Increased Expression of Basement Membrane Collagen in Human Diabetic Retinopathy

Sayon Roy, Michele Maiello, and Mara Lorenzi
Schepens Eye Research Institute and Department of Ophthalmology, Harvard Medical School, Boston, Massachusetts 02114

Abstract

Basement membrane thickening is the most prominent and characteristic feature of early diabetic microangiopathy. Unknown is not only the causative process but also whether the thickening reflects increased synthesis of specific components. Because collagen type IV is uniquely present in basement membranes and represents their predominant structural element, we studied its expression in retinas obtained postmortem from five patients with 8±3 yr of diabetes and six nondiabetic controls. The collagen IV transcript proved to be rare in adult human retina and undetectable by Northern analysis. We thus identified a set of primers and conditions to detect the transcript by the reverse transcriptase polymerase chain reaction and to measure its level relative to an endogenous internal standard (β-actin mRNA). In the diabetic patients the levels of collagen IV mRNA were increased twofold over levels in controls, whereas the actin mRNA levels were similar in the two groups. Hence, the collagen IV/actin ratio was 0.53±0.15 in diabetic samples and 0.24±0.09 in control samples ($P = 0.004$). These results indicate that diabetes induces a twofold increase in the expression of collagen IV by the cells that synthesize basement membranes in the adult retina (vascular cells). Insofar as high ambient glucose in vitro elicits the same effect, it may be proposed that basement membrane thickening in diabetes results from enhanced synthesis of specialized component molecules sustained by hyperglycemia. (*J. Clin. Invest.* 1994. 93:438–442). Key words: basement membrane thickening • diabetic microangiopathy • type IV collagen • polymerase chain reaction

Introduction

Basement membranes are thin extracellular matrices that separate epithelial, endothelial, and parenchymal cells from the underlying connective tissue. Orderly assembly of their unique structural components (type IV collagen, laminin, entactin, and heparan sulfate proteoglycans) provides physical support, a substrate for adhesion and phenotypic modulation of overlying cells, and a selective barrier to filtration. Changes in basement membrane morphology and properties have been reported in several pathologies (1), but in none are the changes so widespread as in diabetes mellitus, where basement membrane thickening is found throughout the vasculature as well as at nonvascular sites (2). The importance of thickened basement membranes in diabetic vascular disease is not clear. However, because they develop within a process that eventually leads to the demise of vascular cells and capillary closure, which in turn trigger proliferative retinopathy (3) and renal failure (4), understanding their pathogenesis should contribute to the broader goal of understanding diabetic microangiopathy.

The development of both thickened basement membranes and microangiopathy requires the presence of the metabolic derangement of diabetes (5, 6), and hyperhexosemia appears to be the abnormality sufficient to cause the lesions (7). The latter observation, coupled with the finding that elevated glucose concentrations in vitro upregulate endothelial cell expression of collagen IV, laminin, and fibronectin (8), proposes a mechanism for basement membrane thickening in diabetes based on hyperglycemia-induced increased synthesis of specific matrix components. But increased synthesis of basement membranes proper has never been clearly documented in diabetic tissues in vivo, and, in fact, it has been suggested that thickening may reflect deposit of basement membrane material by successive generations of cells with shortened life span (9) or accumulation due to decreased susceptibility to degradation (10). In experimentally diabetic animals studies of retinal RNA by Northern analysis have failed to detect increased levels of collagen IV or laminin mRNAs (11), and the renal glomerular preparations showing increased incorporation of collagen precursors (12) could not resolve if the event pertained to synthesis of basement membranes or mesangial matrix. The distinction is important because mesangial matrix differs from vascular basement membranes in several respects, most notably cellular origin and representation of type V collagen (13).

We thus examined if the synthesis of type IV collagen, which is uniquely present in basement membranes and represents their predominant structural component (14), is increased in human diabetes. We performed the experiments in human retinas because thickening of basement membranes is well documented in diabetic retinal vessels (2, 15) and intact RNA can be extracted from these vessels up to 36 h postmortem (16). Having determined that Northern analysis is insufficiently sensitive for detection of the collagen IV transcript, which is rare in the adult retina (17, 18), we designed primers and conditions to detect the transcript by the reverse transcriptase PCR.

