Detection of mRNA for Alpha-3 Chain of Type IV Collagen in the Glomerular Epithelium, and the Effect of Perfused Elastase on Its Expression

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Abstract

Background: The type IV collagen is a complex of trimetrical molecule composed of six genetically distinct polypeptide chains; α1–6(IV). Since α3(IV) distribute specifically in the glomerular basement membrane (GBM) of glomerular capillary, we tried to develop the detection methods for the transcripts of α3(IV) in glomerular epithelial cells (GEC) which produce most of the components for GBM. Then, using these molecular techniques, the influence of elastase, one of the proteases released from activated polymorphonuclear leukocytes at the site of inflammation, on GEC was determined as manifested by expressional alteration of α3(IV) mRNA. Methods: DIG-labeled oligo-DNA probe designating non-collagenous region of α3(IV) was used for in situ hybridization. Semiquantitative measurement of α3(IV) in the renal cortex was performed by PCR reactions, each reaction being normalized by that for GAPDH. Then, the femoral artery of each of 18 Sprague-Dawley rats was catheterized and the left kidney was perfused with saline alone (0.5 ml) or saline containing 100 μg/ml elastase. After collection of urine for 24 h, the left kidney was harvested for analysis of mRNA (4 for in situ hybridization and 5 kidneys for PCR analysis). Results: Antisense cDNA probe and PCR reaction well identified α3(IV) mRNA in the cytoplasm of GEC and in the renal cortex, respectively. Urinary protein excreted by rats with elastase perfusion was 47.2 ± 3.8 mg/24 h but this was only 13.9 ± 1.1 mg/24 h in control rats (mean ± SEM, p < 0.05). In situ hybridization demonstrated that expression of α3(IV) mRNA in GEC was focally or diffusely reduced in the glomeruli of rats with elastase perfusion, whereas the transcripts were well stained in GEC of controls. PCR analysis showed about 25% decrease in transcripts of α3(IV) in the renal cortex of rats with elastase perfusion compared to those of control rats. Conclusions: α3(IV) mRNA was identified specifically in the GEC in the glomeruli. Co-incidence of proteinuria and reduced α3(IV) expression by elastase suggests adverse effects of elastase on GEC and close association between proteinuria and GEC injury.
Introduction

Type IV collagen is the main component of the basement membrane, which is ubiquitous sheet-like extracellular structure separating organ cells from interstitial connective tissue. The basement of renal glomeruli (GBM) is a unique type of basement membrane forming a single, well-defined layer located between endothelial cells and the epithelial podocytes. The GBM has been the focus of intensive research because it is believed to function as the actual renal filtration barrier for macromolecules. Most of the components for GBM including type IV collagen, however, are produced by glomerular epithelial cells (GEC) [1–3]. Therefore, GEC play an important role to maintain the normal structure of GBM, and thus, their condition may be critical especially when GBM was digradated and required to be repaired.

The type IV collagen network forms the structural skeleton of basement membrane [4]. It is a complex of trimetric molecule composed of at least six genetically distinct polypeptide chains; α3(IV), α2(IV), α3(IV), α4(IV), α5(IV), and α6(IV) [5–7]. These assemble into different isoforms of triple-helical molecules, the most abundant combination being [α1(IV)]₃α2(IV). Although little is known about the composition of molecules containing the minor chain α3(IV), α4(IV), α5(IV), and α6(IV), recent studies using specific antibodies demonstrated that α3(IV) and α4(IV) distributed only in GBM of glomerular capillary and in limited part of basement membrane of the proximal tubules [7, 8], whereas α1(IV) and α2(IV) did also in the mesangial matrix, Bowman’s capsule, and tubular basement membrane. These findings indicate that α3(IV) and α4(IV) is considered to be produced specifically by GEC in the glomeruli, and the transcripts of these components may be located only in glomerular epithelium in a glomerulus. Therefore, in the present study, we try to detect the mRNA expression for α3(IV) using in situ hybridization and, then, to semiquantify its transcripts in renal cortex by PCR technique.

