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IN SITU LOCALIZATION OF TYPE III AND TYPE IV COLLAGEN-EXPRESSING CELLS IN HUMAN DIABETIC NEPHROPATHY

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SUMMARY

Nodular intercapillary glomerulosclerosis is the most typical lesion of diabetic nephropathy (DN) and is characterized by increased extracellular matrix (ECM) and amorphous masses of mesangial matrix. The local exaggeration of these deposits results in the formation of the typical diabetic nodule. To clarify the composition of the ECM of sclerotic lesions in DN, we investigated the distribution of type III and type IV collagens and their mRNAs by immunohistochemistry and *in situ* hybridization, respectively. In normal renal tissues, there was no intraglomerular immunostaining for type III collagen, while strongly positive staining was found in the extraglomerular interstitium. Positive immunostaining for type IV collagen was also present in the mesangium, glomerular basement membrane (GBM), Bowman's capsule, and the vascular pole of the normal glomerulus. In DN, the nodular lesions were negative for type III collagen and strongly positive for type IV collagen. On the other hand, in the late stage of global sclerosis, both type III and type IV collagens were diffusely present in the sclerotic matrix. To determine the origins of these type III and type IV collagens in the sclerotic matrix, *in situ* hybridization was performed, utilizing thymine–thymine (T–T) dimerized synthetic oligonucleotides complementary to either pro α1(III) chain or pro α1(IV) chain mRNAs as probes. The signals were detected by enzyme immunohistochemistry using an anti-T–T antibody. Intraglomerular cells (glomerular epithelial and mesangial cells) containing type III collagen mRNA were found in DN with sclerotic lesions, but not in normal glomeruli. At this stage of sclerosis, intraglomerular cells (mainly glomerular epithelial cells and infrequently mesangial cells) were positive for type IV collagen mRNA, but there were few positive cells in globally sclerotic glomeruli. This study provides evidence that both type III and type IV collagens are synthesized by intraglomerular cells during sclerosis and become significant constituents of the sclerotic matrix in DN.

KEY WORDS—Diabetic nephropathy, type III collagen, type IV collagen, immunohistochemistry, non-radioactive *in situ* hybridization.

INTRODUCTION

The pathogenetic mechanisms responsible for the initiation and progression of diabetic nephropathy (DN) are not clearly understood, although about 40 per cent of patients with diabetes mellitus (DM) develop nephropathy.[1] The disease is hallmarked by progressive thickening of the glomerular basement membrane (GBM) and a disproportionate increase in mesangial volume. Morphometrically, the matrix increase is about three times that of mesangial cells in human DN.[2] Falk *et al.*[3] demonstrated increased immunostaining for type IV collagen, laminin, and fibronectin, all of which are normal constituents of the glomerulus. In a separate study, Kim *et al.*[4] described increased α1 and α2 chains of type IV collagen in the mesangial matrix in the early to middle stages of human DN, while at the end stage, these chains were present in diminished amounts. By competitive polymerase chain reaction, Peten *et al.* also reported an elevation

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of mRNA for type α2 IV collagen in human glomerulosclerosis.[5]

It appears to be, however, that not all components of the extracellular matrix (ECM) are equally increased. The mRNA for the B1 chain of laminin and the heparan sulphate proteoglycans (HSPs) in the capillary basement membrane were unchanged, even though type IV collagen was increased.[6] The immunochemically detectable amounts of type IV collagen increased, although the amount of laminin and HSPs present in the GBM of DM was reduced, by 40 per cent for laminin, compared with that found in normal GBM.[7] The uneven composition of the GBM in DN is thought to be a result of hyperglycaemia, which greatly accelerates the non-enzymatic glycosylation of GBM collagen[8] and alters the cross-linking with other glycosylated GBM constituents, such as laminin and HSPs. The alteration leads to degradation of both laminin and HSPs.[9] In addition to the changes in normal constituents, additional ECM components may appear in diabetic glomerular lesions, focal glomerulosclerosis, and several other types of human glomerulonephritis; Glick *et al.*, using immunological and ultrastructural methods, demonstrated type I collagen in the mesangial sclerotic lesions in human DN and focal glomerulosclerosis.[10] On the other hand, in several other types of glomerular disease, type III collagen appeared as an additional component of the ECM in the glomeruli.[11–14] In a separate study, we have also demonstrated *de novo* intraglomerular synthesis of type III collagen in human benign nephrosclerosis.[15]

As DN is characterized by progressive expansion of the mesangial matrix and a thickened GBM, we set out to determine whether the ECM in sclerotic glomeruli in DM also accumulates some extraglomerular connective tissue elements, such as type III collagen. In this study, we have investigated the expression of both intraglomerular collagenous components (type IV collagen) and extraglomerular collagenous components (type III collagen) in the process of sclerosis in DM, using immunohistochemistry and non-radioactive *in situ* hybridization.