Address correspondence to Dr. Mara Lorenzi, Schepens Eye Research Institute and Department of Ophthalmology, Harvard Medical School, 20 Staniford Street, Boston, MA 02114.

Received for publication 29 June 1993 and in revised form 28 September 1993.

J. Clin. Invest.

0021-9738/94/01/0438/05 $2.00
Volume 93, January 1994, 438–442

Methods

Subjects and specimens. Human eyes were provided by certified eye banks and the National Disease Research Interchange; the donors remained anonymous. Criteria for inclusion in the study were < 75 yr old, duration of diabetes < 15 yr in order to address mostly background retinopathy, and the fewest possible chronic pathologies other than diabetes. Criteria for exclusion were the presence of hematologic diseases or uremia, and administration of chemotherapy or life support measures. The clinical characteristics of the five diabetic and six nondiabetic retina donors are reported in Table I.

The eyes were maintained at 4°C and processed within 36 h of death. Under sterile RNase-free conditions, the globe was punctured 2 mm from the limbus, a circumferential incision was made, and the anterior chamber was removed. A vitreous spatula was used to loosen the vitreous adherent to the anterior retina, the retina was cut at the optic nerve and gently separated from the layer of pigmented epithelium. The two retinas were homogenized with five up-and-down strokes of a hand-held glass homogenizer in 3 ml of 6 M guanidine isothiocyanate. Total RNA was isolated by sedimentation through cesium chloride (19) and each sample (5 μg) was subjected to electrophoresis and stained with acridine orange (20) to document absence of degradation.

Reverse transcriptase (RT)[1]-PCR. In each experiment the RNA from a diabetic retina was processed in parallel with the RNA from a control retina. The RNA was reverse transcribed with the Superscript RT (GIBCO BRL, Gaithersburg, MD), which lacks RNase H activity and thus maximizes the yield of full-length cDNAs. The reaction was performed in a 40-μl volume, with 2 μg RNA, 400 U of enzyme, 2.5 μM random hexamers, 1 mM of each dNTP, 5 mM $MgCl_2$, PCR buffer, and RNase inhibitor for 10 min at room temperature followed by 40 min at 42°C. At the end of reverse transcription, samples were boiled for 5 min, cooled on ice, and treated with *Escherichia coli* RNase H (2 U) for 20 min to remove RNA from the hybrid molecules.

The protocol for the PCR was designed to measure the level of collagen IV expression relative to the expression of an endogenous internal standard gene (β-actin) (21, 22). To prevent quantitative inaccuracies deriving from competitive effects and different efficiency and ranges of amplification of the two cDNAs (21, 22), the collagen IV and β-actin cDNAs generated in the same RT reaction were amplified in separate tubes containing increasing volumes of the RT reaction (1, 2, 4, and 8 μl) to document amplification in the linear region for each cDNA. The primers used to amplify the α1 chain of collagen IV and β-actin (Table II) were designed from the known respective cDNA sequences (23, 24) and bridged introns to identify amplification of contaminating genomic DNA. The specificity and yield of the PCR were enhanced by using the "hot start" approach (25). The PCR containing the appropriate aliquot of RT material adjusted to 20 μl, primers (0.2 μM each), AmpliTaq DNA polymerase (2.5 U; Perkin Elmer Cetus, Norwalk, CT), $MgCl_2$ (2 mM), and PCR buffer in a 100-μl volume was performed in a DNA thermal cycler (Perkin-Elmer Cetus) using the following cycle conditions: denaturation for 1 min at 95°C for both collagen IV and actin, annealing for 1 min at 54°C for collagen IV and for 1 min at 50°C for actin, and extension for 2 min at 72°C for both collagen IV and actin. There were 34 cycles for collagen IV and 28 for actin.

Analysis and quantitation of PCR products. PCR products from diabetic and control pairs were always resolved on the same gel (1.2% agarose containing 0.1 μg/ml ethidium bromide) together with molecular weight markers (100-bp DNA ladder; GIBCO BRL). The gel was photographed with Positive/Negative Instant Film (665; Polaroid) and transferred onto nylon membrane for Southern analysis (8). Hybridization was to the HT-21 (23) and β-actin (24) cDNAs.

