Advances in biochemical mechanisms of diabetic retinopathy

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Abstract. – Diabetes mellitus is a major cause of blindness in the working population of the Western World. Numerous large, prospective, randomized clinical trials have delineated the current standard prevention and treatment protocols including intensive glycemic and blood pressure control as well as laser photocoagulation for clinically significant macular edema and/or proliferative retinopathy at a high risk for tractional retinal detachment. However, despite all these interventions, vision loss from diabetic retinopathy still occurs at an alarming rate and no data provide an adequate explanation for the serious and rapid involvement of the retinal microcirculation that may be observed in the disease despite a good metabolic control. In fact, there is now ample of evidence that the development of diabetic retinopathy is a multifactorial process where genetic, metabolic and growth factors play an important role. Some biochemical mechanisms, supposed to be involved in the pathogenesis of diabetic retinopathy, have been highlighted in this review.

Key Words:
Biochemical mechanisms, Diabetes, Diabetic retinopathy, Retina.

Introduction

Diabetes mellitus is a major cause of blindness among young adults in economically developed societies1-5. Its most frequent and potentially blinding complications are represented by exudative maculopathy with clinically significant macular edema (DME), intravitreal or preretinal hemorrhages, macular pigmen-
Pathogenesis

Hyperglycemia

At present, the most effective medical treatment for DR is represented by glycemic control. Two major trials have demonstrated the effect of “intensive” blood glucose in reducing the incidence and progression of DR. The Diabetes Control and Complications Trial (DCCT) has implicated hyperglycemia as a major pathogenetic factor in type 1 patients and a strong correlation has been observed between the glycemic control and the incidence and progression of diabetic microvascular complications. In particular, the adjusted mean risk for development of any retinopathy was reduced by 76% in the intensive therapy, compared with the conventional group. For those with some retinopathy already, the intensive group had a higher incidence of progression during the first year whereas from 3 years onwards, the progression of retinopathy was reduced in the intensive group by 54%. An adverse effect shown by the DCCT is that intensive diabetes therapy reduces plasma levels of LDL cholesterol and triglycerides but increases the risk of major weight gain, which might adversely affect the risk of cardiovascular disease.

Similar results were observed for type 2 diabetics in the United Kingdom Prospective Diabetes Study (UKPDS) group. In this study, patients who were assigned to intensive glucose control had a 25% risk reduction in microvascular endpoints, including the need for retinal photocoagulation. Both studies showed that glycemic control is protective for all levels of control: there is no glycemic threshold below which a reduction in microvascular complications is not observed. The current recommendation is for maintaining the glucose levels as near normal as possible. However, because of some risks associated to hypoglycemia (such as hospitalizations and possibly deaths while operating motor vehicles), the glycemic control targets should be individualized. Therapy should be directed toward achieving the lowest glycemic level that is the safest in terms of hypoglycemic risk for each patient.

Hypermeglycemia is involved in the pathogenesis of diabetic neuropathy, retinopathy, nephropathy, and macrovascular disease via multiple mechanisms, the best studied of which are the following: increased flow through the aldose-reductase pathway (increased aldose reductase activity); nonenzymatic glycation and glycoxidation with formation of advanced glycation end products (AGEs); increased de-novo synthesis of diacylglycerol from glucose, causing protein-kinase C (PKC) activation; oxidative-nitrosative stress with overproduction of reactive oxygen species (ROS); and poly(ADP-ribose) polymerase (PARP), as well as the inflammatory cascade, and these downstream mechanisms are also involved in the pathogenesis of diabetes complications. The interactions among various hyperglycemia-initiated mechanisms are not completely understood, and the relationship between increased aldose reductase activity and the oxidative-nitrosative stress/PARP activation has recently become a focus of interest. According to several studies performed in the diabetic lens, nerve, retina and high-glucose–exposed endothelial cells, increased aldose reductase activity leads to oxidative stress. However, it has also been reported that increased aldose reductase activity is a consequence rather than a cause of oxidative stress (in particular, mitochondrial superoxide production) and PARP activation in the pathogenesis of diabetes complications.