It has long been recognized in animal experiments that the proteases released from polymorphonuclear leukocytes (PMN) adherent to the glomerular capillary wall may induce glomerular injury [9, 10]. Recently, Johnson et al. [11, 12] directly demonstrated that renal perfusion of active elastase, one of active serine proteases released from PMN, results in severe proteinuria. Furthermore, Heeringa et al. [13] showed that such renal perfusion of active elastase degraded GBM, leading to the loss of polyanionic structure of GBM and induction of proteinuria. In addition to degradation of extracellular matrix proteins, other studies have shown that neutral serine proteases can also injure endothelial cells (EC) [14, 15], suggesting the adverse effect of circulating elastase on glomerular EC. However, little is known about the effects of elastase on GEC, even though GEC is thought to reproduce GBM components to repair the GBM when injured by elastase. Therefore, the condition of GEC after glomerular injury by elastase is to be elucidated for the evaluation of progression of glomerular lesions. We assess the condition of GEC, using the detection methods for α3(IV) mRNA developed in the present study, according to the hypothesis that mRNA expression for α3(IV) may remain unchanged or enhanced to repair degraded GBM when the GEC are kept in good condition even after renal perfusion of elastase, whereas, it will be reduced when the GEC themselves are damaged by circulating elastase. The results suggest the adverse effect of elastase on GEC, which may influence on the progression of glomerular lesion.

Materials and Methods

Detection of mRNA for α3(IV)

In situ Hybridization. The antisense oligo-DNAs were designed to frame a part of non collagenous domain (NC1 domain) of α3 chain as follows:

Antisense:

tggtgaatgcttgaagctgctccgagatcacaagtcctgttcctcatgcgtgtcc

Sense:

tgacagccctaggcagcactctggcactgtgctctgcctgcctgtacctcgacacc

Labeling and staining was performed as previously reported [16, 17]. Briefly, these oligo-DNAs were labeled at their 3’-end with DIG-11-dUTP. PFA-fixed specimens were cut into 6 μm thick sections and hybridized with 100 ng/ml DIG-labeled probes. Anti-digoxigenin antiserum conjugated with ALPL (Boehringer Mannheim, Mannheim, Germany) was used to detect the distribution of DIG, and site of ALP was visualized by reaction with NBT/BCIP.

PCR for α3(IV) Transcripts. Semiquantitative PCR was designed by modifying our previous technique of competitive PCR [18]. A pair of PCR primers was designed to frame the part of α3 chain, which is located in the NC domain of the chain. Examination of the nucleotide sequence by the dye-termination method using a genetic analyzer (ABI PRISM™ 310, Perkin Elmer, Conn., USA), showed 100% identical to that of the partial fragment of α3 chain. After confirming the sequential increase in signal densities according to increment in PCR cycles, series of PCRs using different amount of sample cDNA were performed using primer sets for GAPDH as control (25 cycles) or for α3(IV) (35 cycles) to confirm the sensitivity of PCR analysis. The intensity of the bands in an agarose gel was measured using a densitometer. Finally, all five samples from each condition (saline perfusion and elastase perfusion) were analyzed and the mRNA developed in the present study, according to the condition of GEC, using the detection methods for α3(IV) mRNA developed in the present study, according to the hypothesis that mRNA expression for α3(IV) may remain unchanged or enhanced to repair degraded GBM when the GEC are kept in good condition even after renal perfusion of elastase, whereas, it will be reduced when the GEC themselves are damaged by circulating elastase. The results suggest the adverse effect of elastase on GEC, which may influence on the progression of glomerular lesion.
Fig. 1. Localization of transcripts for α3(IV).
A Glomerular section from a representative control saline perfused rat, which was hybridized with the Dig-antisense probe. Note the strong positive signal in the cytoplasm of glomerular epithelial cells (arrow).
B Glomerular section hybridized with the sense probe. No definite signals were observed in glomerular epithelial cells (arrow). x 200.

