The relationship between accumulation of advanced glycation end products and expression of vascular endothelial growth factor in human diabetic retinas

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Summary Both advanced glycation end products and vascular endothelial growth factor are believed to play a role in the pathogenesis of diabetic retinopathy. It is known that vascular endothelial growth factor causes retinal neovascularization and a breakdown of the blood-retinal barrier; how advanced glycation end products affect the retina, however, remains largely unclear. The substance N^ε-(carboxymethyl)lysine is a major immunologic epitope, i.e. a dominant advanced glycation end products antigen. We generated an anti-N^ε-(carboxymethyl)lysine antibody to investigate the relationship between the localization of advanced glycation end products and that of vascular endothelial growth factor in 27 human diabetic retinas by immunohistochemistry. Nine control retinas were also examined. In all 27 diabetic retinas, N^ε-(carboxymethyl)lysine was located in the thickened vascular wall. In 19 of the 27 retinas, strand-shaped N^ε-(carboxymethyl)lysine immunoreactivity was also observed around the vessels. In all

27 diabetic retinas, vascular endothelial growth factor revealed a distribution pattern similar to that of N^ε-(carboxymethyl)lysine. Vascular endothelial growth factor was also located in the vascular wall and in the perivascular area. Neither N^ε-(carboxymethyl)lysine nor vascular endothelial growth factor immunoreactivity was detected in the 9 control retinas. Vessels with positive immunoreactivity for N^ε-(carboxymethyl)lysine and/or vascular endothelial growth factor were counted. A general association was noted between accumulation of N^ε-(carboxymethyl)lysine and expression of vascular endothelial growth factor in the eyes with non-proliferative diabetic retinopathy ($p < 0.01$) and proliferative diabetic retinopathy ($p < 0.05$). [Diabetologia (1997) 40: 764–769]

Keywords Advanced glycation end products, blood retinal barrier, diabetic retinopathy, N^ε-(carboxymethyl)lysine, vascular endothelial growth factor.

Diabetic retinopathy is divided clinically into two main stages, non-proliferative and proliferative. Non-proliferative diabetic retinopathy (NPDR) is

characterized by hyperpermeability of retinal vessels, i.e. blood-retinal barrier (BRB) breakdown as well as retinal oedema, retinal haemorrhage, microaneurysm formation and vessel occlusions [1]. Tolentino et al. [2] reported that all these abnormalities, even vessel occlusions, could be experimentally induced by intravitreal injection of vascular endothelial growth factor (VEGF) in primate eyes. Retinal oedema, caused by BRB breakdown, is a major cause of visual disturbance when it affects the macula [3]. We recently demonstrated a correlation between expression of VEGF and BRB breakdown in diabetic retinas [4, 5]. VEGF has also been postulated to have an adverse effect on proliferative diabetic retinopathy (PDR) [6, 7], since VEGF production is enhanced

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Abbreviations: AGE, Advanced glycation end products; BRB, blood-retinal barrier; CML, N^ε-(carboxymethyl)lysine; GFAP, glial fibrillary acidic protein; NPDR, non-proliferative diabetic retinopathy; VEGF, vascular endothelial growth factor; PDR, proliferative diabetic retinopathy; NIDDM, non-insulin-dependent diabetes mellitus; BSA, bovine serum albumin; PBS, phosphate buffered saline; HSA, human serum albumin.

by ischaemia [8–12]. Ocular neovascularization is almost always associated with the occurrence of capillary non-perfusion.

An increase in the amount of advanced glycation end products (AGE) is seen in the blood of patients with diabetes mellitus [13, 14]. This increase is believed to play a causal role in diabetic neuropathy [15], nephropathy [13, 16] and retinopathy [17–19]. The term AGE is now applied to a broad range of advanced products of the Maillard reaction including N^ε-(carboxymethyl)lysine (CML) protein [20], N^ε-(carboxymethyl)hydroxylysine [21], pyrraline [13], pentosidine [22] and crosslines [23]. Among these, CML has been identified as a major immunologic epitope, i.e. a dominant AGE antigen in tissue proteins [24, 25].