MATERIALS AND METHODS

Kidney tissues

Renal biopsies from ten patients with the typical histological changes of DN as described in the WHO classification of glomerular diseases[16] and ten samples of renal tissue without any noticeable abnormality were used in this study. Portions of the tissues were processed for routine light and electron microscopic examination. For light microscopy, the tissues were fixed in 10 per cent formalin and embedded in paraffin; for electron microscopy, the tissues were fixed in 2·5 per cent glutaraldehyde, osmicated, and embedded in Epon.

Immunohistochemistry

Paraffin sections (4 μm) were deparaffinized and endogenous peroxidase activity was denatured with 0·3 per cent hydrogen peroxide in methanol. After mild treatment with trypsin, the sections were reacted with non-immune rabbit serum to block non-specific binding of monoclonal antibodies. As immunohistochemical controls, the monoclonal antibodies were replaced by normal mouse serum diluted with phosphate-buffered saline (PBS) in the same proportion as that used for the experiment. Monoclonal antibodies against type III collagen and type IV collagen were purchased from Fuji Chemical and Dako, and were diluted to 1:25 (for type III collagen) and 1:75 (for type IV collagen) with PBS (pH 7·2). The sections were reacted with the primary antibodies and processed further using ABC kits (Vector Laboratories, Burlingame, CA). The staining intensity of collagen type III and collagen type IV was graded semi-quantitatively according to the following scale: 0 = no staining; + = focal staining; + + = weak staining; + + + = strong staining.

In situ *hybridization*

Oligo-DNA probes used—A 45-base sequence complementary to the mRNA of human pro α1(III) chain (4081–4126)[17] and a 45-base sequence complementary to the mRNA of human pro α1(IV) chain (1008–1053)[18] were chosen. A computer-assisted search (GenBank) of the above antisense sequences, as well as the sense sequences, revealed no significant homology with any known sequences other than those of pro α1(III) and pro α1(IV) chains. These antisense and sense sequences were used for synthetic oligo-DNA probes but for haptenization of the oligo-DNAs with T–T dimers, two and three TTA repeats of the 5′- and 3′-ends of the sequences

were added, respectively,[19,20] at the time of synthesis. The oligo-DNAs were synthesized by an automatic Applied Biosystem DNA synthesizer (model 391 PCR-MATE, Foster City, CA) and purified on an OPC column and a Nap 10 column (Pharmacia). T–T dimer was introduced into the oligo-DNAs by UV irradiation with a dose of 10 000 J/m^2 and the introduction was verified immunochemically using mouse monoclonal anti-T–T IgG (Kyowa Medics, Japan); the hybridizabilities of the T–T dimerized oligo-DNA probes were confirmed by dot blot hybridization as described previously.[19,20]

In situ *hybridization procedure—In situ* hybridization was carried out as previously described.[19,20] All the *in situ* hybridization experiments were done in duplicate slides. Briefly, deparaffinized sections (4 μm) were treated successively with 0·3 per cent H_2O_2, 0·2 M HCl, 0·2 per cent Triton X-100 and proteinase K. The sections were post-fixed with 4 per cent paraformaldehyde (PFA), immersed in 2 mg/ml glycine, and kept in 40 per cent deionized formamide in 4 × SSC until used.