As a consequence of all this, beside the optimal glycemic control that always needs to be achieved in each patient, pharmacologic inhibition of the above indicated pathways might prevent some of the characteristic lesions of DR, such as loss of retinal pericytes and microaneurysm formation, changes in retinal hemodynamics, and aberrant neovascularization.

Polyol Accumulation

Aldose reductase, the first and rate-limiting enzyme in the polyol pathway, reduces glucose to sorbitol using NADPH as a cofactor; sorbitol is then metabolized to fructose by sorbitol dehydrogenase, which uses NAD⁺ as a cofactor. The polyol (sorbitol) pathway of glucose metabolism is activated in many cell types when intracellular glucose concentrations are very high, and it can generate cellular oxidative stress through a variety of biochemical abnormalities, including myo-inosititol depletion and downregulation of Na/K ATPase activity, NAD⁺/NADH and NADP⁺/NADPH redox imbalances, changes in fatty acid metabolism, impaired neurotrophic support, and upregulation of vascular endothelial growth factor (VEGF). The polyol pathway appears to be both a
“dream” and a “dread” target by devising strategies to prevent DR. The pathway is a dream target because its activation is immediately linked to hyperglycemia, generates various types of cellular stress, and occurs prominently in the tissues that develop complications, thus promising returns beyond retinopathy. In addition, polymorphisms of the aldose reductase gene may help in predicting individual susceptibility to retinopathy and other microvascular complications, and the enzymatic function of aldose reductase can be specifically inhibited. However, the polyol pathway has become a dread target because aldose reductase inhibitors (ARIs) have yielded inconsistent results in the diabetic or diabetic-like retinopathy of experimental animals and only minor benefits in human DR.

However, the polyol pathway seem to be really a rational candidate mechanism for the ganglion cell apoptosis and Müller glial cell activation. Ganglion and Müller cells are the retinal cells most consistently found to contain aldose reductase in all species studied, including humans. Since neuroglial changes may cause vascular changes, and given the general agreement that at least the pericytes of retinal capillaries contain aldose reductase, the inhibition of the polyol pathway could also prevent the vascular abnormalities of DR. In fact, inhibition of aldose reductase was also able to prevent the early activation of complement in the retinal vessel wall as well as the apoptosis of vascular pericytes and endothelial cells and the development of acellular capillaries. Moreover, retinal endothelial cells showed aldose reductase immunoreactivity, and human retinas exposed to high glucose in organ culture increased the production of sorbitol. Finally, experimental evidence exists that defects in the polyol pathway may produce thickening of the capillary basement membrane, loss of mural pericytes and microaneurysm formation, the earliest vascular features of diabetic microangiopathy. In fact, high glucose levels increase flux through the polyol pathway with the enzymatic activity of aldose reductase, thus determining a build-up of intracellular sorbitol concentrations and, consequently, an osmotic damage to the vascular cells. Therefore, it seems possible to conclude that excess of aldose reductase activity might be a mechanism in the pathogenesis of DR.

Positive preliminary results of some ARIs (such as sorbinil, zenarestat or fidarestat) in preventing retinal and neural damage in diabetes have been highlighted, thus justifying further clinical trials of specific, potent, and low-toxic ARI.

**AGEs Accumulation**

Chronically increased amounts of glucose amplify the physiological process of nonenzymatic protein glycosylation (glycation). For example, glycated hemoglobin (HbA1c) is an acknowledged indicator of time-integrated glycaemia.

Glucose forms labile links with the NH₂-terminal and side-chain lysine radicals within proteins, which, after a cascade of molecular rearrangements (Maillard reaction), result in molecules of brown color and specific fluorescence (Amadori products), leading to degradation of both structural and functional proteins and accelerated aging. While most glycated proteins are eliminated in physiological conditions, they accumulate, on the contrary, in the presence of diabetes and undergo further structural arrangements with the formation of AGEs, which, in turn, are implicated in the development of vascular lesions having a proven effect in determining a significant loss of mural pericytes. Moreover, it is also clear that AGEs formation mechanisms are diverse and complex, encompassing both non-oxidative (glycation) and oxidative (glycoxidation) pathways. These reactions, together with intra- and intermolecular cross-link formation, are able to modify structure and function of target molecules in such a way that they do not respond anymore to biological signals. Interaction of AGEs with their receptors (RAGE) has also been implicated in enhanced ROS formation and inflammatory vascular complications. For example, N(carboxymethyl)lysine-protein (CML-protein), macrophage colony stimulating factors (M-CSF) and soluble vascular cell adhesion molecule-1 (sVCAM-1) have been found to be increased in patients with diabetic micro-angiopathy and CML-human serum protein (CML-HSP) levels, which are at variance with the HbA1c index for blood glucose, have been proposed as a good biomarker both for glycoxidation and for the development of microvascular complications in type II diabetes.