There was excellent correlation ($r = 0.99$) between the densitometric readings of signals on Polaroid negatives and autoradiography films. Hence, the former were used throughout the study to minimize variations in transfer and hybridization efficiency. The densitometric values for the amount of PCR products generated by the increasing volumes of RT reaction were averaged to yield the collagen IV and actin signals for each sample (densitometric units/μl RT reaction). The data are summarized with the mean±SD. The values obtained in diabetic and control samples were compared with the Student's t test.

1. *Abbreviation used in this paper:* RT, reverse transcriptase.

Results

Collagen IV mRNA, which could not be detected in adult human retina by Northern analysis, was identified by RT-PCR. The expected 436-bp PCR product was present on agarose gel and yielded a unique band in Southern blot (Fig. 1). The reproducibility of the collagen IV and actin signals obtained with RT-PCR was assessed by studying two diabetic and two control samples in two different experiments. The interassay coefficient of variation was 6±3%. The accuracy of the quantitative information obtained with RT-PCR was verified by comparing this method with Northern analysis in the quantitation of the effects of high glucose on collagen IV expression in human umbilical vein endothelial cells. In four experiments the results obtained with the two methods showed a correlation of 0.90.

We compared collagen IV and actin expression in diabetic and control retinas. Fig. 2 shows the band pattern in agarose gel for the collagen IV and actin PCR products from a diabetic and control RNA studied in the same experiment (diabetes mellitus subject 2 and control subject 2 in Tables I and III). Band intensity increased linearly with the amount of RT reaction subjected to amplification; whereas the actin signal was comparable in the diabetic and control sample, the collagen IV signal was clearly more prominent in the diabetic sample. The quantitation of results for all RNA studied is presented in Table III. Because the actin signal was similar in the diabetic and control group, actin could be used appropriately as an internal reference providing for each sample a control for the yield and reverse transcription of RNA. The level of collagen IV gene expression could thus be determined relative to the expression of the actin gene, and the collagen IV/actin ratios observed at the different amounts of cDNA subjected to amplification are presented in Fig. 3. At each point the ratio in diabetic samples was greater ($P < 0.05$) than in control samples; the mean collagen IV/actin ratio was 0.53±0.15 in diabetic samples and 0.24±0.09 in control samples ($P = 0.004$). Actin mRNA was more abundant than the collagen IV mRNA in all samples despite the relative underestimation due to a lesser number of amplification cycles, and in accordance with the detectability of the retinal actin transcript in Northern analysis (16).

Discussion

We have proposed that understanding the lesions of diabetic angiopathy and their pathogenesis will require knowing whether and how the cell types involved modify their differentiated functions (26). This study indicates that cells that synthesize basement membranes in the retina increase by twofold their collagen IV mRNA levels in diabetes.

The results were obtained with RT-PCR, a technique whose quantitative capabilities have limitations based on the lack of an optimal strategy for controlling internally all steps of the procedure (22). To rely on an internal standard reporting on possible losses during RNA processing and different efficiencies of transcription, we normalized collagen IV gene ex-

Table I. Clinical Characteristics of Study Subjects

Subjects	Sex	Age	DM duration	DM therapy	Cause of death	Other known pathologies
		yr	*yr*			
DM						
1	F	65	10	Insulin	Uterine Ca	None
2	M	71	10	Insulin	Colon Ca	None
3	F	56	10	Insulin	MI	HTN, CVA
4	M	73	10	Insulin	CVA	CAD
5	M	67	2	SU	MI	CAD
Mean		66	8			
SD		6	3			
Control						
1	F	55			CAD	None
2	F	57			CVA	Cardiomyopathy
3	F	52			Gallbladder Ca	None
4	M	57			MI	None
5	M	62			MI	CAD
6	F	66			Lung Ca	None
Mean		58				
SD		5				

DM, diabetes mellitus; Ca, cancer; CAD, coronary artery disease; CVA, cerebrovascular accident; HTN, hypertension; MI, myocardial infarction; SU, sulfonylurea drug.

pression to an internal standard gene expressed at a constant level in diabetic and control retinas. This strategy is less effective than other methods (e.g., competitive PCR) in controlling for efficiency of the amplification reaction. However, it has been used successfully by several investigators (21, 22) and showed in our study the desired accuracy and reproducibility (see Results).