Glucose reacts non-enzymatically with proteins to form Schiff base and Amadori products, which are early stage products. Although macular oedema is discrete and not manifested at all times, the incidence of macular oedema was associated with higher levels of glycated haemoglobin (Hb) [3], which is one of the early stage products. Further incubation of early stage products leads to the formation of AGE [26]. AGE increases VEGF expression in cultured retinal cells [27]; however the relationship between AGE and VEGF in vivo has not been elucidated. To investigate the relation between accumulation of AGE and expression of VEGF in diabetic retinas, we performed an immunohistochemical study for CML and VEGF in human diabetic retinas.

Materials and methods

Preparation of CML-proteins and hippuryl-CML. CML-BSA (bovine serum albumin) was prepared as described previously [25]. Briefly, 175 mg BSA was incubated at 37°C for 24 h with 0.15 mol/l glyoxylic acid and 0.45 mol/l NaCNBH₃ in 1 ml of 0.2 mol/l sodium phosphate buffer (pH 7.8), followed by dialysis against phosphate buffered saline (PBS). Other CML-proteins were also prepared from human serum albumin (HSA), ribonuclease (RNase) and Hb. In the same manner, hippuryl-CML was prepared as described previously [24]. Briefly, 40 mg/ml hippuryllysine was incubated with 0.13 mol/l glyoxylic acid in the presence of 0.65 mol/l NaCNBH₃ in 0.5 ml of 0.1 mol/l sodium carbonate buffer (pH 10).

Preparation of anti-CML antibody. All experiments conformed to the Association for Research in Vision and Ophthalmology Resolution on the Use of Animals in Ophthalmic and Vision Research and were also reviewed and accepted by the ethics committee on animal experiments, Faculty of Medicine, Kyushu University. To raise the polyclonal anti-CML antibody, 1.0 mg of CML-BSA in 50% Freund's complete adjuvant was injected intradermally into a rabbit at 20 skin sites, followed by five booster injections with the same amount of CML-BSA in 50% Freund's incomplete adjuvant. The serum was taken 10 days after the final immunization for further affinity purification. Formyl-cellulofine gels were coupled to BSA or CML-BSA as described previously [26]. The anti-CML-BSA antiserum (10 ml) was passed over a column (2.0 × 12 cm) of

formyl-cellulofine-BSA gel. The non-adsorbed fraction was then passed over a column (1.2 × 9.0 cm) of formyl-cellulofine-CML-BSA. The fraction adsorbed to CML-BSA was eluted with 0.1 mol/l citric acid buffer (pH 3.0). Pooled antibody fraction was neutralized, concentrated, and dialysed against PBS. The resultant affinity purified polyclonal anti-CML antibody was used for the experiments.

Enzyme-linked immunosorbent assay. Competitive enzyme-linked immunosorbent assay (ELISA) was performed at room temperature. Each well of a 96-well microtitre plate was coated with 0.1 ml of 1.0 µg/ml of CML-BSA in 50 mmol/l sodium carbonate buffer (pH 9.6) and incubated for 60 min. This was followed by triplicate washing with PBS containing 0.1% Tween 20 (buffer A); each well was then blocked with 0.5% gelatin and incubated for 60 min. After washing three times with buffer A, 50 µl of test samples and 50 µl of anti-CML antibody (5 µg/ml) were added to each well and incubated for 60 min. After three washes with buffer A, horseradish peroxidase-conjugated anti-rabbit IgG was added to each well, and visualized with 1,2-phenylenediamine dihydrochloride. Absorbance at 492 nm was then determined.