The use of compounds that inhibit AGE formation (such as pimagedine and aminoguanidine) has been investigated as a possible therapeutic intervention, highlighting promising results in the prevention of DR in animal models. Preliminary results for human diabetic nephropa-
PKC Inhibitors

There is a large body of evidence to support the hypothesis that PKC plays a major role in hyperglycemia-induced microvascular dysfunction in diabetes. In fact, hyperglycemia leads to persistent de novo synthesis and activation of PKC, induced by increased glucose availability through de novo synthesis of diacylglycerol (DAG), which, in turn, is associated with a number of biochemical and metabolic abnormalities, including increased expression of matrix proteins, such as collagen and fibronectin, and increased expression of vasoactive mediators, such as endothelin. The net effect of these changes may be manifested as basement membrane thickening and changes in vessel permeability and/or blood flow. Although the activity of multiple PKC isoforms (α, β, γ, δ, ε, and θ) is increased in vascular diabetic tissues, studies suggest that the PKC-β is preferentially activated. Moreover, PKC-β has been shown to be an integral component of cellular signaling by vascular endothelial growth factor (VEGF), an important mediator of ocular neovascularization, secondary to retinal ischemia and DME, thus stimulating retinal pericyte proliferation.

A selective inhibitor of PKC-β, ruboxistaurin mesylate (LY333531), was initially reported to prevent the increase in leukostasis and the decrease in blood flow in the retinas of transgenic diabetic rats. However, multicenter clinical trials in humans did not show a same effectiveness on DR progression. A possible reason of that might be the observation that PKC inhibition augments pro-apoptotic effects of high glucose on cultured pericytes. Therefore, it seems possible to conclude that the potential effectiveness of PKC-β inhibitors on DR progression is at this time still controversial.

Oxidative Damage

Diabetes and hyperglycemia are associated with increase in oxidative stress, and overproduction of ROS (free radicals) are thought to be responsible for microvascular damage, being consistent with increased malondialdehyde, isoprostanes, nitrotyrosine or 8-hydroxy-2′deoxyguanosine levels as well as an overall decreased antioxidant status. Production of ROS may result from various mechanisms, including glucose auto-oxidation, protein glycation, increased flux through the polyol pathway, and prostanoïd production. These high ROS levels are thought to determine structural and functional changes in all cellular components, leading to DNA and protein modification and lipid peroxidation. In particular, pericytes are highly sensitive to the oxidative stress not only directly but also indirectly, due to significantly decreased levels of scavenging enzymes and increased rate of apoptosis. Pericyle loss or functional deficiency have been found to reduce the inhibition of endothelial proliferation in vivo. As damage progresses, the blood vessel wall becomes more porous, letting proteins and other materials leak out abnormally, thus determining the typical features of nonproliferative DR (e.g. hard exudates and clinically significant DME).

Furthermore, animal studies suggest that antioxidants such as vitamin E may prevent some of the vascular dysfunction associated with diabetes by means of several different mechanisms: reduced retinal DAG levels; normalized PKC-β activation; normalized retinal blood flow; restored nitric oxide-mediated endothelium-dependent relaxation. The use of anti-oxidants is promising, but further studies are needed to determine appropriate doses, and/or whether this approach will translate into long-term benefits of reduced DR and DME.

