

Regulation of Type IV Collagen α Chains of Glomerular Epithelial Cells in Diabetic Conditions

An early feature of diabetic nephropathy is the alteration of the glomerular basement membrane (GBM), which may result in microalbuminuria, subsequent macroproteinuria, and eventual chronic renal failure. Although type IV collagen is the main component of thickened GBM in diabetic nephropathy, cellular metabolism of each α chains of type IV collagen has not been well studied. To investigate the regulation of α (IV) chains in diabetic conditions, we examined whether glucose and advanced glycosylation endproduct (AGE) regulate the metabolism of each α (IV) chains in the diabetic tissue and glomerular epithelial cells (GEpC). Glomerular collagen α 3(IV) and α 5(IV) chains protein were higher and more intense in immunofluorescence staining according to diabetic durations compared to controls. In vitro, mainly high glucose and partly AGE usually increased total collagen protein of GEpC by [³H]-proline incorporation assay and each α (IV) chain proteins including α 1(IV), α 3(IV), and α 5(IV) in time-dependent and subchain-specific manners. However, the changes of each α (IV) chains mRNA expression was not well correlated to the those of each chain proteins. The present findings suggest that the metabolism of individual α (IV) chains of GBM is differentially regulated in diabetic conditions and those changes might be induced not only by transcriptional level but also by post-translational modifications.

Key Words : Glycosylation End Products, Advanced; Diabetic Nephropathies; Collagen Type IV; Glucose; Podocytes

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INTRODUCTION

Diabetic nephropathy and vascular changes remain major complications of diabetes, accounting for up to 40% of cases with end-stage renal disease and 75% of cases with cardiovascular disease (1). Many studies suggest a key role for hyperglycemia in the pathogenesis of these complications (2, 3). Indeed, elevated glucose is known to activate a variety of cells to stimulate extracellular matrix (ECM) synthesis including collagen mediated by stimulating transforming growth factor (TGF)- β (2, 4, 5).

Pathologic changes in the glomerular ECM lead to abnormal protein excretion as well as to impairment of glomerular filtration in diabetic nephropathy. Alterations in the diabetic glomerular basement membrane (GBM) include increase in its thickness and decrease in the density of anionic sites (6). The former corresponds to increase in GBM content of collagen forming mesh-like barrier structure which regulates the size-selective aspects of glomerular permselectivity (1-3).

GBM are assembled through an interweaving of type IV

collagen (collagen IV) with laminins, nidogen, and sulfated proteoglycans (7-9). Collagen IV belongs to a family of collagenous proteins that has at least 25 distinct members. The *COL4A1*, *COL4A2*, *COL4A3*, *COL4A4*, *COL4A5*, and *COL4A6* genes encode the six chains of collagen IV, α 1(IV) through α 6(IV) (9-11). Type IV collagen chains have a highly regulated distribution in the glomerulus; α 1 and α 2 chains are localized to the mesangium, whereas α 3 through α 6 chains are found mainly in the GBM (9-13). Type IV collagen of GBM is produced by and attached to podocytes, therefore, type IV collagen of GBM is closely related to podocytes. Dysregulation of GBM collagen chains produces proteinuria and hematuria as seen in Alport's syndrome and Goodpasture syndrome clinically (10, 11). However, the dysregulation of cellular metabolism of individual chains of type IV collagen in podocytes (glomerular epithelial cells; GEpC) has not been well studied in diabetic nephropathy. Our aim in this study was to investigate the regulation of α chains of type IV collagen of podocytes that is responsible for the synthesis of GBM in diabetic condition.