Results

Detection of mRNA for α3(IV)

In situ Hybridization. Figure 1 shows the localization of transcripts for α3(IV). When glomerular sections from control rats perfused with saline were hybridized with the DIG-antisense probe, a strong positive signal was clearly detected in the cytoplasm of glomerular epithelial cells, but not in those of mesangial cells or endothelial cells (fig. 1A). In contrast, no definite staining was found even in GEC with the sense probe (fig. 1B), which was used as a negative control in every experiment.

PCR Analysis. Figure 2a demonstrates a series of different amounts of RNA with 25 cycles of PCR reaction, showing the linear increase in the GAPDH signal density between 25 and 75 ng RNA. Then, for the analysis of GAPDH transcripts signals we used 50 ng RNA and 25 cycles of PCR reaction. Figure 2b depicts a series of different amount of RNA (35 cycles) for the analysis of transcripts for α3(IV), showing again a linear increase in signal density between 12.5 and 37.5 ng RNA. Thus, we compared the transcript signals for α3(IV) using 25 ng RNA with 35 cycles.

Effect of Elastase Perfusion

Urinalysis, Renal Function, Plasma Albumin Concentration, and Plasma Electrolytes. Although urinary protein excretion was observed in control rats that were perfused with saline only, rats perfused with elastase showed marked proteinuria (table 1). There were no significant changes in plasma concentrations of urea and creatinine, suggesting that elastase did not have an immediate effect on renal function. Furthermore, plasma albumin concentrations were maintained at baseline levels even in rats perfused with elastase. No effects of renal elastase perfusion on plasma electrolytes were observed.

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Nephron 2002;92:853–859
Fig. 2. PCR signals for GAPDH and α3(IV) with series of different amounts of RNA. a GAPDH signals (25 cycles of PCR) showing linear increase from 25 to 75 ng RNA. b Signals for α3(IV) (35 cycles) showing a linear increase from 12.5 to 37.5 ng RNA.

Table 1. Effects of renal perfusion

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<thead>
<tr>
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<th>Saline perfusion</th>
<th>Elastase perfusion</th>
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<tr>
<td>n</td>
<td>8</td>
<td>8</td>
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<tr>
<td>Change in body weight, %</td>
<td>8.7 ± 0.6</td>
<td>8.0 ± 0.7</td>
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<tr>
<td>Plasma</td>
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<tr>
<td>Urea, mg/dl</td>
<td>17.8 ± 2.0</td>
<td>15.2 ± 1.9</td>
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<tr>
<td>Creatinine, mg/dl</td>
<td>0.4 ± 0.1</td>
<td>0.5 ± 0.1</td>
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<tr>
<td>Albumin, g/dl</td>
<td>1.0 ± 0.1</td>
<td>1.0 ± 0.1</td>
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<tr>
<td>Sodium, mEq/l</td>
<td>140 ± 1.0</td>
<td>139 ± 1.0</td>
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<tr>
<td>Potassium, mEq/l</td>
<td>4.5 ± 0.1</td>
<td>4.9 ± 0.3</td>
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<tr>
<td>Urine</td>
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<tr>
<td>Volume, ml/24 h</td>
<td>17.2 ± 2.6</td>
<td>16.7 ± 2.2</td>
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<tr>
<td>Protein, mg/24 h</td>
<td>13.9 ± 1.1</td>
<td>47.2 ± 3.8*</td>
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Values are means ± SEM. *p < 0.05 compared with saline perfusion. Blood sample was obtained 24 h after renal perfusion.

Discussion

In the present study, we examined the expression level of transcripts of type IV collagen, which is thought to represent the process of production of basement membrane components in the glomerular epithelium. We selected the cDNA probe for α3 chain of IV collagen, since this unit of type IV collagen is a unique component in GBM in glomeruli [7, 8]. As expected, cDNA probe designating the carboxyl terminal of the α3(IV) clearly stained the cytoplasmic area of glomerular epithelial cells (GEC), indicating that α3(IV) is specifically produced in GEC and analysis of its transcripts may show ability of epithelial cells to construct the basement membrane. Renal elastase perfusion, which induced proteinuria without any significant influences on renal function, reduced expression for α3(IV) mRNA in some of the GEC of rats with renal elastase perfusion. Semiquantitative PCR analysis confirmed the decreased level of expression of the transcripts in the renal cortex of these rats.

