Increased Expression of Lysyl Oxidase mRNA in Progressive Mesangial Proliferative Nephritis in the Rat

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Abstract: Glomerulosclerosis is the final event of many chronic renal diseases. It is characterized by the accumulation of extracellular matrix (ECM) proteins. Collagen IV is one of the major ECM proteins that accumulate in glomeruli in chronic renal disease. Lysyl oxidase (LOX)-induced cross-linking of collagen may be involved in these processes. Therefore, we studied LOX mRNA expression by reverse transcriptase-polymerase chain reaction (RT-PCR) in progressive proliferative glomerulonephritis (anti-Thy 1.1 nephritis induced in unilaterally nephrectomized rats) and reversible proliferative glomerulonephritis (anti-Thy 1.1 nephritis induced in normal rats). Segmental glomerulosclerosis was observed in about 10% of glomeruli in rats with anti-Thy 1.1 nephritis induced in unilateral nephrectomy at eight weeks after disease induction. A marked increase in glomerular LOX mRNA expression was also demonstrated. In contrast, the glomerulus at 7 days after anti-Thy 1.1 nephritis induced in normal unmanipulated rats was characterized by marked glomerular cell proliferation and matrix expansion. However, there was no evidence of sclerosis in the glomerulus in this model and there was no LOX mRNA expression in glomeruli. These results suggest that expression of LOX in glomeruli may be a marker for the occurrence of glomerulosclerosis, and that LOX may also play a key role in the development of irreversible glomerular fibrosis by cross-linking of collagen fibrils.

Key words: glomerulosclerosis, extracellular matrix, lysyl oxidase, collagen crosslink

Introduction

Mesangial cell proliferation and subsequent accumulation of extracellular matrix (ECM) proteins are the major histologic features of many progressive glomerular diseases. These processes may precede the development of glomerulosclerosis that is characterized by increased amounts of extracellular matrix and an irreversibly progressive fibrotic process. Therefore the regulation of glomerulosclerosis (glomerular fibrosis) may provide useful insight into the future therapeutic strategies for human progressive glomerular diseases. Abnormal ECM accumulation in the development of glomerulosclerosis is the result of an imbalance between deposition and removal of components of ECM proteins. Collagen IV is...
one of the major ECM proteins that accumulates in the diseased glomeruli, leading to
glomerulosclerosis\(^1\)\(^-\)\(^4\). It was reported that collagen cross-linking induced by LOX is critical
in the development of organ fibrosis such as liver cirrhosis or idiopathic lung fibrosis\(^5\)\(^,\)\(^6\). Donato \textit{et al.} demonstrated that LOX expression is increased in the fibrotic process in
chronic adriamycin nephropathy\(^7\) and that these processes preceded glomerular fibrosis.
Therefore, in the present study, we examined whether or not LOX mRNA is increased in
the glomerulus in progressive mesangial proliferative glomerulonephritis.

\textbf{Materials and Methods}

\textit{Experimental protocol and disease model}

A single injection of anti-Thy-1.1 antibody causes transient proteinuria and mesangial
lesions, with mesangiolysis, mesangial hypercellularity and mesangial matrix expansion which
are characteristic of human acute glomerulonephritis\(^8\)\(^-\)\(^13\). Recently, a new chronic animal
model of progressive glomerulosclerosis was developed by administration of a similar
antibody to unilaterally nephrectomized rats\(^9\). In the present study, we studied anti-Thy 1.1
nephritis induced in unilaterally nephrectomized rats. All experimental procedures involving
animals were approved by the Institutional Animal Care and Use Committee of Showa
University. Right uninephrectomy was performed on five Wistar male rats weighing 200g
(Nippon Ikagaku Doubutsu, Tokyo, Japan). On the next day of unilateral nephrectomy,
anti-Thy 1.1 nephritis was induced in all rats by a single intravenous injection of rat-anti
Thy 1.1 monoclonal antibody (Cedarlane Laboratories Limited, Hornby, Ontario, Canada),
as reported previously\(^8\)\(^,\)\(^14\). Eight weeks after disease induction, rats were sacrificed and the
left kidney was removed after 24-hour urine collection and blood sampling. As a control,
24-hour urine collections, blood samples and kidney tissues were obtained from normal
unmanipulated rats (\(n=5\)), and rats with anti-Thy 1.1 nephritis (\(n=5\)) at seven days after
disease induction were also studied.

\textit{Isolation of glomeruli and preparation of glomerular RNA}

The glomeruli were isolated by differential sieving\(^15\). To preserve the integrity and
stability of the glomerular RNA, all isolation steps were done at 4 ℃ using
diethylpyrocarbonate (Sigma, St. Louis. MO)-treated phosphate-buffered saline, autoclaved
or baked sieves, and glassware\(^7\)\(^,\)\(^16\). Less than 20 minutes elapsed from nephrectomy to
dissolution of the isolated glomeruli in RNAzol\(^R\) solution (Cinna/Biotex Laboratories,
Houston, TX). Total RNA was extracted from isolated glomeruli from all groups of rats
with RNAzol\(^R\) following the manufacturer's instructions\(^8\).