MATERIALS AND METHODS

Diabetic animals

Diabetes was induced by a single intravenous injection of streptozotocin (STZ; Sigma Chemical Co., St. Louis, MO, U.S.A.) at 45 mg/kg body weight, freshly dissolved in 0.1 M sterile sodium citrate, pH 4.5, in 6-week-old (180–220 g) male Sprague-Dawley rats. The rats were considered diabetic if blood glucose levels were above 200 mg/dL at 48 hr after STZ injection. The rats remained hyperglycemic throughout experiment were sacrificed at 48 hr, 4 weeks and 10 weeks after the induction of diabetes. The right kidney was removed, weighed, cut into portions, and sectioned at 5 μ m for the assessment of immunofluorescence (IF).

Cell culture

Rat GEPCs, cloned from primary rat glomerular cultures, were characterized and provided by Kreisberg (14). They were characterized by sensitivity to puromycin aminonucleoside, positive staining for Heymann antigen (gp330) and podocalyxin, whereas negative staining for factor VIII (15). GEPCs were maintained as previously described (15). Experiments were performed with cells between passages 15 and 18.

Preparation of culture additives

Cells were serum-deprived to reduce background for 24 hr before each experiment, then exposed to glucose and/or AGE. Rat GEPCs were incubated in culture media containing either 5 mM glucose with 66 unit/mL insulin or 30 mM glucose (high glucose, HG) without insulin in order to mimic the metabolic environment of type I diabetes mellitus. Advanced glycosylation endproducts (AGE) was produced by the technique previously described by Ha *et al.* (15). To imitate the long-term diabetic condition, AGE was added (5 μ g/mL) and controls were established using unmodified bovine serum albumin (BSA) (5 μ g/mL). To exclude the effect of additionally produced glycated proteins in culture conditions, no longer than 48 hr of incubation was used. Fetal bovine serum (FBS) was reduced to 0.5% on the last media change to reduce background before protein and RNA extraction. For identification purposes, coating with AGE or BSA was denoted as 'A' or 'B', and addition of glucose at 5 or 30 mM by '5' or '30', respectively. Their meaning of each condition described before (15).

[³H]-proline incorporation

Collagen synthesis was assessed by measurement of cellular [³H]-proline uptake. For all experiments, [³H]-proline (PerkinElmer, Boston, MA, U.S.A.) was added to each well at a final concentration of 1 μ Ci/mL for the last 24 hr in trip-

licate. After incubation, the media was removed and the cells were washed with ice-cold 10% trichloroacetic acid (TCA) for 30 min at 4°C. After two rinses with cold 10% TCA, the acid-precipitable material was solubilized overnight in 1N NaOH at 37°C and neutralized with 1N HCl per well. Total protein was determined from an aliquot of each well by Bradford assay (Sigma Chemical Co., St. Louis, MO, U.S.A.). Incorporated radioactivity (dpm) from the resultant supernatant was measured in a liquid scintillation counter and was expressed as percent of control.

Cellular protein extraction

For measuring collagen, cell extracts were prepared from rat GEPCs grown for each duration period under different conditions as described before. At the end of incubation, the confluent grown cell layers were washed twice with phosphate-buffered saline and subsequently extracted in 4 M guanidinium-HCl, 2% CHAPS, and protease inhibitors containing 100 mM 6-aminohexanoic acid, 10 mM benzamidine HCl, and 1 mM phenylmethylsulfonyl fluoride (PMSF) at 4°C overnight and stored at -20°C till further analysis. Protein concentrations were determined with a Bio-Rad kit (Bio-Rad Laboratories, Hercules, CA, U.S.A.).

Western blotting of collagen α chains

Thirty μ g of boiled extracts was applied on 10% SDS-PAGE gels and transferred to polyvinylidene fluoride membranes (Bio-Rad Laboratories). Then, the membranes were air-dried and blocked in 3% fat-free milk before incubation with monoclonal goat anti-rat collagen α 1, α 3, and α 5 chains (Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.). After incubation with horseradish peroxidase-conjugated secondary antibodies, bands were detected by using the ECL chemiluminescence system (Amersham Biotech Ltd., Bucks, U.K.). Each band density was measured using densitometry program (LabWorks 4.0, UVP Inc., Upland, CA, U.S.A.) and controlled by β -actin amounts.