Hybridization was performed overnight at 37°C with 2 μg/ml T–T dimerized antisense oligo-DNA probes in the hybridization solution[19,20] with 10 per cent dextran sulphate and 40 per cent deionized formamide. Then the slides were washed in 50 per cent formamide in 2 × SSC, 2 × SSC, and PBS. Immunohistochemistry was performed on the sections as previously described.[19,20] The sites of peroxidase were visualized using a solution containing 3,3′-diaminobenzidine·4 HCl (DAB), H_2O_2, $CoCl_2$, and $NiSO_4(NH_4)_2SO_4$;[21] one of the duplicate slides was counterstained by periodic-acid Schiff (PAS). Adjacent sections were reacted either with the hybridization solution containing T–T dimerized sense oligo-DNA probes or the solution without probes as the negative controls. Sections consecutive to these sections were also hybridized with T–T dimerized oligo-DNA complementary to 28S rRNA, to demonstrate the degree of RNA retention and the accessibility of the probes to cytoplasmic RNA. To establish the specificity of the hybridization, some consecutive sections were pretreated with RNase or hybridized with a solution containing a 50-fold excess of non-haptenized antisense oligo-DNA probes, in addition to the T–T dimerized antisense oligo-DNA probes.

RESULTS

Immunohistochemical localization of type III collagen and type IV collagen in normal kidney

No intraglomerular staining for type III collagen was found, while strongly positive (++ to +++) staining was noted in the extraglomerular interstitium of the control cases (Fig. 1A). As for the type IV collagen, the mesangium (+ to ++), GBM (+), Bowman's capsule (+), and vascular pole (+) were positively stained in the normal glomeruli (Fig. 1B).

Immunohistochemical localization of type III collagen and type IV collagen in DN

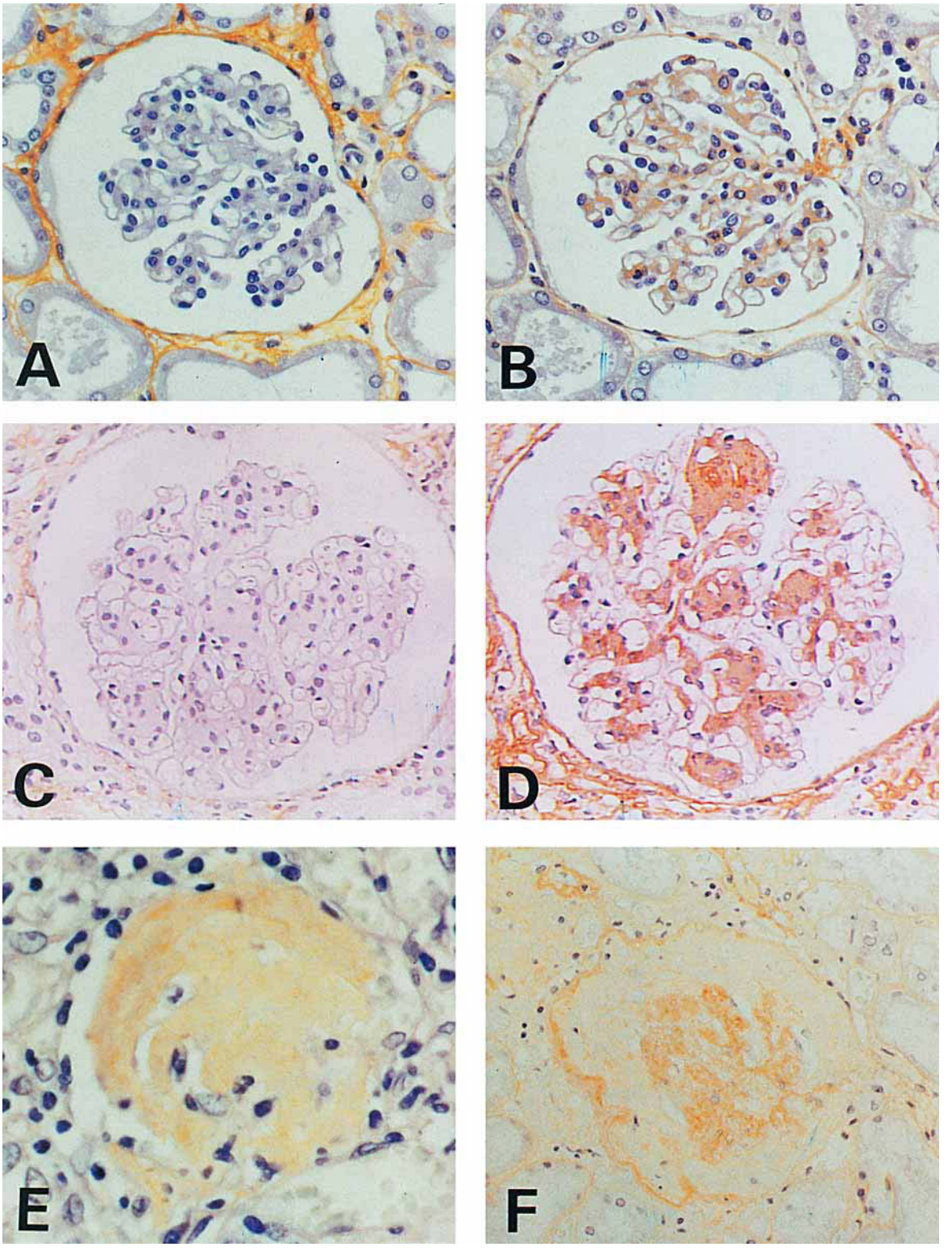
In nodular lesions of DN, collagen type IV was recorded as ++ to +++ in the sclerotic matrix (Fig. 1D), but no intraglomerular staining for type III collagen was found in the same nodular lesions (Fig. 1C). In the late stage of glomerulosclerosis, i.e., in the hyalinized acellular glomerulus, staining for type III collagen (+) and type IV collagen (+ to ++) was diffusely present in the sclerotic matrix (Figs 1E and 1F).