\textit{RT-PCR Analysis for lysyl oxidase expression}

LOX mRNA levels were determined by reverse transcriptase-polymerase chain reaction
(RT-PCR). 1 µg of total RNA extracted from purified glomeruli using RNA zol\(^R\)
(TEL-TEST, Inc., TX, U.S.A.) was then reverse-transcribed by Avian Myeloblastosis Virus
transcriptase using random 9- mers as primers and amplified by PCR (RNA PCR kit,
Takara Biochemicals, Japan). The PCR reaction was performed with a set of primers
specific for rat LOX mRNA that amplify a 636-bp region (R-LOX/5′, 5′-CTA TGA CAC
TTA TGA AGA ACC GGT CCG GG-3′; R-LOX/3′, 5′-CAC AGC GTA CGA CAT
TGT TAC TGT AGT CTG-3′)\(^7\). The PCR amplification conditions were: 1 minute
denaturation at 94°C, 1 minute annealing at 55°C, 2.5 minutes elongation at 72°C, for 30 cycles. Level of cDNA were normalized against levels of the house-keeping gene glyceraldehyde-3-phosphate-dehydrogenase (G3PDH), using previously described primers: forward 5'-TGA AGG TCG GAG TCA ACG GAT-3'; reverse 5'-CAT GTG GGC CAT GAG GTC C-3'7). PCR conditions were: 1 min at 95°C, 1.5 min at 59°C, 2 min at 72°C, for 27 cycles. The PCR products were quantified by densitometric analysis (NIH Image v. 1.61 for Macintosh, freeware) and expressed in arbitrary units as the LOX/G3PDH expression ratio7).

Renal morphology

Kidney tissues were fixed in buffered formalin and embedded in paraffin. 4-μm sections were stained with Periodic acid-Schiff (PAS) or Periodic acid-methenamine-silver (PAM) reagent and counterstained with hematoxylin17). The expansion of mesangial matrix was observed in the PAS-stained sections and glomerulosclerosis was evaluated in the PAM-stained sections.

Measurement of urine protein excretion

Twenty-four-hour urine collection was obtained from all rats that were individually housed in metabolic cages. Rats were fasted during the collection period, but were allowed free access to water. Urine protein excretion was measured by a sulfosalicylic acid method15) using a whole serum standard (Lab Trol, Dade Diagnostics, Aquado, Puerto Rico).

Statistical analyses

All values are expressed as mean ± SD unless stated otherwise. Statistical significance (defined as p<0.05) was evaluated using the t test18).

Results

Renal morphology

The glomerulus at 8 weeks after anti-Thy 1.1 nephritis induced in unilaterally nephrectomized rats was characterized by mesangial matrix expansion with fibrosis. Segmental glomerulosclerosis was evident in a small portion of glomerular tuft, and also in about 10% of glomeruli. Several glomerular capillary lumens showed evidence of obliteration and the adhesive lesion of the glomerular tuft to the capsule was also observed (Fig. 1B). The early phase of glomerulosclerosis was demonstrated in PAM-stained sections (Fig. 2C). Mild glomerular cell proliferation was also present. In contrast, the glomerulus at 7 days after anti-Thy 1.1 nephritis induction in normal unmanipulated rats was characterized by marked glomerular cell proliferation and matrix expansion (Fig. 1A). However, there was only minimal PAM-positivity in the glomerulus. Therefore this model was characterized by no sclerotic process in to say, There was no matrix expansion, cell proliferation or sclerosis in the glomerulus in normal rats (Fig. 2A).

Increased expression of LOX mRNA in progressive glomerulonephritis

There was no LOX mRNA expression in glomeruli in unmanipulated normal rats or in rats with anti-Thy 1.1 nephritis on day 7 (Fig. 3). In contrast, LOX mRNA levels were dramatically elevated at 8 weeks after anti-Thy 1.1 nephritis induction on unilaterally
Fig. 1. Periodic acid-Schiff-stained sections in anti-Thy 1.1 nephritis induced in normal rat (1A) and induced in unilateral nephrectomized rat (1B). 1A is at 7 days and 1B is at 8 weeks after disease induction. 1A shows marked matrix expansion and hypercellularity in the glomerulus. Glomerular cellularity in 1B is not so prominent as 1A, but segmental matrix expansion is observed. Periodic acid-Schiff staining, $\times 400$

Fig. 2. Periodic acid-methenamine-silver-stained sections in normal rat (2A), in anti-Thy 1.1 nephritis induced in normal rat (2B) and in anti-Thy 1.1 nephritis induced in unilateral nephrectomized rat (2C). Segmental glomerulosclerosis (arrows) is observed in about 10% of glomeruli in 2C. However, there is minimal PAM-positivity in the glomerulus in 2B, as is seen in 2A, instead of prominent expansion of mesangial matrix. Periodic acid-methenamine-silver staining, $\times 400$
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Fig. 3. RT-PCR analysis of glomerular LOX mRNA expression in normal rat (lane 1), in anti-Thy 1.1 nephritis induced in normal rat (lane 2) and in anti-Thy 1.1 nephritis induced in unilateral nephrectomized rat (lane 3). The figure shows a typical PCR amplification of LOX mRNA in lane 3. There is no LOX mRNA expression in lanes 1 and 2.