RT-PCR analysis of collagen α chains

Total RNA was extracted from cultured rat GEPC as previously described (15). After estimating its concentration by UV spectrophotometry, 5 μ g of total RNA was used for first-strand cDNA synthesis. Aliquots of the cDNA were amplified using primers for collagen α 1(IV): sense 5'-TCGGCTA-TTCCTTCGTGATG-3' and anti-sense 5'-TCTCGCTTC-TCTCTATGGTG-3'; α 3(IV): sense 5'-ATCCTGGTCTTC-CTGGACCT-3' and anti-sense 5'-CCATCTCACCAGGCAACCT-3'; collagen α 5(IV): sense 5'-ATGCTGACTAG-CAACCATGA-3' and anti-sense 5'-GCCAGATCACATC-TAAATGA-3'. The expression of GAPDH as RNA control was analyzed employing the following primers: sense 5'-CT-

CTACCCACGGCAAGTTCAA-3' and anti-sense 5'-GGA-TGACCTTGCCCACAGC-3'. PCR products were visualized on 2% agarose gels, and band density was measured using densitometry program as Western blotting.

Statistics

The data (cpm per mg cell protein) in each condition of incubation from at least three experiments were expressed as mean \pm SD if not remarked and compared by Student's t test; $P < 0.05$ was considered to be statistically significant.

RESULTS

Increased intensity of α (IV) chains in diabetic glomeruli

Double-staining of $\alpha 5$ (IV) with P-cadherin and $\alpha 3$ (IV) showed colocalization in podocyte foot processes around capillary loops in rat glomeruli (Fig. 1A). The observed tissues were obtained at 48 hr, 4 weeks and 10 weeks after the induction of diabetes, of them, urinary albumin excretion increased significantly only in the prolonged diabetic rat (24.5 mg/day at 10 weeks vs. less than 4 mg/day at 48 hr and 4 weeks). IF microscopy of kidney stained with anti- $\alpha 3$ (IV) and $\alpha 5$ (IV) antibodies showed that the increased and more intense stainings of glomerular capillary $\alpha 3$ (IV) and $\alpha 5$ (IV) chains were observed according to diabetic durations compared to controls (Fig. 1B). There were no glomerular stainings in primary antibodies-negative controls (figures not shown). We also found that there were glomerular hypertrophy and sclerosis in the advanced diabetic kidneys.

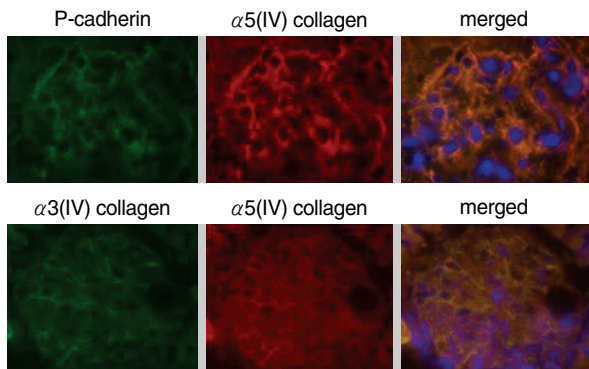


Fig. 1. Immunofluorescence staining of $\alpha 3$ (IV) and $\alpha 5$ (IV) in rat tissues. (A) Double-staining of $\alpha 5$ (IV) with P-cadherin and $\alpha 3$ (IV) showed colocalization in podocyte foot processes around capillary loops in rat glomeruli at 48 hr of diabetes (magnification $\times 1,000$). (B) Immunofluorescence microscopy of rat kidney tissue stained with anti- $\alpha 3$ (IV) and $\alpha 5$ (IV) antibodies at each experimental time (magnification $\times 400$). The further diabetic nephropathy advanced, the more intense stainings of $\alpha 3$ (IV) and $\alpha 5$ (IV) chains along the glomerular capillaries were observed compared with those of age-matched controls.