Endothelium-Related Dysfunction of the Coagulant and Anticoagulant Pathways

Besides but strictly associated with these metabolic vascular changes, there are many reports that extensively document the presence of a remarkable endothelium-related dysfunction of the coagulant and anticoagulant pathways in diabetics, but it is not yet clear if this condition is due to hyperglycemia only. Abnormal coagulation is manifested by enhanced prothrombin conversion to thrombin – as demonstrated both by increased activated factor VII (FVII:c) and prothrombin degradation products (F1 + 2) plasma levels and downregulation of the anticoagulant pathway, caused by reduced antithrombin III activity and thrombomodulin endothelial receptors. In particular, it has been suggested that in patients without retinal lesions, despite the hyperglycemic condition, the vascular endothelium preserves its physiological thromboresistance. On the contrary, in the cases of PDR a hypercoagulable state is present as a consequence of a...
functional conversion of the endothelium from a thromboresistant to a thrombogenic surface with activation of the extrinsic haemostatic pathway. In fact, a higher platelet and erythrocyte sedimentation rate, with a elevated erythrocyte sodium-lithium countertransport activity, has also been observed in diabetics, as well as: increased plasma concentrations of tissue plasminogen activator (tPA) inhibitor (PAI-1), von Willebrand-antihaemophilic factor A and fibrinogen; decreased concentrations of endothelium-derived relaxing factor, prostacyclin and tPA; increased fibronectin and thrombomodulin levels and reduced fibrinolytic potential of vascular endothe- lia.25.

The endothelin system is probably of great impor-
tance in mediating the diabetes-induced retinal vascular dysfunction as suggested by increased endothelin-1 levels (see below). Moreover, the finding of antipericyte and phospholipid-binding autoantibodies (e.g. Lupus Anticoagulant)26, as well as the presence of several immuno-competent cells (activated T lymphocytes, B lymphocytes, macrophages, HLA DR/DQ expressing cells and immunoglobulin deposits) in the vitreous and in preretinal membranes of PDR, suggests that the immune system might play an important, but still unknown, role in the early pathophysiology of DR25.

**Thiamine and Benfotiamine**

Blockade of the unifying mechanism of the pathogenesis of diabetic microangiopathy may be also obtained through another, more radical, approach in order to correct the metabolic imbalances induced by hyperglycemia. Thiamine (vitamin B1) is the cofactor of three enzymes involved in glycolysis and Kreb’s cycle: transketolase, which shifts 2,3-diphosphoglycerate (2,3-DPG) into the pentose phosphate shunt; pyruvate dehydrogenase, which links pyruvate to acetyl-CoA and channels them into the Kreb’s cycle; α-ketoglutarate dehydrogenase, which accelerates the Kreb’s cycle by turning α-ketoglutarate into succinyl-CoA. The net result is facilitation of glucose metabolism, with accumulation of highly reactive metabolites that, like 2,3-DPG, play a key role in the synthesis of AGE and the activation of DAG/PKC.

It has been demonstrated that thiamine and benfotiamine (a lipophilic analogue that can be administered orally) are able to correct increased lactate and AGE formation in cultured endothelial cells and found to prevent retinopa-thy and nephropathy in animals with experimental diabetes.27-30. In particular, it has been recently reported that thiamine and benfotiamine reduce aldose reductase mRNA expression and activity, sorbitol concentrations, and intracellular glucose while increasing the expression and activity of transketolase in human endothelial cells and bovine retinal pericytes cultured in high glucose.31 Therefore, thiamine and benfotiamine correct the polyl pathway activation induced by high glucose in vascular cells: in fact, the activation of transketolase may shift excess glycolytic metabolites into the pentose phosphate cycle, accelerate the glycolytic flux, and reduce intracellular free glucose, thereby preventing its conversion to sorbitol. These effects on the polyl pathway, together with other beneficial effects reported for thiamine in high glucose, could justify testing thiamine as a potential approach to the prevention and/or treatment of diabetic complications.