Urinary protein excretion

As we previously reported\textsuperscript{14}, induction of anti-Thy 1.1 nephritis in normal rats was associated with a transient increase in the urinary protein excretion. Urinary protein excretion markedly increased at day 2, and then decreased gradually\textsuperscript{14}. In contrast, urinary protein excretion gradually increased in anti-Thy 1.1 nephritis induced in unilaterally nephrectomized rats at 8 weeks after disease induction (22.77 ± 14.18 mg/day, p<0.01 v.s. day 0; 3.9 ± 2.1 mg/day).

Discussion

Renal fibrosis is the final event of a complex pathogenic cascade originating from immunologic, toxic or infective renal injuries\textsuperscript{1,2,18,19}. Therefore, the prevention or delay of progression to glomerular fibrosis is critical in treatment strategies for glomerulonephritis. Type IV collagen is one of the most important ECM proteins in the progression of glomerulosclerosis. While the mechanisms of the early stage of renal damage, in which renal cells are stimulated to produce collagen components have been well characterized\textsuperscript{5,20}, very little is known about the processes of progression of disease that involve collagen metabolism. In recent years, some progress has been made in our understanding of the mechanisms involved in the regulation of ECM deposition and removal in the kidney\textsuperscript{5,20}. The LOX enzyme catalyzes collagen cross-linking through oxidative deaminaton of lysyl \(\varepsilon\)-amino groups, the final products of which are stable and insoluble collagen molecules. The structural abnormality associated with the alterations in the physico-chemical properties of collagen, including increased insolubility and stiffness, is found in various fibrotic disorders that are characterized by increased deposition of collagen such as liver cirrhosis, lung fibrosis, skin, tendon, bone and arteries\textsuperscript{6,21-23}. In all these cases the complex fibrogenic events culminate in the formation of cross-links between the \(\varepsilon\)-amino groups of lysine residues contained in the collagen chains, an enzymatic process known as maturation of collagen, which is catalyzed by LOX. The formation of cross-links between collagen chains
from enzymatic activity, or from nonenzymatic glycosylation\textsuperscript{24, 25} is considered to be a central step in the generation of irreversible organ lesions, since the cross-linked regions cannot be degraded by enzymatic cleavage.

Based on the assumption that LOX activation could also take place in glomerulosclerosis, Donato \textit{et al.} recently examined LOX mRNA expression in chronic adriamycin nephropathy, which is characterized by glomerulosclerosis and interstitial fibrosis and showed that the level of LOX progressively increased\textsuperscript{7}. In this study, we analyzed LOX mRNA expression in the glomerulus in progressive anti-Thy 1.1 nephritis induced in unilaterally nephrectomized rats. Our results showed a significant increase of LOX mRNA expression in glomeruli at 8 weeks in this model. Urinary protein excretion was also significantly increased at this time (22.77 ± 14.18 mg/day v.s. day 0: 3.9 ± 2.1 mg/day in controls). As previously reported\textsuperscript{26}, glomerulosclerosis, mainly expressed by focal or segmental fibrosis, appeared in about 10 percent of glomeruli in this model. We also examined LOX expression in anti-Thy 1.1 nephritis induced in normal rats, which show reversible acute mesangial cell proliferative nephritis. At day 7 after disease induction in this model, there was a marked increase in mesangial matrix. However there was minimal PAM-positivity in this expanded mesangial matrix, indicating that glomerular fibrosis had not occurred in this model. Furthermore there was no LOX expression, as was seen in normal unmanipulated rats. From these results, we propose that glomerular expression of LOX mRNA may be a marker for the occurrence of glomerulosclerosis, and may also play a key role in the development of irreversible glomerular fibrosis by cross linking of collagen fibrils.

These asynchronous patterns of LOX expression between the acute and chronic phase is of particular interest since possible preventive efforts to block maturation of collagen chains must precede the formation of irreversible fibrosis. It has been reported that the administration of the lathyrogen \(\beta\)-aminopropionitrile (\(\beta\ APN\))\textsuperscript{27}, an irreversible inhibitor of LOX, reduced the mechanic collagen strength and prevented scar formation in other animal models of organ fibrosis\textsuperscript{28, 29}. To define the role of LOX mRNA expression in the pathogenesis of progressive proliferative glomerulonephritis, the effect of the lathyrogen \(\beta\)-aminopropionitrile should be studied.

References

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