Several criteria suggest that the increased expression of collagen IV was caused by diabetes and was a concomitant of background retinopathy. Although the group of diabetic patients was slightly older than the control group (the need to process eyes no later than 36 h after death imposes some limitation on the availability of specimens), even in the patients exactly age matched with controls (patients 1, 3, and 5 in Tables I and III) the collagen IV transcript and the collagen IV/actin ratio were increased above control values. Moreover, in humans age appears to be a determinant of basement membrane thickening only until the fourth decade of life (27). It has been calculated that in patients with type II diabetes such as those we studied, the onset of the disease antedates clinical diagnosis by 4–7 yr (28). Hence, our patients would have had diabetes for ~ 15 yr, time at which proliferative retinopathy is present in < 15% of the patients, while the prevalence of background retinopathy exceeds 70% (29). Indeed, in two groups of patients with characteristics similar to those of the present group and studied with techniques permitting examination of retinal vascular morphology (30, 31), we encountered microaneurysms, microvessels with focal areas of pericyte loss, occasional acellular capillaries, and no foci of neovascularization.

Although we studied RNA isolated from the whole retina, the data should reflect events occurring mostly in vascular cells. In situ hybridization studies in mice have shown that the postnatal retina contains collagen IV mRNA only at the level of blood vessels (18). Even if glial cells contributed to the collagen IV mRNA measured by the more sensitive method of RT-PCR, their role in the increased expression observed in diabetic patients is likely to be minor since the Müller cell basement membranes appear much less affected than the subendothelial and pericytic basement membranes in diabetic retinal microvessels (15). Insofar as the in vitro biosynthetic profile of retinal endothelial cells and pericytes indicates that only the endothelial cells synthesize substantial amounts of collagen IV (32), it may be extrapolated that our observations reflect increased synthesis of collagen IV by vascular endothelial cells in diabetic

Table II. Sequence of PCR Primer Pairs Used to Amplify Collagen IV [α1(IV)] and β-Actin cDNAs

cDNA	Exon	Sense upstream primer	Antisense downstream primer	Fragment size
α1(IV)	49–51	AGCACAATGCCCTTC (148–163)	TTGAACATCTCGCTC (570–584)	436 bp
β-Actin	1–3	ATGGATGATGATATCGCC (1–18)	ATCACGATGCCAGTGGTA (1014–1032)	457 bp

Primer sequences are reported in the 5'-to-3' orientation. Number in parentheses indicate location of primer in the sequence reported in reference 23 for α1(IV) and reference 24 for β-actin.

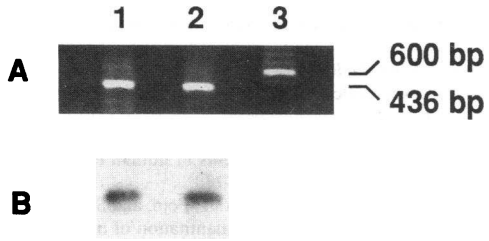

Figure 1. PCR amplification products for human retinal α1 (IV) collagen as detected on ethidium bromide-stained agarose gel (*A*) and Southern blot (*B*). Lanes *1* and *2* correspond to samples from two retinas, lane *3* to molecular weight markers

patients. The possibility that an increased number of endothelial cells in the diabetic retinas may contribute to the finding cannot be categorically excluded but is unlikely for three reasons. As indicated earlier, morphological examination of a total of 21 retinas obtained from diabetic patients with clinical characteristics matched to those of the present group failed to reveal focal areas of hypercellularity interpretable as intraretinal neovascularization (30, 31). Immunocytochemical and in situ hybridization studies of these retinas showed that neither the abundance nor the synthesis of von Willebrand factor (an endothelial cell marker) are increased in diabetic vessels (31). In addition, numerical estimates of microvascular cells in human diabetic retinopathy provide indication of decreased number of pericytes but not of increased number of endothelial cells (33).

