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EDITORIAL

High dietary fructose intake: Sweet or bitter life?

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Abstract

Epidemiological data show that the consumption of added sugars as ingredients in processed or prepared foods and caloric beverages has dramatically increased. Fructose and fructose-based sweeteners are the most commonly added sugars and high-fructose corn syrup (HFCS-55: 55% fructose, 42% glucose and 3% higher saccharides) accounts for over 40% of all added caloric sweeteners. Concerns regarding the health risk of added sugar follow the demonstration that the consumption of foods and beverages high in sugars is associated with an increased prevalence of obesity, insulin resistance, dyslipidemia and, more recently, ischemic heart and kidney diseases. The molecular mechanism(s) underlying the detrimental effects of sugar are not completely understood and their elucidation is critical to provide new insights on the health risk of fructose-based sweeteners. A better understanding of the key role of fructose overconsumption in the development of metabolic disorders may contribute to planning new strategies for preventing deleterious dietary behaviors from becoming established and, thus, curbing the rise in the number of insulin-resistant, obese and diabetic populations worldwide.

Key words: Fructose; High-fructose corn syrup; Insulin resistance; Metabolic syndrome

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INTRODUCTION

The consumption of large amounts of added sugars, a prominent source of low nutrient calories used as ingredients in processed or prepared foods and caloric beverages (i.e., soft-drinks, colas) is a relatively new phenomenon. It was not until the mid-19th century that these sweeteners became widely available and consumption began to increase dramatically[1]. Today, one of the most commonly consumed added sugars is high-fructose corn syrup (HFCS-55). Normal sugar, known as saccharose, contains equimolar quantities of fructose and glucose whereas HFCS-55 syrup, synthesized by refining corn starch, contains 55% fructose and 41% glucose. HFCS-55 appeared in 1977, opening a new frontier for the sweetener and soft drink industries. In the USA where more accurate data are available, HFCS-55 accounts for the 42% of total consumption versus 16% in 1977-1978 and soft drinks account for over 70% of this intake[2]. Bray *et al*[3] noted that the average intake of HFCS-55 (the sole sweetener in US soft drinks) rose 1000% between 1970 and 1990. In other parts of the world, the problem has been less investigated than in the USA. According to a recent survey on over 3300 subjects[4], in Italy the mean daily consumption of soft drinks is 132 g/d in female teenagers, 203 g/d in male teenagers (10 to 17.9 years), 114 g/d in adult women and 147 g/d in adult men (18 to 64.9 years). It has been recently confirmed that the consumption of foods and beverage with a high content in added sugars, mainly fructose, is associated with insulin resistance, dyslipidemia and an increased rate in obesity[5-7]. In addition to effects on weight, soft drinks have been demonstrated to displace essential nutrients and contribute to overall poorer diets. Several cross-sectional studies have shown that increased soft drink intake is

related to lower consumption of milk and calcium, although the average effect sizes were small[8-10]. Soft drink consumption was also related to higher intake of carbohydrates, lower intakes of fruit and dietary fiber and lower intakes of a variety of macronutrients in cross-sectional, longitudinal and longer-term experimental studies[11-13]. The main diet issues involve a general lack of education and/or understanding of the implications with recent consumption patterns. Despite education programs to prevent obesity and diabetes worldwide, there has been little focus on the reduction of fructose and HFCS-55 in beverages.

FRUCTOSE METABOLISM

Fructose is readily absorbed and rapidly metabolized by human liver. Fructose enters the cells predominantly through the transporters GLUT5 and/or GLUT2[14]. GLUT2 has high affinity for glucose and a moderate affinity for fructose, whereas GLUT5 predominantly transports fructose with very low affinity for glucose. GLUT5 is essential for the absorption of fructose in the intestine and its expression is increased in rats or mice fed a fructose-enriched diet[15]. Both transporters are expressed in the liver, the primary site for fructose metabolism. In the liver, fructose undergoes a specific metabolism which differs markedly from that of glucose. Fructose is phosphorylated by fructokinase (ketohexokinase) to yield fructose-1-phosphate which is then cleaved by aldolase B to glyceraldehyde and dihydroxyacetone phosphate which directly forms methylglioxale[16]. Fructose can also be phosphorylated by hexokinase but the Km for fructose is much higher than for glucose which minimizes fructose phosphorylation through this pathway. Unlike hexokinase, the fructokinase pathway of fructose metabolism bypasses tightly regulated glycolytic checkpoints, especially phosphofructokinase. Thus, while glucose metabolism is *negatively* regulated by phosphofructokinase, fructose can *continuously* enter the glycolytic pathway. Therefore, fructose can uncontrollably produce glucose, glycogen, lactate and pyruvate, providing both the glycerol and acyl portions of acyl-glycerol molecules. These particular substrates and the resultant excess energy flux due to unregulated fructose metabolism will promote the over-production of triglycerides.

Another unique characteristic of fructose metabolism is the ability to raise uric acid levels. As fructokinase has no negative feedback, all fructose entering the cell is rapidly phosphorylated which can result in ATP depletion which has been well documented *in vitro* and *in vivo* in animal models and humans. ATP depletion activates enzymes of purine metabolism which degrade adenine nucleotides to uric acid via xanthine oxidoreductase with the development of hyperuricemia[17].