High glucose increases [3 H]-proline incorporation

Compared to incubation of GEpC with 5 mM glucose (B5), 30 mM glucose without insulin (B30), AGE with 5 mM glucose (A5), and AGE with 30 mM glucose (A30) for 48 hr resulted in 55.5%, 20.6, and 50.3% increase in [3 H]-proline incorporation, respectively (Fig. 2). Therefore, the increase in [3 H]-proline incorporation meaning collagen synthesis might be induced mainly by high glucose.

High glucose increases α chains of type IV collagen by western analysis

The protein amounts of novel glomerular collagen α chains proteins, $\alpha 3$ (IV) and $\alpha 5$ (IV) chains, were compared with that

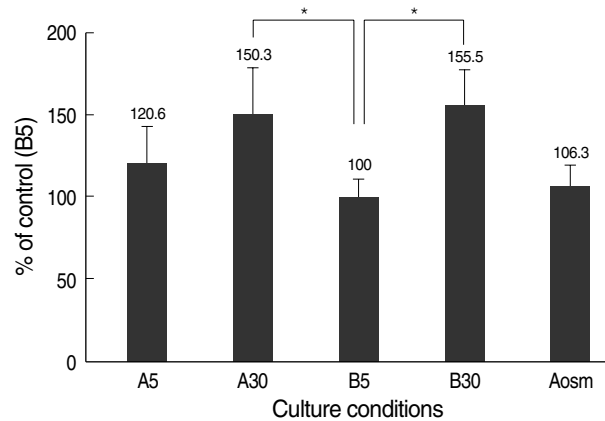
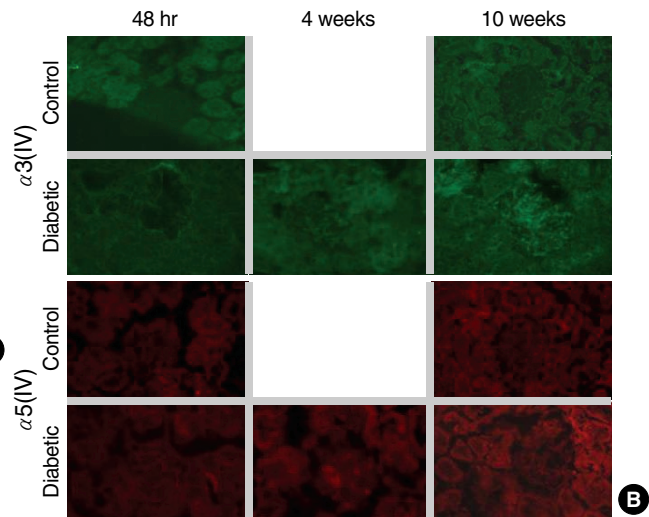


Fig. 2. [3 H]-Proline incorporation by high glucose and AGE. High glucose increased [3 H]-proline incorporation of GEpC significantly ($*P < 0.05$).



A

B

of condition B5 at 2, 8, 24, and 48 hr incubation time. Both $\alpha 3(\text{IV})$ and $\alpha 5(\text{IV})$ chains, were not changed initially, then, increased at 48 hr by high glucose (Fig. 3A, B). AGE did not showed any additive effect on the novel α chains proteins, although $\alpha 5(\text{IV})$ chain protein increased somewhat since 24 hr incubation. Then, we observed the changes of $\alpha 1(\text{IV})$ and $\alpha 5(\text{IV})$ chains proteins at 48 hr incubation.