Exposure of human vascular endothelial cells to high glucose levels in vitro results in increased transcription of the collagen IV gene (8). The present finding that in diabetes upregulation of collagen IV expression occurs at the pretranslational level is consonant with a causative role of hyperglycemia, but does not prove it. However, it provides evidence against which contender mechanisms can be measured. It also provides a novel perspective for comparative and functional analysis of the thickened basement membranes of diabetes. Studies with

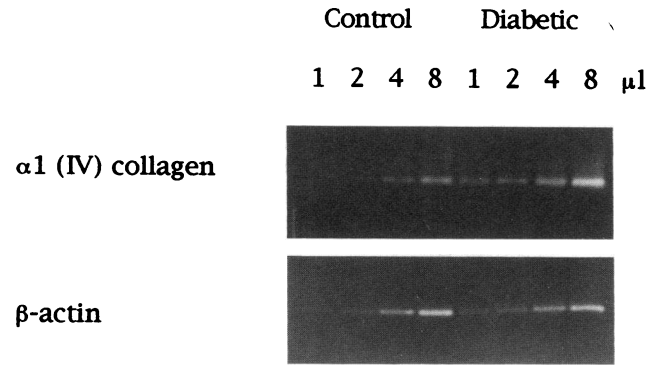

Figure 2. PCR amplification products for human retinal α1 (IV) collagen and β-actin showing linearity of amplification and increased expression of α1 (IV) collagen in the diabetic sample. The RNAs from the diabetic and control retinas were studied in parallel. The α1 (IV) collagen and β-actin mRNAs were reverse transcribed in the same reaction, and the indicated volumes of RT reaction were amplified in separate tubes as described in Methods. The PCR products were resolved on the same ethidium bromide-stained agarose gel.

Table III. Collagen IV and Actin mRNA Levels in Human Retinas

Subjects	Collagen IV	β-Actin
	Densitometric units/µl RT reaction	
DM		
1	127	182
2	62	167
3	66	94
4	57	93
5	54	147
Mean	73	136
SD	30	41
Control		
1	42	181
2	37	199
3	29	193
4	46	110
5	26	127
6	23	101
Mean	34	151
SD	9	44

Numbering of diabetic and control subjects corresponds to that in Table I.

sensitive molecular techniques can be extended to other matrix components and other tissues at the early stages of human diabetes; they will help define the pattern and consistency of biosynthetic abnormalities and help assess the likelihood of correct supramolecular assembly. The latter is critical for proper function of extracellular matrices, and likely to require secretion of the component protomers in correct relative and absolute concentrations (34, 35).

In addition to collagen IV overexpression we have recently observed in retinal microvessels of diabetic patients increased abundance of the receptors (β1 integrins) for extracellular matrix components (30). In vitro, changes of this nature are associated with increased cell matrix adhesion and decreased replication (30, 36, 37). It is thus possible that the thickened base-

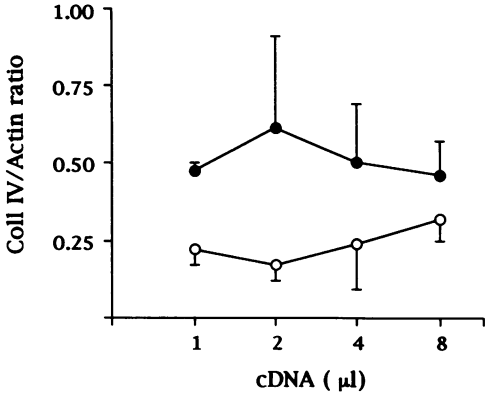

Figure 3. Collagen IV/β-actin ratios at varying amounts of cDNA (microliters of RT reaction) subjected to PCR amplification. Each point depicts the mean and SD of the ratios computed in control (○) and diabetic (●) samples.

ment membranes of diabetes have consequences on important cellular functions, and that the event responsible for initiating the phenotypic changes is increased synthesis of specific matrix components.

Acknowledgments

This work was supported by a grant (EY-09122) from the National Institutes of Health, a Mentor-Based Postdoctoral Fellowship Award from the American Diabetes Association, and the George and Frances Levin Endowment (to M. Lorenzi) and by a grant (9200706 CT 04) from the Consiglio Nazionale delle Ricerche (Italy) (M. Maiello).