Detection of T–T dimerized oligo-DNAs blotted or hybridized on nitrocellulose filter by immunochemistry

At least 10 pg of all probes dotted on a nitrocellulose filter was detectable with the immunochemical procedures. When T–T dimerized antisense pro α1(III) oligo-DNA was hybridized with unhaptenized sense pro α1(III) oligo-DNA on a nitrocellulose filter, at least 10 pg of the sense oligo-DNA was detected; for T–T dimerized antisense pro α1(IV) oligo-DNA at least 1 pg of unhaptenized sense pro α1(IV) oligo-DNA on a nitrocellulose filter was detected. There was, however, no detectable hybridization signal between T–T dimerized antisense pro α1(III) oligo-DNA and unhaptenized sense pro α1(IV) oligo-DNA on a nitrocellulose filter, or between T–T dimerized antisense pro α1(IV) oligo-DNA and unhaptenized sense pro α1(III) oligo-DNA on a nitrocellulose filter. These results indicate that each antisense probe recognizes its respective sense DNA and that there is no cross-hybridization between them.

In situ *hybridization to localize type III and type IV collagen mRNAs*

In order to identify the cells synthesizing type III and type IV collagens, sections of the normal and


A
B
C
D
E
F

DN kidneys were hybridized *in situ* with T–T dimerized antisense oligo-DNA probes complementary to pro α1(III) or pro α1(IV) mRNA. No cells in the normal glomerulus hybridized with T–T dimerized antisense pro α1(III) oligo-DNA consistent with the immunohistochemical observations, whereas glomerular epithelial cells and occasional mesangial cells hybridized with T–T dimerized antisense pro α1(IV) oligo-DNA in normal kidney. These results indicate that in normal glomeruli, mRNA for type IV collagen is expressed but not for type III collagen. In diabetic glomeruli, proportionally more glomerular cells (mainly glomerular epithelial cells and infrequently mesangial cells) hybridized with T–T dimerized antisense pro α1(IV) oligo-DNA (Figs 2E and 2F). As expected from the results of immunohistochemistry, T–T dimerized antisense pro α1(III) oligo-DNA hybridized with the intraglomerular cells (both glomerular epithelial and mesangial cells) in diabetic glomeruli (Figs 2B and 2C). In the diabetic glomerulus, hybridization signals for type III and type IV collagen mRNAs were detected in both glomerular epithelial and mesangial cells, but no signal was detected with the sense probes (Figs 2A and 2D).

DISCUSSION

In this study, the synthesis of type III and type IV collagens by intraglomerular cells was examined by immunohistochemistry and non-radioactive *in situ* hybridization. The results demonstrate increased synthesis of type IV collagen and *de novo* synthesis of type III collagen, as an additional constituent of the ECM, by both glomerular epithelial and mesangial cells during the progression of sclerosis in DN.

