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Deficient degradation of homotrimeric type I collagen, $\alpha1(I)_3$ glomerulopathy in *oim* mice

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ABSTRACT

Col1a2-deficient (*oim*) mice synthesize homotrimeric type I collagen due to nonfunctional proα2(I) collagen chains. Our previous studies revealed a postnatal, progressive type I collagen glomerulopathy in this mouse model, but the mechanism of the sclerotic collagen accumulation within the renal mesangium remains unclear. The recent demonstration of the resistance of homotrimeric type I collagen to cleavage by matrix metalloproteinases (MMPs), led us to investigate the role of MMP-resistance in the glomerulosclerosis of *Col1a2*-deficient mice. We measured the pre- and post-translational expression of type I collagen and MMPs in glomeruli from heterozygous and homozygous animals. Both the heterotrimeric and homotrimeric isotypes of type I collagen were equally present in whole kidneys of heterozygous mice by immunohistochemistry and biochemical analysis, but the sclerotic glomerular collagen was at least 95–98% homotrimeric, suggesting homotrimeric type I collagen is the pathogenic isotype of type I collagen in glomerular disease. Although steady-state MMP and *Col1a1* mRNA levels increased with the disease progression, we found these changes to be a secondary response to the deficient clearance of MMP-resistant homotrimers. Increased renal MMP expression was not sufficient to prevent homotrimeric type I collagen accumulation.

1. Introduction

Type I collagen is predominantly found in tissues as a heterotrimeric isotype consisting of two α1(I) chains and one α2(I) chain, [$\alpha1(I)_2\alpha2(I)$]. However the homotrimers consisting of three α1(I) chains, [$\alpha1(I)_3$] [1–4] have been shown to be present during embryogenesis [5], in tumors [6–8], fibrotic tissues [9–12] and in stressed mesangial cells [13].

Haralson et al. demonstrated that cultured wildtype rat mesangial cells synthesized the homotrimeric type I collagen in what was postulated to be a wound response [13]. They hypothesized that the synthesis of the homotrimeric isotype may contribute to sclerotic accumulation of type I collagen within the renal mesangium. Historically, glomerulosclerosis has been attributed to an imbalance of collagen synthesis and degradation [14–17]. Mesangial cells are thought to be responsible for the excessive expression of collagen in the glomerular mesangium during disease progression [18–21]. Although the resulting matrix accumulation leads to glomerulosclerosis [22–25], the initiating mechanisms of the pathology and the specific role of homotrimeric type I collagen still remain unclear.

A clue to answering the latter question may be contained in the recent finding that type I collagen homotrimers are much more resistant to degradation by matrix metalloproteinases (MMPs) than the heterotrimers [8,26]. MMPs are known regulators of the extracellular matrix (ECM) and are of keen interest in glomerulosclerotic disease [27–30]. To maintain ECM homeostasis, human kidneys express collagenases (MMP-1, -13 and -14), gelatinases (MMP-2 and -9) and stromelysin-1 (MMP-3) [31]. MMP-2 has been most widely studied across species

Abbreviations: +/+, wildtype mouse; +/−, heterozygous *oim* mouse; −/−, homozygous *oim* mouse; BSA, bovine serum albumin; DAB, 3,3′ diaminobenzidine tetrahydrochloride; ECM, extracellular matrix; EMT, epithelial-to-mesenchymal-transition; ER, endoplasmic reticulum; HBSS, Hanks Balanced Salt Solution; HPRT, hypoxanthine-guanine phosphoribosyltransferase; IHC, immunohistochemistry; MMP, matrix metalloproteinase; OI, osteogenesis imperfecta; *oim*, osteogenesis imperfecta murine; PBS, phosphate buffered saline; PMSF, phenylmethyl sulfonyl fluoride; PSR, picrosirius red; TRS, target retrieval solution; UPR, unfolded protein response.

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and shown to be constitutively expressed by both mesangial and glomerular epithelial cells [31–33] and is postulated to act as a pro-inflammatory activator of mesangial cells [34,35] while possibly contributing to epithelial-to-mesenchymal-transition (EMT) [36]. MMP-9 [31,37,38] is also synthesized by glomerular epithelial cells, but unlike MMP-2, has been shown to have restricted temporal expression during development and in adult rodent tissues [39,40] and is differentially regulated in several animal models [41–44]. Though less well studied in kidneys, MMP-3 is an important activator of MMP-2, -9 and -13, degrading many of the same substrates including gelatin [45]. It has also been shown to be elevated in the serum of human renal transplant patients with chronic nephropathy [46]. MMP-13 is synthesized by mesangial cells, activated by MMP-2, -3 and -9, and plays an important role in cancer progression [8,31]. The membrane collagenase MMP-14 (also known as MT1-MMP) has multiple functions, including but not limited to pericellular collagen cleavage and activation of MMP-2 [47]. It is known to be crucial for general collagen turnover [48] as well as wound healing and tumor invasion [49]. MMP-3, -13 and -14 have also been identified as primary players in fibrosis due to EMT [50].

Mice constitutively expressing the MMP-resistant homotrimers provide an unprecedented opportunity to examine the potential pathogenic role of this collagen isotype in glomerulosclerotic disease without confounding factors of artificially induced renal injury. The *Col1a2*-deficient mouse has a single nucleotide deletion that causes a frame-shift and disrupts the carboxyl terminus (C-propeptide) of the α2(I) chain, required for the association with α1(I) procollagen chains to form the heterotrimeric triple helix [51]. The resulting exclusion of the α2(I) chain from the type I collagen triple helix leads to synthesis and secretion of only the homotrimeric isotype in homozygous *Col1a2*-deficient (−/−) mice. The genetic defect itself, reduced bone mineral density and increased bone fragility in the *Col1a2*-deficient mouse [52–54] resemble a patient with an autosomal recessive form of osteogenesis imperfecta [55–57], which is why this mouse is also known as the osteogenesis imperfecta murine (*oim*) model. In addition to the bone phenotype, our previous studies of the *Col1a2*-deficient mouse revealed postnatal accumulation of homotrimeric type I collagen in the renal mesangium, resulting in progressive glomerulosclerosis, podocyte foot process effacement and proteinuria [12,58]. These findings and the synthesis of the homotrimers by mesangial cells that have no genetic *Col1a2* deficiency [13] beg the question whether this isotype is the primary pathogenic collagen in glomerulosclerotic lesions.