Diabetic and control retinas. Twenty seven retinas from subjects with diabetic retinopathy were used. Twenty retinas were from subjects with NPDR (non-insulin-dependent diabetes mellitus (NIDDM); 11 male, 9 female, age range 57–81 years, duration of diabetes range 9–19 years) and seven (NIDDM; 3 male, 4 female, age range 64–78 years, duration of diabetes range 11–18 years) from subjects with PDR. Of these, four retinas with PDR were surgically obtained and were fixed immediately after enucleation with 4% paraformaldehyde. The others, obtained from autopsy cases were fixed with 10% formaldehyde. Nine eyes obtained from autopsy cases (5 male, 4 female, age range 55–83) without diabetes or any known ocular disease were used as controls.

Antibodies used. To locate CML, 3 µg/ml of anti-CML antibody described above was used. To locate VEGF, polyclonal rabbits anti-VEGF IgG against N-terminal amino acid residues 1–21 of human VEGF (Santa Cruz Biotechnology Inc., Santa Cruz, Calif., USA), 1 µg/ml, was used. To identify the types of CML or VEGF positive cells, we performed immunohistochemistry for glial fibrillary acidic protein (GFAP), which is a marker for glial cells, and for a smooth muscle actin, which is a marker for smooth muscle cells and pericytes in the serial sections. For this purpose, anti-GFAP IgG (Dakopatts, Glostrup, Denmark) and anti smooth muscle actin IgG (Sigma, St. Louis, Mo., USA) were used. Immunohistochemistry was performed with each antibody using the avidin-biotin complex method [28].

Controls. The immunohistochemical specificity of this antibody was confirmed by three types of negative controls: (a) substituting rabbit non-immune IgG for the primary antibody; (b) omitting the primary antibody in the staining protocol; and (c) using anti-CML antibody (3 µg/ml) in the presence of an excess of CML-BSA (100 µg/ml).

Investigation of the relationship between expression of CML and VEGF. In each pair of two serial sections immunohistochemically stained for CML and VEGF, two corresponding posterior and two corresponding peripheral areas of the retinas were videotaped at high power fields (×100). The vessels which could be identified in both sections were counted. Subsequently, to investigate the relation between expression of CML and VEGF, the vessels with the following characteristics were counted: 1) vessels with both CML and VEGF immunoreactivity; 2) vessels with CML immunoreactivity but not

Fig. 1. Immunoreactivity of anti-CML antibody to CML-proteins. Each well was coated with 0.1 ml of 1.0 μ g of CML-BSA, blocked with 0.2 ml of 0.5% gelatin, and washed three times with buffer A. To each well 50 μ l of samples and 50 μ l of anti-CML antibody (5 μ g/ml) were added, followed by incubation for 60 min. The wells were then washed three times with buffer A, and the antibodies bound to wells were detected by HRP-conjugated anti-rabbit IgG. Samples were CML-BSA (●), CML-HSA (◆), CML-RNase (▲), CML-Hb (▼), hippuryl-CML (□), BSA (○), HSA (◇), RNase (△) and Hb (▽)

VEGF immunoreactivity; 3) vessels with VEGF immunoreactivity but not CML immunoreactivity; 4) vessels with neither CML immunoreactivity nor VEGF immunoreactivity.

Statistical analysis. The number of vessels of each group was compared and the effect of CML on VEGF expression was statistically analysed using the Cochran-Mantel-Haenszel test.

Results

Immunological reactivity of anti-CML antibody. To confirm the specificity of anti-CML antibody used in this experiment, we examined the immunological reaction of anti-CML antibody with CML-proteins by competitive ELISA. As shown in Figure 1, anti-CML antibody significantly reacted with CML-BSA, CML-HSA, CML-RNase and CML-Hb, whereas the unmodified proteins were not recognized by the antibody. Furthermore, hippuryl-CML, a carboxymethylated low-molecular-weight compound, was effectively recognized by anti-CML antibody. It is evident, therefore, that anti-CML antibody specifically reacts with the CML moiety.