**Hypercholesterolemia Lipoproteins**

The beneficial effect on DR of a medical treatment for hypercholesterolemia is still unclear: the Early Treatment Diabetic Retinopathy Study (ETDRS) and the Wisconsin Epidemiologic Study of Diabetic Retinopathy (WESDR) pointed out a significant association between elevated serum lipid levels and increased risk of retinal hard exudates2-4,25. However, it was also reported that lowering cholesterol by therapeutic means may not be indicated for the sole purpose of decreasing the incidence and progression of these diabetic retinal lesions. In one recently published report only, the severity of retinopathy was positively associated with triglycerides and negatively associated with HDL cholesterol, being retinopathy positively associated with small and medium VLDL and negatively with VLDL size.32 In men only, DR was positively associated with small LDL, LDL particle concentration, apolipoprotein B concentration, small HDL and was negatively associated with large LDL, LDL size, large HDL, and HDL size. No associations were found with apolipoprotein A1, Lipoprotein(a), or susceptibility of LDL to oxidation.33 Elevated apolipoprotein C-III (apoC-III) levels have been reported to be associated with increased macrovascular disease risk and an independent positive association of apoC-III level with microvascular complications of type 1 diabetes was documented.34 However, neither gender nor glycemia influence LDL oxidation in vitro.35
In conclusion, the exact role of serum lipoprotein concentrations in the development of DR needs to be elucidated by further investigations.

**Cytokines Interleukines**

Functional damage and necrosis of the retinal capillaries are probably the main contributing factors to the breakdown of the blood-retina barrier and the onset of nonproliferative DR and DME in diabetic patients\(^2\)\(^-\)\(^4\),\(^3\)\(^6\). However, high levels of serum interleukine (IL)-6 seem also to influence the development of DME while other measured serum cytokines (e.g. TGF-\(\beta\), AGE, TNF-\(\alpha\)) have not been correlated to the severity of DR\(^3\)\(^6\). In particular, Funatsu et al. observed not only that a significant relationship is present between VEGF and IL-6 but also that their aqueous levels are significantly correlated with the severity of fundus findings. In these cases, both concentrations were found to be higher in the aqueous than in the plasma\(^3\)\(^7\).

In different studies, these Authors observed that the IL-6 plasma levels and the degree of the posterior vitreous detachment (PVD) may be significantly correlated with the severity of macular edema whereas the aqueous levels of VEGF and IL-6 may predict the postoperative exacerbation of macular edema in patients with NPDR after phacoemulsification for cataract surgery\(^3\)\(^8\),\(^3\)\(^9\). On the contrary, the vitreous levels of angiotensin II (AII), VEGF and IL-6 were found to be elevated in DME patients irrespective of the status of PVD, suggesting that they may promote an increase of vascular permeability without PVD\(^4\)\(^0\),\(^4\)\(^1\). However, the vitreous itself should play a still unknown role, as subjects who have posterior vitreous detachment rarely develop DME or PDR\(^2\)\(^5\).

A significant relationship between VEGF and IL-6 levels in aqueous humor and in vitreous fluid has been recently reported\(^4\)\(^2\), thus concluding that these measurements may be useful to analyze the pathogenesis of DR and to predict the progression of the retinal disease. An increased expression of IL-6 has also been observed in surgically removed epiretinal membranes\(^4\)\(^3\).

What’s more, serum and vitreous leptin, a pleiotropic cytokine with reported angiogenic activity, have been found to be higher in patients with diabetes than in those without, and vitreal concentrations were especially elevated in patients with PDR. Leptin and leptin receptors were detected in fibrovascular epiretinal membranes of diabetic subjects\(^4\)\(^3\).

Vitreal elevation of matrix metalloproteinases (MMPs)-2, -9 and their tissue inhibitors (TIMPs)-1, -4 has also been reported in diabetic patients and correlated to the severity of retinopathy\(^4\)\(^4\),\(^4\)\(^5\). MMPs are a tightly regulated family of zinc-dependent endopeptidase, capable of degrading all components of the extracellular matrix and basement membranes and affecting the cell-cell and cell-matrix interactions. Given the early histopathological features of DR (thickening of the capillary basement membrane and loss of pericytes with microaneurysm formation), these matrix degrading enzymes might play a role in the pathogenesis of DR. In fact, MMP activity seems to represent a “final common pathway” in the process of retinal neovascularization, from whatever cause\(^4\)\(^6\) and a therapeutical inhibition at this level could be potentially more attractive than targeting individual systems such as VEGF. In fact, even if this could be effectively achieved, there might be some other “escapes” by different inducers of neovascularization employing this final common pathway. MMP-9 might also be involved in the hemorrhagic transformation of the vitreous in patients with PDR\(^4\)\(^6\).