It is hard to analyse the mRNA-positive cell types of the glomeruli in the hybridized sections. To define the location of the cells, the renal sections were counterstained by PAS after *in situ* hybridization. Occasionally it was difficult to recognize mRNA-positive cells when they were in the midst of PAS-positive mesangial and sclerotic matrices. In such instances, mRNA expression patterns were compared in serial sections with and without the PAS counterstain, to identify mRNA-positive mesangial cells. Both the glomerular epithelial cells and the mesangial cells were found to contain mRNAs for both type III and type IV collagens in DN.

Increased type IV mRNA positive cells and their protein in diabetic glomeruli reflected the increased synthesis of type IV collagen in DN. Our results correlate with the findings of Peten *et al.*,[5] who reported elevated levels of α2(IV) collagen mRNA in human glomerulosclerosis. Similarly, Oomura *et al.*[14] demonstrated increased immunostaining for various types of collagen, including type III and type IV collagen, in human glomerular diseases.

Unlike the mRNA for type IV collagen, type III collagen exhibited a different pattern. We could not detect type III collagen mRNA or protein in the normal glomerulus. Our observation is similar to that of Prigent *et al.*,[22] who also could not detect mRNA signals for type I and type III collagens in the normal human glomerulus. However, the appearance of pro α1(III) mRNA-positive cells, with accumulation of type III collagen protein in the diabetic sclerotic matrix, demonstrated that interstitial type III collagen is also synthesized by the intraglomerular cells in DN. The accumulation of type III collagen protein in the glomeruli of various human renal diseases has already been reported by several investigators, including our own group.[11–14]

Infiltration of interstitial type III collagen through the damaged Bowman's capsule has been postulated to explain the presence of type III collagen in sclerotic ECM in several types of glomerulonephritis. Recent studies, however, have demonstrated the presence of type I and type III mRNA-positive cells in human hypertensive and transplanted glomeruli,[15,22] when there was no apparent disruption of Bowman's capsule or pericapsular fibrosis. In this study, we also observed the appearance of pro α1(III) mRNA-positive cells in diabetic glomeruli with an intact Bowman's capsule, suggesting that intraglomerular synthesis of type III collagen does occur.

It is not clearly understood what initiates the *de novo* synthesis of type III collagen and the increased synthesis of type IV collagen in diabetic glomeruli. Human studies on this subject are

Fig. 1—Immunohistochemical staining of type III (A) and type IV (B) collagens in normal glomerulus. In diabetic nodular lesions, there was no immunostaining for type III collagen (C), while intense staining for type IV collagen (D) was found. In the globally sclerotic glomerulus of DN, type III (E) and type IV (F) collagens were diffusely distributed. (Immunoperoxidase technique)