Immunohistochemistry for CML, VEGF and cell markers. In all 27 diabetic retinas examined, CML

was located in the thickened vascular wall of the large vessels in the nerve fiber layer (Fig. 2A). In 19 of 27, strand shaped CML immunoreactivity was also observed around the vessels (Fig. 2B). VEGF showed a similar pattern of distribution to that of CML (Fig. 2C). Although smooth muscle cells are major constituents of vascular walls, positive immunoreactivity for smooth muscle actin was attenuated and the stained portion was thinner than that of CML (Fig. 2D).

Controls. None of the negative controls including anti-CML antibody (3 μ g/ml) adsorbed with an excess of CML-BSA (100 μ g/ml) showed a positive reaction (Fig. 2E). In control non-diabetic retinas, neither VEGF nor CML immunoreactivity was observed.

Relationship between expression of CML and VEGF

A total of 274 vessels could be observed in both sections in all 108 pairs of serial sections. Of these, 63 (NPDR 39, PDR 24) vessels showed both CML and VEGF immunoreactivity; 18 (NPDR 14, PDR 4) vessels showed CML immunoreactivity but no VEGF immunoreactivity; 7 (NPDR 2, PDR 5) vessels showed VEGF immunoreactivity but no CML immunoreactivity; and 186 (NPDR 135, PDR 51) vessels showed neither VEGF immunoreactivity nor CML immunoreactivity. Statistical analysis using the Cochran-Mantel-Haenszel test showed a general association between accumulation of CML and expression of VEGF in both non-proliferative ($p < 0.01$) and proliferative specimens ($p < 0.05$). VEGF expression was enhanced in the eyes with PDR ($p < 0.05$), while there was no significant difference in AGE accumulation between the eyes with NPDR and PDR.

Discussion

VEGF is one of the major growth factors that has an adverse effect on ocular neovascular diseases such as diabetic retinopathy. To find a new modality of therapy, the effect of VEGF inhibitors on retinal neovascularization has been investigated [29–31]. Investigation of the factors that promote VEGF expression is also important because their inhibitors are potential drugs for treatment of diabetic retinopathy. Ischaemia [8–12], adenosine [32], basic fibroblast growth factor [33], platelet derived growth factor [34] and AGE [27] have been reported to have a promoting effect on VEGF expression. To investigate the relationship between the expression of AGE and VEGF in human diabetic retinas, we performed an immunohistochemical study using 27 human diabetic retinas.

Both CML and VEGF immunoreactivities were distributed in the diabetic retinas and both were accentuated around the blood vessels. We recently

Fig. 2 A. Immunohistochemistry for N^ε-(carboxymethyl)lysine (CML) that is a dominant advanced glycation end product (AGE) antigen in tissue proteins. In all 27 diabetic retinas examined, CML was located in the thickened vascular wall in the nerve fiber layer, $\times 200$. **B)** Immunohistochemistry for CML. In 19 of 27 retinas examined, strand-shaped CML was also located in the retina, mainly around the vessels in diabetic retinas (arrow), $\times 100$. **C)** Immunohistochemistry for VEGF in a serial section of the retina shown in Figure 2B. VEGF is also located around the vessels in diabetic retinas $\times 100$. **D)** Immunohistochemistry for a smooth muscle actin. Attenuated positive immunoreactivity (arrow) is much thinner than that of CML, $\times 100$. **E)** An immunohistochemical negative control using anti-CML antibody (3 $\mu\text{g}/\text{ml}$) adsorbed with an excess of CML-BSA (100 $\mu\text{g}/\text{ml}$). No immunoreactivity is observed, $\times 100$

reported that Müller cells are one site of VEGF location in rat diabetic retinas. GFAP, which is expressed by astrocytes and Müller cells in the diabetic retina, and VEGF showed a similar distribution pattern in

rat diabetic retinas [5]. In the 27 human diabetic retinas examined in the present study, GFAP showed a pattern of distribution similar to that of CML and VEGF (data not shown). Since AGE formation has been reported with glial proteins [35], we concluded that Müller cells were one site of CML location in diabetic retinas.