References

1. Martinez-Hernandez, A., and P. S. Amenta. 1983. The basement membrane in pathology. *Lab. Invest.* 48:656–677.

2. Osterby, R. 1990. Basement membrane morphology in diabetes mellitus. *In* Diabetes Mellitus. Theory and Practice. H. Rifkin and D. Porte, Jr., editors. Elsevier Science Publishing Co., Inc., New York. 220–233.

3. Engerman, R. L. 1989. Pathogenesis of diabetic retinopathy. *Diabetes.* 38:1203–1206.

4. Osterby, R. 1992. Glomerular structural changes in type 1 (insulin-dependent) diabetes mellitus: causes, consequences, and prevention. *Diabetologia.* 35:803–812.

5. Steffes, M. W., D. E. R. Sutherland, F. C. Goetz, S. S. Rich, and S. M. Mauer. 1985. Studies of kidney and muscle biopsy specimens form identical twins discordant for type I diabetes mellitus. *N. Engl. J. Med.* 312:1282–1287.

6. Feingold, K. R., T. H. Lee, M. Y. Chung, and M. D. Siperstein. 1986. Muscle capillary basement membrane width in patients with vacor-induced diabetes mellitus. *J. Clin. Invest.* 78:102–107.

7. Engerman, R. L., and T. S. Kern. 1984. Experimental galactosemia produces diabetic-like retinopathy. *Diabetes.* 33:97–100.

8. Cagliero, E., T. Roth, S. Roy, and M. Lorenzi. 1991. Characteristics and mechanisms of high-glucose-induced overexpression of basement membrane components in cultured human endothelial cells. *Diabetes.* 40:102–110.

9. Vracko, R. 1974. Basal lamina layering in diabetes mellitus. *Diabetes.* 23:94–104.

10. Brownlee, M., A. Cerami, and H. Vlassara. 1988. Advanced glycosylation end products in tissue and the biochemical basis of diabetic complications. *N. Engl. J. Med.* 318:1315–1321.

11. Poulsom, R., D. J. Prockop, and R. P. Boot-Handford. 1990. Effects of long-term diabetes and galactosaemia upon lens and retinal mRNA levels in the rat. *Exp. Eye Res.* 51:27–32.

12. Brownlee, M., and R. G. Spiro. 1979. Glomerular basement membrane metabolism in the diabetic rat. *Diabetes.* 28:121–125.

13. Mene, P., M. S. Simonson, and M. J. Dunn. 1989. Physiology of the mesangial cell. *Physiol. Rev.* 69:1347–1425.

14. Vuorio, E., and B. de Crombrugghe. 1990. The family of collagen genes. *Annu. Rev. Biochem.* 59:837–872.

15. Carlson, E. C., and N. J. Bjork. 1990. SEM and TEM analyses of isolated human retinal microvessel basement membranes in diabetic retinopathy. *Anat. Rec.* 226:295–306.

16. Cagliero, E., M. B. Grant, and M. Lorenzi. 1991. Measurement of gene expression in human retinal microvessels by solution hybridization. *Invest. Ophthalmol. & Visual Sci.* 32:1439–1445.

17. Stone, C. M., and G. W. Laurie. 1991. Cellular expression of collagen IV mRNA in eye. *Exp. Eye Res.* 53:677–680.

18. Sarthy, V. 1993. Collagen IV mRNA expression during development of the mouse retina: an in situ hybridization study. *Invest. Ophthalmol. & Visual Sci.* 34:145–52.

19. Chirgwin, J. M., A. E. Przybyla, R. J. MacDonald, and W. J. Rutter. 1979. Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. *Biochemistry.* 18:5294–5299.

20. Cagliero, E., M. Maiello, D. Boeri, S. Roy, and M. Lorenzi. 1988. Increased expression of basement membrane components in human endothelial cells cultured in high glucose. *J. Clin. Invest.* 82:735–738.

21. Murphy, L. D., C. E. Herzog, J. B. Rudick, A. T. Fojo, and S. E. Bates. 1990. Use of the polymerase chain reaction in the quantitation of mdr-1 gene expression. *Biochemistry.* 29:10351–10356.