Densitometric analysis of $\alpha 1(\text{IV})$ chain by Western blot-

ting revealed a 46% increase in high glucose condition (B30) and 50% increase in high glucose and AGE condition (A30) at 48 hr incubation compared to B5 ($n=3, P<0.05$, both, Fig. 3C). However, these changes were not found in the A5 and osmotic control (Aosm). $\alpha 5(\text{IV})$ chain also revealed a 66% increase in high glucose condition (B30) and 2.2 fold increase in high glucose and AGE condition (A30) compared to B5 ($n=3, P<0.05$, both, Fig. 3D). However, these changes were

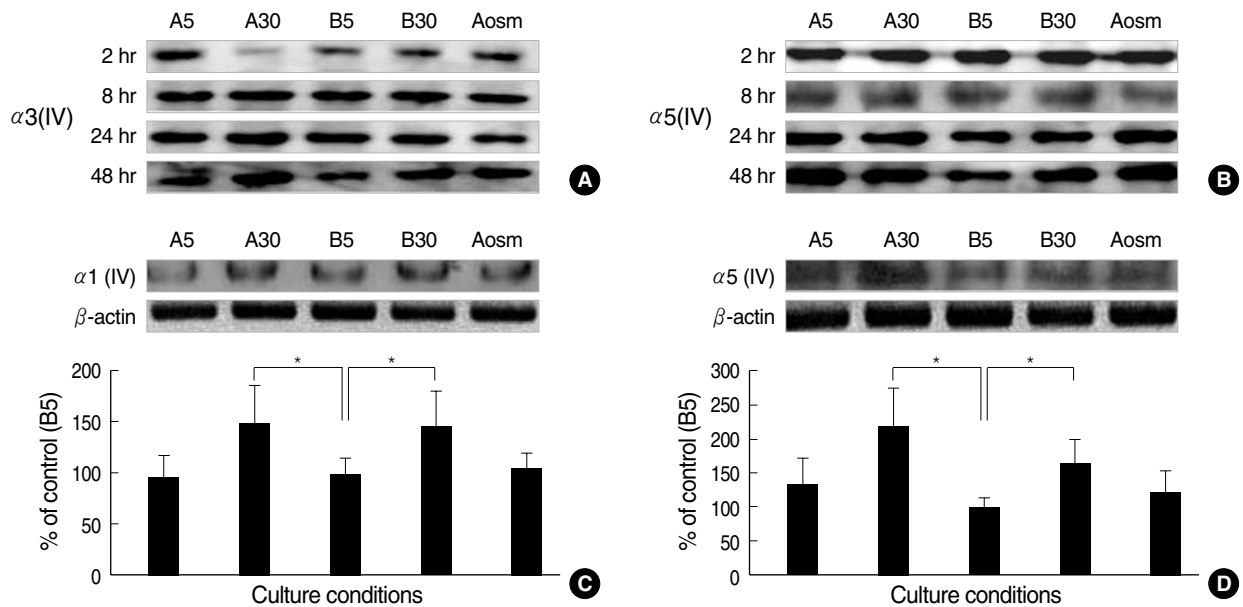


Fig. 3. Effects of glucose and AGE on the $\alpha(\text{IV})$ chains protein in cultured GEpC assayed by Western blotting. (A) Sequential changes of $\alpha 3(\text{IV})$ proteins, (B) Sequential changes of $\alpha 5(\text{IV})$ proteins ; Both $\alpha 3(\text{IV})$ and $\alpha 5(\text{IV})$ chains were not changed initially, then, increased at 48 hr by high glucose, (C) High glucose (B30 and A30) increased the amounts of $\alpha 1(\text{IV})$ proteins significantly compared with group B5, (D) High glucose (B30 and A30) increased the amounts of $\alpha 5(\text{IV})$ proteins significantly compared with group B5. Data on the densitometric analysis of $\alpha 1(\text{IV})$ and $\alpha 5(\text{IV})/\beta$ -actin ratio are expressed as mean \pm SD. Control (100%); the value of B5. * $P<0.05$ versus control.