Another site of CML location in the diabetic retina was the vascular wall. In some vessels, CML immunoreactivity was observed in the entire thickness of the vascular wall, while immunoreactivity for a smooth muscle actin, a marker for smooth muscle cells, was attenuated. Smooth muscle cells are a main constituent of the vascular wall; however, they are damaged and replaced by collagen bundles in the large vessels of retinas with advanced diabetic changes [36]. These findings indicate that CML was located in the extracellular matrix, e.g. collagen rather than in the cytoplasm of cellular components of the vascular walls.

AGE accumulation is believed to play a causative role in diabetic complications [13, 16] including retinopathy [17–19, 37]. We previously reported that VEGF expressed in diabetic retinas induces retinal oedema [5]. In human diabetic retinas, the present study demonstrated a co-localization of AGE and VEGF. There was a significant association between AGE accumulation and VEGF expression in the eyes with NPDR ($p < 0.01$) and PDR ($p < 0.05$). Together with the following evidence, these results suggest that AGE could be involved in VEGF-induced diabetic retinal oedema: 1) AGE is known to enhance VEGF expression in cultured retinal cells [27]. 2) AGE can promote thrombogenesis by inhibiting the expression of anticoagulant factors such as prostacyclin [37] and thrombomodulin [38] or by inducing the expression of procoagulant factor such as tissue factor [38]. Ischaemia caused by thrombi formation could then upregulate VEGF expression in retinal tissue. 3) VEGF itself is thrombogenic inducing tissue factor [39] or von Willebrand factor production [40] by endothelial cells. Tolentino et al. [2] reported that intravitreal injections of VEGF could produce retinal vessel occlusions and other diabetic retinal changes such as haemorrhage, microaneurysm, and intraretinal neovascularization in primate eyes.

These data suggest that AGE accumulation in the diabetic retina could initiate a positive feedback loop for increasing VEGF levels, leading to a further development of diabetic retinopathy. This mechanism could explain why VEGF expression was enhanced in the eyes with PDR, while there was no significant difference in AGE accumulation between the eyes with NPDR and PDR in the present study.

The available data described above suggest that AGE can promote the progression of diabetic retinopathy. An upregulation of VEGF expression in the retinal tissue by AGE accumulation is one possible mechanism for this effect.