Finally, increased levels of macrophage migration inhibitory factor (MIF) have been observed in the vitreous of patients with PDR together with a significant association between MIF levels and grades of fibrous proliferation\(^4\)\(^7\),\(^4\)\(^8\). MIF is the first T cell-derived soluble lymphokine reported to prevent random migration of macrophages, to recruit them at inflammatory loci and to enhance their activity such as adherence, motility and phagocytosis. Aqueous MIF levels are significantly correlated with aqueous monocyte chemo tactic protein-1 (MCP-1) levels and the clinical stage of DR, suggesting that both MIF and MCP-1 might have a co-operative role in the progression of DR\(^4\)\(^8\).

**Endothelin-1**

Endothelin-1 (ET-1) is a peptide produced by endothelial cells that induces vasoconstriction by interacting with endothelin A receptors (ETA) on the vascular smooth muscle cells and vasodilatation by interacting with the endothelin B receptors (ETB) on the vascular endothelial cells, resulting in the release of endothelium derived nitric oxide and prostacyclin. It has been shown that hypoxia induces the ET-1 gene expression in endothelial cells\(^4\)\(^9\). The presence of an ET-1 system in the eye is well established and plays a role on ocular blood flow, glial proliferation, and col-
lager matrix contraction by the retinal pigmented epithelial cells. Immunoreactive ET-1 in human vitreous has been found to be elevated in the presence of proliferative vitreoretinopathy (PVR) (such as PDR), RD, and idiopathic epiretinal membranes. ET-1 and its receptors ETA and ETB are present in epiretinal tissue of both idiopathic and PVR membranes, thus suggesting an involvement of ET-1 in these retinal diseases. Moreover, as a result of the demonstrated association between enhanced ET-1 expression and PKC activation in early diabetes, a PKC inhibitor could be able to reverse the upregulation of ET-1. The therapeutical effect of a long-term selective blockade of the ETA receptor has also been recently evaluated in a genetic mouse model of nonobese type 1 diabetes (NOD), in order to suggest a new strategy for preventing the development of retinopathy. In this study, an associated upregulation of ET-1 and adrenomedullin (an angiogenic factor) was documented. This novel approach to DR has also been confirmed by another study, in which an ETA/ETB dual receptor antagonist was found to reverse the VEGF expression levels in the diabetic rat retina.

**Conclusion**

DR is the most severe ocular complication of diabetes, its earliest clinical signs being represented by microaneurysms and haemorrhages. Later signs include dilated, tortuous irregular veins and retinal non-profusion, leading to retinal ischaemia that ultimately results in neovascularisation. DME, which is caused by the breakdown of the blood-retinal barrier, also occurs and is responsible for a major part of vision loss, particularly in type 2 diabetes. The pathogenesis of DR is very complex. Many biochemical mechanisms have been proposed as potential explanations for the development and progression of DR. Chronic hyperglycaemia leads to oxidative injury, microthrombi formation, cell adhesion, molecule activation, leukostasis and cytokine activation. Last but not least, the retinal ischaemia induces a demonstrated overexpression of growth factors (VEGF, IGF-1, angiopoietin-1 and -2, stromal-derived factor-1, fibroblast growth factor-2, tumour necrosis factor and several others), which act in synergy in mediating the steps toward angiogenesis, including protease production, endothelial cell proliferation, migration and tube formation. Therefore, because of this complex interplay, targeting a single growth factor will be unlikely to result in therapeutic inhibition of angiogenesis. However, this important topic will be specifically analysed in a further review.

Within the near future, pharmacologic treatment will probably be available for treating and preventing the progression of DR. Antioxidant administration such as high-dose vitamin E may help to reduce the oxidative stress from diabetes and hyperglycemia, but further studies are needed to determine whether retinopathy progression can be reduced. Aldose reductase inhibitors have been developed for the prevention of retinal and neural damage in diabetes, but additional clinical trials are needed to assess whether they can delay or stop the progression of DR and macular edema effectively. A long-term selective blockade of the ET-1 receptors, once tested in humans, could also disclose a novel therapeutical approach of DR in the future.

The increasing use of medical therapies such as pharmacologic agents will also require greater communication between ophthalmologists, diabetologists, and primary care physicians. This interdisciplinary cooperation will aid in identifying those patients most at risk for vision loss and those most likely to benefit from new treatments once they become available.

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