Clinical studies examining the effect of oral serine protease inhibitors in patients with nephropathies have demonstrated the antiproteinuric effect of this medication in chronic glomerulonephritis (CGN) [20, 21] and diabetic nephropathy (DN) [20, 22]. In addition to CGN where an inflammatory process is thought to be continuously involved in the glomeruli, enhanced leukocyte adhesion to the peripheral capillary of diabetic rats has been noted in association with the onset of diabetic retinopathy [23]. Thus, the clinical findings by protease inhibitor suggest
**Fig. 3.** In situ hybridization of α3(IV) in glomeruli of rats with renal elastase perfusion (E-1,2,3, and 4). Arrows point to glomerular epithelial cells in which signal density for α3(IV) transcripts is lower and irregular. × 200.

**Fig. 4.** a PCR signals of each rat for GAPDH and α3(IV). b The average of five determinations of signal density for α3(IV) mRNA, which was normalized to that of GAPDH. *p < 0.05.
that proteinuria in CGN and DN is induced, in part, by the effects of protease released from activated neutrophils in the glomeruli. Besides, several in vivo studies have demonstrated the direct evidence that circulating elastase induces proteinuria [11–13]. Especially, GBM damage by activated polymorphonuclear leukocytes (PMN) [9, 10], which release protease, or by circulating elastase [13] have been well examined. Therefore, our hypothesis before the study was that the production of GBM components would be promoted in GEC after renal elastase perfusion in order to repair injured basement membrane, and the enhanced production would be manifested by an increase in the transcripts for GBM component. Accordingly, we applied our new techniques of detecting the transcripts for the components of GBM. The results of in situ hybridization, however, clearly demonstrated reduced expression for α3(IV) mRNA in some of the GEC of rats perfused with elastase, suggesting that production of GBM components may be impaired in these cells. This reduced expression of the α3(IV) mRNA in the cortex from rats with renal elastase perfusion was further confirmed by semi-quantitative PCR.

Besides the ability of protease to degrade extracellular matrix like GBM, several studies have shown that neutral serine protease can also injure endothelial cells (EC) [14, 15]. Although no histological findings of endothelial cell injury was observed in the glomeruli of the rats with renal elastase perfusion in the present study, histological evidence for the degradation of GBM has not been noted [11, 12] until Heeringa et al. [13] examined using immunohistochemical staining. Thus, the glomerular endothelium, which was directly exposed to circulating elastase, was possibly damaged by circulating elastase. In combination with GBM degradation, EC injury in the glomeruli may also allow the direct exposure of elastase to some of GEC. Focal defects of α3(IV) mRNA staining observed in rats with renal elastase perfusion may result from degradation of GEC which were exposed to elastase.

Activation of PMN adherent to the glomerular capillary wall leads to the production of reactive oxygen species and the release of their granule constituents, including serine protease. At the site of degranulation high concentration of these proteolytic enzymes may be reached which may cause glomerular injury as observed in PMN-dependent GN [9, 10]. Although the effects of these enzymes on the glomerular mesangial cells are still unknown, our present findings indicate that enzymes released from activated PMN may damage GEC in addition to GBM and glomerular EC, influencing on the process of reconstruction of injured GBM.

In conclusion, in situ hybridization using cDNA probe for α3(IV) mRNA clearly identified cytoplasmic region of the glomerular epithelium. The staining intensity was modified in the pathological condition induced by renal elastase perfusion, suggesting the adverse effects of elastase on glomerular epithelial cells.

References

6 Zhou J, Ding M, Zhao ZH, Reeder ST: Complete primary structure of the sixth chain of human, basement membrane collagen, alpha 6(IV): Isolation of the cDNAs for alpha 6(IV) and comparison with five other type IV collagen chains. J Biol Chem 1994;269:13193–13199.