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References

1. Davis MD (1990) Diabetes mellitus. In: Gold DH, Weingeist TA (eds) *The eye in systemic disease*. Vol 1. J.B. Lippincott Company, Philadelphia, pp 79–85
2. Tolentino MJ, Miller JW, Gragoudas ES, et al. (1996) Intravitreal injection of vascular endothelial growth factor produce retinal ischaemia and microangiopathy in adult primate. *Ophthalmology* 103: 1820–1828
3. Klein R, Klein BEK, Moss SE, Cruickshanks KJ (1995) The Wisconsin Epidemiologic Study of Diabetic Retinopathy XV. The long-term incidence of macular oedema. *Ophthalmology* 102: 7–16
4. Murata T, Ishibashi T, Khalil A, Hata Y, Yoshikawa H, Inomata H (1995) Vascular endothelial growth factor plays a role in hyperpermeability of diabetic retinal vessels. *Ophthalmic Res* 27: 48–52
5. Murata T, Nakagawa K, Khalil A, Ishibashi T, Inomata H, Sueishi K (1996) The relation between expression of vascular endothelial growth factor and breakdown of blood-retinal barrier in diabetic rat retinas. *Lab Invest* 74: 819–825
6. Aiello LP, Avery RL, Arrigg PG et al. (1994) Vascular endothelial growth factor in ocular fluid of patients with diabetic retinopathy and other retinal disorders. *N Engl J Med* 331: 1480–1487
7. Pe'er J, Shweiki D, Itin A, Hemo I, Gnessin H, Keshet E (1995) Hypoxia-induced expression of vascular endothelial growth factor by retinal cells is a common factor in neovascularizing ocular diseases. *Lab Invest* 72: 638–645
8. Dvorak HF, Brown LF, Detmar M, Dvorak AM (1995) Vascular permeability factor/vascular endothelial growth factor, microvascular hyperpermeability, and angiogenesis. *Am J Pathol* 146: 1029–1039
9. Ferrara N, Houck K, Jakeman L, Leung DW (1992) Molecular and biological properties of the vascular endothelial growth factor family of proteins. *Endocr Rev* 13: 18–32
10. Minchenko A, Bauer T, Salceda S, Caro J (1994) Hypoxic stimulation of vascular endothelial growth factor expression in vitro and in vivo. *Lab Invest* 71: 374–379
11. Senger DR, Van de Water L, Brown LF et al. (1993) Vascular permeability factor (VPF, VEGF), in tumor biology. *Cancer Metastasis Rev* 12: 303–324
12. Shweiki D, Itin A, Soffer D, Keshet E (1992) Vascular endothelial growth factor induced by hypoxia may mediate hypoxia-initiated angiogenesis. *Nature* 359: 843–845
13. Hayase F, Nagaraj RH, Miyata S, Njoroge FG, Monnier VM (1989) Aging of proteins: immunological detection of a glucose-derived pyrrole formed during Maillard reaction in vivo. *J Biol Chem* 263: 3758–3764
14. Makita Z, Vlassara H, Cerami A, Bucala R (1992) Immunochemical detection of advanced glycosylation end products in vivo. *J Biol Chem* 267: 5133–5138
15. Brownlee M, Cerami A, Vlassara H (1988) Advanced glycosylation end products in tissue and the biochemical basis of diabetic complications. *N Engl J Med* 318: 1315–1321
16. Makino H, Shikata K, Hironaka K et al. (1995) Ultrastructure of nonenzymatically glycosylated mesangial matrix in diabetic nephropathy. *Kidney Int* 48: 517–526
17. Brownlee M (1994) Lilly lecture 1993. Glycation and diabetic complications. *Diabetes* 43: 836–841
18. Hammes HP, Weiss A, Hess S, Araki N, Horiuchi S, Brownlee M, Preissner T (1996) Modification of vitronectin by advanced glycation alters functional properties in vitro and in the diabetic retina. *Lab Invest* 75: 325–338
19. Vlassara H, Bucala R, Striker L (1994) Pathogenic effects of advanced glycosylation: biochemical, biologic, and clinical implications for diabetes and aging. *Lab Invest* 70: 138–151
20. Ahmed MU, Thorpe SR, Baynes JW (1986) Identification of N epsilon-carboxymethyllysine as a degradation product of fructoselysine in glycated protein. *J Biol Chem* 261: 4889–4894
21. Dunn JA, McCance DR, Thorpe SR, Lyons TJ, Baynes JW (1991) Age-dependent accumulation of N epsilon-(carboxymethyl)lysine and N epsilon-(carboxymethyl)hydroxylysine in human skin collagen. *Biochemistry* 30: 1205–1210
22. Sell DR, Monnier VM (1989) Structure elucidation of a senescence cross-link from human extracellular matrix. Implication of pentoses in the aging process. *J Biol Chem* 264: 21597–21602

