mouse models.

Objective 3. To examine relationships between expression of proliferating cell nuclear antigen (PCNA), α-smooth muscle actin (α-sma), transforming growth factor-β (TGF-β) receptors, and Smad proteins in mesangial cells of wildtype, heterozygous, and COL1A2 deficient animals.

Rationale. As described in Chapter I, mesangial cells are a major component of the glomerulus and are affected by the type I collagen glomerulopathy. Based on electron microscopy studies, collagen deposition in mesangial cells is a consequence of the glomerulopathy and it can be hypothesized that mesangial cells may be secreting the collagen deposited within the glomeruli (Phillips, Pfeiffer et al. 2002). In addition, it has been shown that cultured mesangial cells mount a wound healing response and secrete homotrimeric type I collagen in mice and rats upon being cultured (Haralson, Jacobson et

al. 1987). There is growing evidence that mesangial cells respond to glomerular injury in other ways, as well. Mesangial hypercellularity is a characteristic of many chronic renal diseases. PCNA is a nuclear protein that is expressed in late G1 through the M phase of the cell cycle and is expressed during mesangial cell proliferation (Johnson, Floege et al. 1992). Mesangial cells have also been postulated to develop myofibroblast-like activity and function similar to smooth muscle cells in response to glomerular injury. Such myofibroblast-like activity includes synthesis of interstitial collagens, including type I collagen, and upregulation of α-sma. α-sma is a contractile protein normally expressed in smooth muscle cells but not in mesangial cells and its presence in mesangial cells indicates activation, or acquisition of myofibroblast-like properties. Mesangial production of interstitial collagens and development of myofibroblast-like activities are cellular events that lead to the histologic presentation of glomerulosclerosis and are hypothesized to play a major role in progression of glomerular injury to glomerulosclerosis in the absence of intervening factors that allow for restoration of normal glomerular architecture (Johnson, Floege et al. 1992). At the molecular level, there is growing evidence that TGF-β and Smad signaling are mediators of such processes. TGF-β has been found to be upregulated in numerous mouse and human models of glomerulosclerosis and chronic renal disease. It has also been shown that TGF-β can activate mesangial cells and signal the transition to a myofibroblast-like state as well as stimulate extracellular matrix production by activated cells *in vitro* (Liu 2006). Based on these relationships, it is apparent that a clear understanding of the proteins produced by mesangial cells in the COL1A2 deficient model will allow for a better

understanding of mechanistic and therapeutic relationships as well as further comparison with other disease models of glomerulosclerosis.

Approach 1. As an initial step, immunohistochemical techniques, previously described by Phillips et. al, will allow for examination of protein expression in mesangial cells of wildtype, heterozygous, and COL1A2 deficient animals (Phillips, Pfeiffer et al. 2002).

Anticipated Result: We postulate that immuhistochemical examination of glomeruli from COL1A2 deficient, wildtype, and heterozygous mice will demonstrate differences in PCNA, α-sma, TGF-β receptors, and Smad proteins based on previous accounts of such findings in human and mouse models.

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VITA

Amanda Christine Brodeur was born March 3, 1978 in Columbia, MO. She was the firstborn to Michael and Krista Cully of Springfield, MO. After attending Kickapoo High School in Springfield, MO, she chose to attend the University of Missouri-Columbia to study biochemistry. As a sophomore at the university, Amanda was granted admission to the University of Missouri-School of Medicine through the Conley Scholars program. In the same year, she also decided to pursue her interests in biomedical research and was truly blessed when she met Dr. Charlotte Phillips. It was through interaction with Dr. Phillips that Amanda decided to pursue a combined M.D. and Ph.D. in biochemistry. In the summer of 2000, Amanda made the decision to stay at the University of Missouri for her postgraduate training. It was in that same summer that she traveled to Europe and met her now husband, Christian Brodeur, also a graduate of the University of Missouri-Columbia. Amanda has decided to stay in Columbia following graduation to pursue an integrated research residency in Child Health. Amanda and Chris are currently awaiting the birth of their first child in April of 2006.