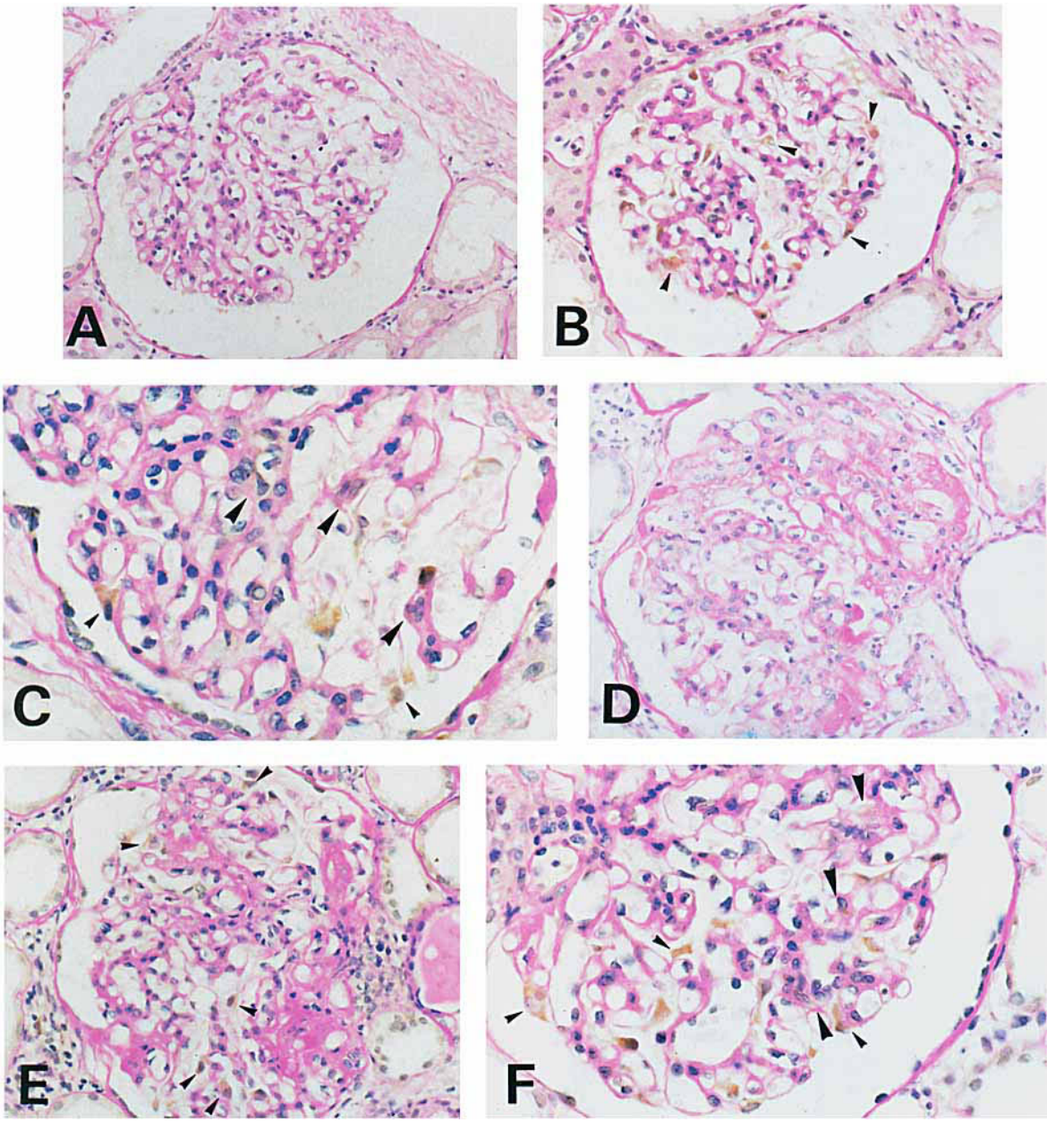


Fig. 2—In situ hybridization using type III collagen sense (A), type III collagen antisense (B, C), type IV collagen sense (D), and type IV collagen antisense (E, F) oligo-DNA probes, showing positive epithelial cells (small arrow-head) in the glomerulus of DN. High power view of the glomerulus (C and F) reveals mRNA positive mesangial cells, within the PAS-positive mesangial matrix (large arrow-head). (PAS counterstain)

sparse. From experimental studies, however, there are indications that some glomerular cells in altered environments can produce both types of collagen. Rat mesangial cells, when placed in an *in vitro* environment, produce interstitial collagens, including type III collagen.[23] Rat glomerular epithelial cells also produce increased matrix components, such as type IV and fibronectin, following

exposure to TGF-β1.[24] An elevated glucose environment is also known to influence the composition of the ECM.[25] Hyperglycaemia elevates the levels of glomerular mRNAs, including that encoding type IV collagen,[6] TGF-β,[26] and TIMP 2 mRNA,[27] in experimental diabetic kidneys. Possibly through the elevation of mRNAs, mesangial cells grown in a high glucose medium accumulate more fibronectin, laminin, and type IV collagen than cells grown in a low glucose medium.[28] All these observations, together with the current results, suggest that the hyperglycaemic environment of DM induced the synthesis of type III collagen as well as increasing the synthesis of type IV collagen and subsequently contributed to the sclerosis in DN.

To localize the specific mRNA sequence at the individual cell level, we found that non-radioactive *in situ* hybridization utilizing T–T dimerized synthetic oligo-DNA as a probe on renal biopsy material was a reliable and sensitive technique. As histological examination of the renal biopsy is already the most common diagnostic method, the use of *in situ* hybridization on renal biopsies should be able to provide valuable information at the molecular level in a variety of pathological conditions.

In conclusion, the cells in diabetic glomeruli synthesize both type III and type IV collagens. Overproduction of these collagens contributes significantly to the sclerotic process in DN.

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