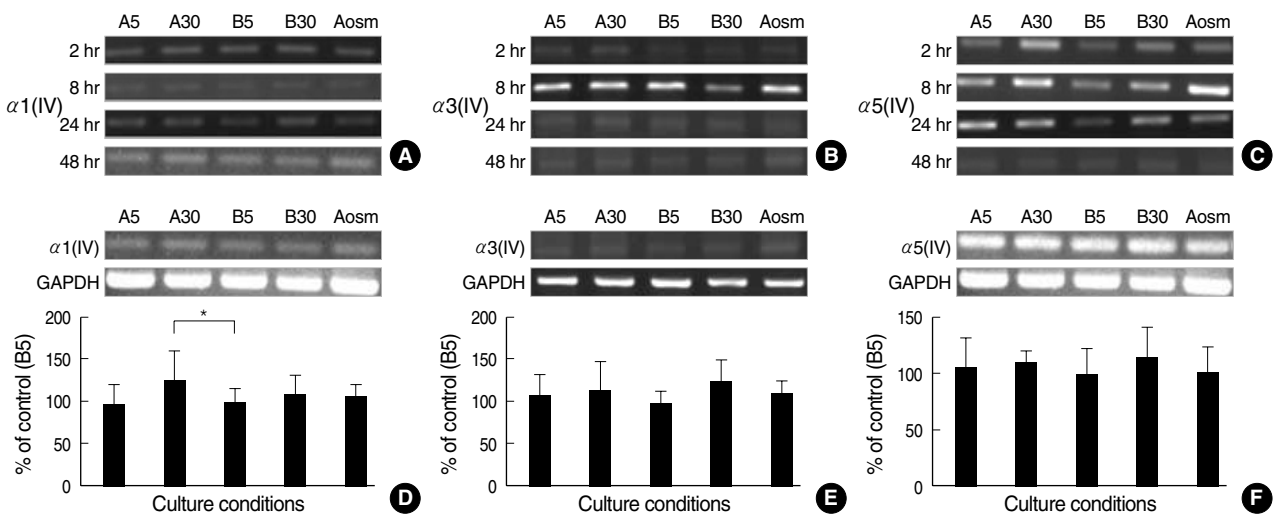


Fig. 4. Regulation of $\alpha(\text{IV})$ chains mRNA in cultured GEpC assayed by RT-PCR. Sequential changes of $\alpha 1(\text{IV})$ mRNA (A), $\alpha 3(\text{IV})$ mRNA (B), $\alpha 5(\text{IV})$ mRNA (C). (D) High glucose and AGE (A30) increased the amounts of $\alpha 1(\text{IV})$ mRNA significantly at 48 hr incubation compared with group B5. Change of $\alpha 3(\text{IV})$ mRNA (E) and $\alpha 5(\text{IV})$ mRNA (F) at 48 hr incubation. Data on the densitometric analysis of each $\alpha(\text{IV})/\text{GAPDH}$ ratio are expressed as mean \pm SD. Control (100%); the value of B5. * $P<0.05$.

not found in the A5 and osmotic control (Aosm) as the results of $\alpha 1$ (IV) chain. The increased amount of $\alpha 1$ and $\alpha 5$ chains protein in high glucose-added conditions (B30 and A30) was much more than that of A5 and Aosm, implying that high glucose might have more influence on the amount of $\alpha 1$ and $\alpha 5$ chains protein of GEpC than AGE and osmolality.

Gene expression of α chains of type IV collagen by high glucose and AGE

The expression levels of mRNA for $\alpha 1$ (IV), $\alpha 3$ (IV), and $\alpha 5$ (IV) in each condition were compared with that of condition B5 at 2, 8, 24, and 48 hr incubation time. GAPDH mRNA was not significantly different in all PCR products. According to incubation time, mRNA expression of each α chain showed the slightly increasing tendency in chain-different manners, however, their significances were rare (Fig. 4A-C). Then, we observed the changes of each chains expression at 48 hr incubation.