22. Horikoshi, T., K. D. Danenberg, T. H. W. Stadlbauer, M. Volkenandt, L. C. C. Shea, K. Aigner, B. Gustavsson, L. Leichman, R. Frosing, M. Ray, N. W. Gibson, C. P. Spears, and P. V. Danenberg. 1992. Quantitation of thymidylate synthase, dihydrofolate reductase, and DT-diaphorase gene expression in human tumors using the polymerase chain reaction. *Cancer Res.* 52:108–116.

23. Pihlajaniemi, T., K. Tryggvason, J. C. Myers, M. Kurkinen, R. Lebo, M. C. Cheung, D. J. Prockop, and C. D. Boyd. 1985. cDNA clones coding for the pro-α1(IV) chain of human type IV procollagen reveal an unusual homology of amino acid sequences in two halves of the carboxyl-terminal domain. *J. Biol. Chem.* 260:7681–7687.

24. Nakajima-Iijima, S., H. Hamada, P. Reddy, and T. Kakunaga. 1985. Molecular structure of the human cytoplasmic β-actin gene: interspecies homology of sequences in the introns. *Proc. Natl. Acad. Sci. USA.* 82:6133–6137.

25. Erlich, H. A., D. Gelfand, and J. J. Sninsky. 1991. Recent advances in the polymerase chain reaction. *Science (Wash. DC).* 252:1643–1651.

26. Lorenzi, M., and E. Cagliero. 1991. Pathobiology of endothelial and other vascular cells in diabetes mellitus. *Diabetes.* 40:653–659.

27. Steffes, M. W., J. Barbosa, J. M. Basgen, D. E. R. Sutherland, J. S. Najarian, and S. M. Mauer. 1983. Quantitative glomerular morphology of the normal human kidney. *Lab. Invest.* 49:82–86.

28. Harris, M. I., R. Klein, T. A. Welborn, and M. W. Knuiman. 1992. Onset of NIDDM occurs at least 4–7 yr before clinical diagnosis. *Diabetes Care.* 15:815–819.

29. Klein, R., B. E. K. Klein, S. E. Moss, M. D. Davis, and D. L. DeMets. 1984. The Wisconsin epidemiologic study of diabetic retinopathy. *Arch. Ophthalmol.* 102:527–532.

30. Roth, T., F. Podesta, M. A. Stepp, D. Boeri, and M. Lorenzi. 1993. Integrin overexpression induced by high glucose and by human diabetes: potential pathway to cell dysfunction in diabetic microangiopathy. *Proc. Natl. Acad. Sci. USA.* 90:9640–9644.

31. Boeri, D., E. Cagliero, F. Podesta, and M. Lorenzi. Vascular wall von Willebrand factor in human diabetic retinopathy. *Invest. Ophthalmol. & Visual Sci.* In press.

32. Canfield, A. E., A. M. Schor, D. J. Loskutoff, S. L. Schor, and M. E. Grant. 1989. Plasminogen activator inhibitor-type I is a major biosynthetic product of retinal microvascular endothelial cells and pericytes in culture. *Biochem. J.* 259:529–535.

33. Speiser, P., A. M. Gittelsohn, and A. Patz. 1968. Studies on diabetic retinopathy. III. Influence of diabetes on intramural pericytes. *Arch. Ophthalmol.* 80:332–337.

34. Yurchenco, P. D. 1990. Assembly of basement membranes. *Ann. NY Acad. Sci.* 580:195–213.

35. Garofalo, S., M. Metsaranta, J. Ellard, C. Smith, W. Horton, E. Vuorio, and B. de Crombrugghe. 1993. Assembly of cartilage collagen fibrils is disrupted by overexpression of normal type II collagen in transgenic mice. *Proc. Natl. Acad. Sci. USA.* 90:3825–3829.

36. Massague, J. 1990. The transforming growth factor-β family. *Annu. Rev. Cell Biol.* 6:597–641.

37. Giancotti, F. G., and E. Ruoslahti. 1990. Elevated levels of the $\alpha_5\beta_1$ fibronectin receptor suppress the transformed phenotype of chinese hamster ovary cells. *Cell.* 60:849–859.