In the present study, we addressed the potential role of delayed degradation and clearance of this MMP-resistant collagen isotype from renal mesangium. We compared pre- and post-translational expression and distribution of type I collagen isotypes and MMPs in whole kidneys and isolated glomeruli from 1 month old and 3 month old heterozygous and *Col1a2*-deficient mice. Since mouse MMP-1 is only synthesized during embryogenesis [31,59,60], we limited the study to MMP-2, -3, -9, -13 and -14.

2. Materials and methods

2.1. Animals

Heterozygous B6C3Fe *a/a-Col1a2*$^{oim/J}$ (+/−) mice were purchased from Jackson Laboratory (Bar Harbor, ME) and bred to produce wildtype (+/+), heterozygous (+/−) and homozygous (−/−) animals. Animals were housed and fed (Purina 5008 Formulab Diet; Purina Mills Inc., Richmond, IN) ad libitum in an AAALAC accredited animal facility in accordance with an approved University of Missouri Animal Care and Use protocol. Animal genotypes were determined as previously described [61] and aged to 1 (n = 168 mice) or 3 months (n = 151) of age. Animals were sacrificed and kidneys or glomeruli harvested as described below.

2.2. Glomerular isolation

Wildtype, heterozygous and *Col1a2*-deficient mice were aged to 1 month [−/−, n = 20; +/+, n = 21; and +/−, n = 38] and 3 months [−/−, n = 13; +/+, n = 22; and +/−, n = 23] of age and anesthetized prior to kidney perfusion [62]. Perfusion of 8×10^7 tosylactivated Dynabeads® magnetic beads (deactivated according to the manufacturer's instructions) in 1 XPBS were perfused through the body via the heart. Perfused kidneys were removed, weighed and minced followed by a 30 minute digestion in 1 mg/ml collagenase A (Invitrogen Corporation, Carlsbad, CA), 100 units/ml DNase (Invitrogen Corp., Carlsbad, CA) and Hanks Balanced Salt Solution (HBSS) (GibCo-Invitrogen Corporation, Carlsbad, CA) at 37 °C. The digested slurry was sieved twice through 100 μm cell strainers (BD Bioscience, San Jose, CA) with the addition of HBSS, followed by centrifugation at 1500 rpm for 15 min. The pellet was resuspended in HBSS and placed onto a magnetic particle concentrator for 1 min to separate glomeruli from extraneous tissue, and repeated 5 times. Remaining glomeruli were resuspended in HBSS and assessed for purity (greater than 98% purification) and yield using a hemocytometer, followed by snap-freezing and storage at −80 °C.

2.3. Microscopy and glomerular counting

Longitudinal sections (5 μm) of formalin-fixed kidneys from 1 to 3 month wildtype, heterozygous and *Col1a2*-deficient [n = 8 of each genotype] were embedded in paraffin and stained with picrosirius red (PSR) fibrillar collagen stain. The PSR-stained sections were examined by conventional light microscopy and with polarized light. Glomeruli within individual sections were evaluated blindly to obtain a glomerular lesion score for each kidney as previously described [58], and to assess the average glomerular number within longitudinal sections. Mean glomerular number was calculated as the average of the number of glomeruli within 4 sections.

2.4. Immunohistochemistry (IHC) for α1(I) and α2(I) collagen

Longitudinal sections (10 μm) of zinc fixed kidneys were embedded in paraffin and placed on slides. Heat-induced epitope retrieval in target retrieval solution (1X TRS) (Dako, Carpinterea, CA) was followed by quenching of endogenous biotin using an avidin/biotin block. Endogenous peroxidase was removed by treating slides with 3% hydrogen peroxide and non-specific antibody binding was blocked with a 5% bovine serum albumin (BSA) solution. Next, kidneys were incubated in either rabbit polyclonal anti-α1(I) collagen primary antibody (MD Biosciences, St Paul, MN) diluted 1:600 or rabbit polyclonal anti-α2(I) collagen primary antibody diluted 1:3000. The anti-α2(I) collagen primary antibody was produced by the antibody production core at UT-Southwestern Medical Center. In brief, two rabbits (U6410 and U6425) were immunized genetically with 1 mg each of cDNA encoding murine α2(I) collagen chain three times over 42 days. The rabbits were boosted every 2 weeks (five times total) with collagen purified from homozygous *Col1a2*-deficient mice. Pre-immune serum and sera collected after the final boost were evaluated by ELISA for reactivity with collagen isolated from wildtype and *Col1a2*-deficient mice. Following the addition of the primary antibodies, biotinylated swine anti-rabbit secondary antibody diluted 1:300 was added, followed by a streptavidin horseradish peroxidase conjugate, and 3,3′ diaminobenzidine tetrahydrochloride (DAB) substrate with hematoxylin counterstaining. Staining was performed on a Dako Autostainer Universal Staining System. Anti-α1(I) and anti-α2(I) collagen antibody specificity was confirmed by western blot analysis of wildtype and *Col1a2*-deficient mouse tail tendon collagen. The anti-α2(I) collagen also specifically binds acid-solubilized and pepsin treated rat and human α2(I) collagen chains by western blot analyses.