There were significantly increased $\alpha 1$ (IV) chain mRNA only in A30 condition (25%, compared with B5 ($P < 0.05$)), however, no significant changes in the other conditions (Fig. 4D). And, there are no significant changes of $\alpha 3$ (IV) and $\alpha 5$ (IV) chains mRNA in any conditions (Fig. 4E, F). Fig. 4C showed that the expression levels of mRNA for $\alpha 5$ (IV) in each condition increased compared with those of condition B5 at early stages, 2, 8, and 24, however, not at 48 hr incubation time in Fig. 4F.

DISCUSSION

Diabetic glomerulopathy is characterized by thickening of the GBM and expansion of the mesangial matrix (6, 16). An early feature of diabetic nephropathy is the alteration of the glomerular basement membrane (GBM), which may result in microalbuminuria, subsequent overt proteinuria, and eventual renal failure (1, 3). As for the GBM, its conspicuous thickening in diabetes, perhaps due to accumulation of collagen IV and alterations in its architecture and composition (6, 16, 17), would seem to constitute a more effective barrier to the filtration of proteins but is in fact more porous to proteins (18). The glomerular visceral epithelial cell, called podocyte is also affected by the early pathologic changes in diabetic glomerulopathy, including overproduction of cytokines such as VEGF and TGF β , widened foot processes width (hypertrophy), podocytopenia, and the dysregulation of GBM components, all of which contribute to the initiation of proteinuria and progression of renal disease (18-20). Because the onset and progression of diabetic glomerulosclerosis are delayed in patients who achieve an adequate level of glycemic control (21), hyperglycemia is thought to be an important regulator of glomerular lesion development.

GBM is composed of several macromolecules, including

type IV collagen, laminin, proteoglycan, and entactin (7-9). Type IV collagen, which includes six genetically distinct isoforms named $\alpha 1$ (IV) through $\alpha 6$ (IV) (9-11), is the most abundant constituent of the GBM (7-9, 12). These isoforms organize themselves into a unique network, which provides GBM structural stability and unique functionality (7, 12). Different isoforms of type IV collagen are specific for each glomerular compartment. The novel $\alpha 3$ (IV), $\alpha 4$ (IV), and $\alpha 5$ (IV) chains are predominantly associated with the GBM, while classical $\alpha 1$ (IV) and $\alpha 2$ (IV) chains are present in the mesangial matrix (9-13, 16).

At all developmental stages of kidney morphogenesis, $\alpha 1$ and $\alpha 2$ (IV) chains were seen in basement membranes. Collagen $\alpha 3$ -5(IV) chains, on the other hand, were not found in fetal nephron at the primary vesicle, S-shaped body and capillary loop stages but emerged during later development of the nephron. This is in line with earlier studies on rat tissues suggesting that in the rat, $\alpha 3$ -5(IV) chains are first expressed and start replacing $\alpha 1$ and $\alpha 2$ (IV) chains in glomerulus at the capillary loop stage (22). In the human kidney, however, this switch seems to appear at a distinctly later stage of development, reflecting species-specific differences in kidney development. Human kidney also shows expression of $\alpha 1$ and $\alpha 2$ (IV) chains in GBM, which is not seen in developing rat kidney (22, 23).

Dysregulation of GBM collagen chains produces proteinuria and hematuria genetically or acquired as seen in Alport's syndrome and Goodpasture syndrome, respectively (10, 11). However, the dysregulation of cellular metabolism of individual chains of type IV collagen has not been well studied in diabetic nephropathy.