23. Nakamura K, Hasegawa T, Fukunaga Y, Ienaga K (1992) Crosslines A and B as candidates for the fluorophores in age- and diabetes-related cross-linked proteins, and their diacetates produced by Maillard reaction of α -N-acetyl-L-lysine with D glucose. *J Chem Soc Commun* 7: 992–994
24. Ikeda K, Higashi T, Sano H et al. (1996) N^ε-(Carboxymethyl)lysine protein adduct is a major immunological epitope in proteins modified with advanced glycation end products of the Maillard reaction. *Biochemistry* 35: 8075–8083
25. Reddy S, Bichler J, Wells-Knecht KJ, Thorpe SR, Baynes JW (1995) N^ε-(Carboxymethyl)lysine is a dominant advanced glycation end product (AGE) antigen in tissue proteins. *Biochemistry* 34: 10872–10878
26. Horiuchi S, Araki N, Morino Y (1991) Immunochemical approach to characterize advanced glycation end products of the Maillard reaction. Evidence for the presence of a common structure. *J Biol Chem* 266: 7329–7322
27. Adamis AP, Kuroki M, Tolentino MJ, Kim I (1996) Advanced glycation end products (AGE) increase retinal VEGF gene expression. *Invest Ophthalmol Vis Sci* 37: 975 (Abstract)
28. Guesdon JL, Ternynck T, Avrameas S (1979) The use of avidin-biotin interaction in immunoenzyme techniques. *J Histochem Cytochem* 27: 1131–1139
29. Adamis AP, Shima DT, Tolentino MJ et al. (1996) Inhibition of vascular endothelial growth factor prevents retinal ischaemia-associated iris neovascularization in a nonhuman primate. *Arch Ophthalmol* 114: 66–71
30. Aiello LP, Pierce EA, Foley ED et al. (1995) Suppression of retinal neovascularization in vivo by inhibition of vascular endothelial growth factor (VEGF) using soluble VEGF-receptor chimeric proteins. *Proc Natl Acad Sci USA* 92: 10457–10461
31. Robinson GS, Pierce EA, Rook SL, Foley E, Webb R, Smith LE (1996) Oligodeoxynucleotides inhibit retinal neovascularization in a murine model of proliferative retinopathy. *Proc Natl Acad Sci USA* 93: 4851–4856
32. Fischer S, Sharma HS, Karliczek GF, Schaper W (1995) Expression of vascular permeability factor/vascular endothelial growth factor in pig cerebral microvascular endothelial cells and its upregulation by adenosine. *Brain Res Mol Brain Res* 28: 141–148
33. Stavri GT, Zachary IC, Baskerville PA, Martin JF, Erusalimsky JD (1995) Basic fibroblast growth factor upregulates the expression of vascular endothelial growth factor in vascular smooth muscle cells. Synergistic interaction with hypoxia. *Circulation* 92: 11–14
34. Stavri GT, Hong Y, Zachary I et al. (1995) Hypoxia and platelet-derived growth factor-BB synergistically upregulate the expression of vascular endothelial growth factor in vascular smooth muscle cells. *FEBS Lett* 358: 311–315
35. Dominiczak MH (1991) The significance of the products of the Maillard (Browning) reaction in diabetes. *Diabet Med* 8: 505–516
36. Gardiner TA, Stitt AW, Anderson HR, Archer DB (1994) Selective loss of vascular smooth muscle cells in the retinal microcirculation of diabetic dogs. *Br J Ophthalmol* 78: 54–60
37. Yamagishi S, Yamamoto Y, Harada S, Hsu CC, Yamamoto H (1996) Advanced glycosylation end products stimulate the growth but inhibit the prostacyclin-producing ability of endothelial cells through interactions with their receptors. *FEBS Lett* 384: 103–106
38. Esposito C, Gerlach H, Brett J, Stern D, Vlassara H (1989) Endothelial receptor-mediated binding of glucose-modified albumin is associated with increased monolayer permeability and modulation of cell surface coagulant properties. *J Exp Med* 170: 1387–1407
39. Clauss M, Gerlach M, Gerlach H et al. (1990) Vascular permeability factor: a tumor-derived polypeptide that induces endothelial cell and monocyte procoagulant activity, and promotes monocyte migration. *J Exp Med* 172: 1535–1545
40. Brock TA, Dvorak HF, Senger DR (1991) Tumor-secreted vascular permeability factor increases cytosolic Ca²⁺ and von Willebrand factor release in human endothelial cells. *Am J Pathol* 138: 213–221