In STZ-induced diabetic rats, the mRNA levels for the $\alpha 1$ (IV) collagen chain increased significantly with age even before morphological thickening of basement membrane occurred (24). Treating the diabetic rats with insulin for 4 weeks ameliorated the abnormally regulated ECM gene expression in the glomeruli (24). These data suggest that hyperglycemia may play a role in the abnormal ECM gene expression in vivo. The mRNA levels for the $\alpha 5$ (IV) collagen chain also increased significantly in STZ-induced diabetic rats at month 1 but not at month 4, whereas collagen $\alpha 5$ (IV) protein was higher at both 1 and 4 months (25). We also found that both novel collagen chains, $\alpha 3$ (IV) and $\alpha 5$ (IV) chains, increased in the GBM of experimental overt diabetic tissues as seen in Fig. 1. In the *db/db* mice, there was a significant reduction of $\alpha 3$ (IV) collagen protein in renal cortex but not of $\alpha 5$ (IV) collagen subchain in compared with the *db/m* controls (26). In human diabetic kidney tissue, there was intense staining for $\alpha 3$ (IV) NC and $\alpha 4$ (IV) NC domain in the GBM but not in the mesangial matrix of type I and type II diabetic patient tissue with overt diabetic nephropathy (27, 28). In contrast, staining with antibodies to $\alpha 1$ (IV) NC and $\alpha 2$ (IV) NC domain reacted with mesangial matrix but appeared to diminish significantly in the GBM in the patients with overt diabetic

nephropathy (27, 28).

In vitro study, Kang et al. (25) reported that collagen $\alpha 5$ (IV)/GAPDH mRNA ratio and protein of cultured mouse podocytes were higher in high glucose for 10 days incubation than in low (normal) glucose. These results are similar to our results on the change of $\alpha 5$ chain mRNA levels at early stages although not significant later, as cultured cells might not be identical. On the other hand, Bai et al. (29) demonstrated using cultured mouse podocytes that high glucose decreased the protein levels of $\alpha 5$ (IV) chain significantly during 3–4 days incubation, whereas, increased MMP-9 activity and mRNA levels, suggesting that the change of GBM collagen would be caused by an imbalance in ECM synthesis and degradation. We also found that high glucose induced the increased amount of $\alpha 1$ (IV), $\alpha 3$ (IV), and $\alpha 5$ (IV) chains protein at late stage. On the other hand, high glucose induced the mRNA expression of $\alpha 5$ (IV) and partly $\alpha 1$ (IV) at early stage but not at late stage. The incoordination between α (IV) chains protein and their mRNA expression at each stage suggest that the increased amount of $\alpha 5$ (IV) chains protein in high glucose-added conditions may be induced by transcriptional metabolic imbalance at early stage but much more by post-translational modification including structural changes by glycosylation mostly at late stage. On the other hand, the other α (IV) chains might be regulated much more by post-translational modification including structural changes by glycosylation and/or metabolic imbalance in time-dependent and isoform-specific manners. Similarly, AGE also induced the increased amount of $\alpha 1$ (IV), $\alpha 3$ (IV), and $\alpha 5$ (IV) chains protein at late stage and the mRNA expression of $\alpha 5$ (IV) at early stage and $\alpha 1$ (IV) at late stage. These changes induced by AGE also suggest that α (IV) chains might be regulated not only by transcriptional level but also by post-translational modification including structural changes by glycosylation and/or metabolic imbalance in time-dependent and subchain-specific manners. Additive effects by AGE were not evident.

Although the mechanisms of increased each α chains of type IV collagen would not be clearly elucidated because of their different expressions, many researchers suggested the roles of angiotensin II (30), TGF- β , PKC-p38 MAPK signaling (25), insulin (24), decreased matrix degradation (28), etc. as the initiator and modulator of type IV collagen dysregulation in diabetic environments. According to the our present and previous studies, diabetic conditions induced the dysregulation of glomerular α (IV) chains by transcriptional and post-translational modification with a discrepancy in the species-, strains-, time-, cell-, and/or subchain-dependent manners. The mechanisms of the changes of type IV collagen α chains would be further elucidated for the regulation of type IV collagen biosynthesis.

In conclusion, we suggest that the metabolism of individual α (IV) chains of GBM is differentially regulated in diabetic conditions and the change of each α chains would be induced by transcriptional level and post-translational modifi-

cation including structural changes by glycosylation and/or metabolic imbalance in time-dependent and isoform-specific manners.

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