CLINICAL CONSEQUENCES OF HIGH DIETARY FRUCTOSE CONSUMPTION

Excessive intake of fructose, primarily in the form of added dietary sugars, has been linked epidemiologically with the development of metabolic syndrome, a cluster of clinical and biochemical features that includes abdominal obesity, insulin resistance, hypertension and dyslipidemia. It is well documented that the administration of fructose to humans induces all of the features of metabolic syndrome. A ten week trial of 32 overweight or obese individuals from 42 to 70 years demonstrated that plasma lipid and lipoprotein concentrations increased markedly during fructose consumption and were unchanged in subjects consuming glucose[18]. In addition, subjects consuming fructose developed visceral obesity (measured by computed tomography scan) and insulin resistance. Interestingly, fasting plasma glucose and insulin levels increased and insulin sensitivity decreased in subjects consuming fructose-sweetened beverages but not in those consuming glucose. Recently Le *et al*[19] reported that just one week of a high-fructose diet increased ectopic fat deposition in the liver and skeletal muscle in healthy young men without a family history of diabetes. Interestingly, healthy normal-weight offspring of patients with type 2 diabetes who are prone to develop metabolic disorders have a higher accumulation of intrahepatocellular lipids and VLDL-triacylglycerols, thus suggesting that they may be more susceptible to the development of dyslipidemia and related metabolic disorders when consuming significant amounts of fructose. A recent analysis of liver biopsies combined with survey answers from more than 400 people found a link between daily fructose consumption and increased hepatic inflammation and fibrosis[20]. A statistically significant correlation between caloric sweeteners, mainly HFCS-55, and blood lipid levels has been also assessed in a cross-sectional study among over 6000 US adults from the National Health and Nutrition Examination Survey[5]. Fructose ingestion has also been associated with higher blood pressure levels in both adolescents and adults with no previous history of hypertension[21-23].

A clinical study performed in young, healthy male volunteers found that ingestion of 3 g of fructose per kilogram of body weight per day (as a 20% fructose solution for 6 d) led to a substantial increase in plasma triglycerides and an impaired insulin-induced suppression of adipose tissue lipolysis[24]. Furthermore, a positive correlation was observed between plasma triglyceride concentration and hepatic de novo lipogenesis. These observations support the hypothesis that fructose-induced stimulation of hepatic de novo lipogenesis is indeed instrumental in increasing plasma triglycerides[24]. In a crossover study, Hallfrisch *et al*[25] fed 12 hyperinsulinemic men and 12 male controls with diets containing 0%, 7.5% and 15% of energy from fructose for 5 wk each. Total plasma cholesterol and low-density lipoprotein cholesterol concentrations were higher when the men consumed 7.5% or 15% of energy as fructose compared with starch. Plasma triacylglycerol concentrations in the hyperinsulinemic men increased as the amount of fructose increased.

TOWARDS MECHANISTIC INSIGHTS

Although detrimental effects of fructose have been established, the mechanisms whereby this happens are only now

being discovered. There is some experimental evidence that fructose enhances the production of tumor necrosis factor (TNF)-α[26,27], a potent pro-inflammatory cytokine that has been demonstrated to induce insulin resistance and lipoprotein production. Fructose may also evoke alteration of post-receptor insulin signaling. In skeletal muscle of rodents, a high-fructose diet decreased insulin receptor activation and the phosphorylation of insulin receptor substrate-1[28]. Fructose is also known to induce oxidative stress and mitochondrial dysfunction, resulting in a stimulation of peroxisome proliferator-activated receptor gamma coactivator 1-α and β (PGC1-α and PGC-1β) that drive both insulin resistance and lipogenesis[29]. Fructose effects on lipogenesis seem to be related to its ability to alter the activity of key lipogenic enzymes and transcription factors in the liver, such as pyruvate dehydrogenase kinase and sterol-regulatory-element-binding protein-1c (SREBP-1c), the principal inducer of hepatic lipogenesis[30,31]. Fructose feeding has been reported to dramatically induce SREBP1c expression, compared with feeding with regular chow. This effect was largely diminished by treating fructose fed rats with PGC1β antisense oligonucleotides[29]. The decrease in SREBP1c expression led to decreased induction of lipogenic enzymes such as fatty acid synthase, which in turn accounted for the decreased accumulation of di- and triacylglycerol within the livers of fructose fed rats. This decrease in tissue lipid content was associated with improvements in insulin action. Another protein involved in the regulation of fructose-mediated lipogenesis is the X-box binding protein (XBP)1 which regulates the expression of many proteins that are involved in endoplasmic reticulum membrane expansion, including the lipogenic enzymes[32]. XBP1 protein expression in mice is elevated after fructose feeding and is associated with the induction of critical genes involved in fatty acid synthesis[33]. In contrast, deletion of XBP1 lowers expression of SREBP1 and key lipogenic enzymes, decreases rates of hepatic de novo lipogenesis and cholesterol synthesis and, *in vivo*, decreases plasma triglyceride concentration and secretion.

Another important molecular mechanism involved in the deleterious effects of high fructose intake is the biochemical process of non-enzymatic advanced glycation. The formation of advanced glycation end products (AGEs), also named Maillard reaction, starts from Schiff bases and the Amadori product, a 1-amino-1-deoxyketose, obtained by the reaction of the carbonyl group of a sugar, like glucose, with proteins or lipids amino groups. Glycation of proteins and lipids causes molecular rearrangements that lead to AGEs. The general mechanisms of AGEs effects are: (1) the formation of cross-links between key molecules in the basement membrane of the extracellular matrix; (2) the alteration of intracellular proteins; and (3) the interaction of AGEs with specific receptors on the cell surfaces, thus altering intracellular signaling and disrupting cell function. Although an elevated level of glucose exerts a primary role in the Maillard reaction, glucose has a low chemical reactivity in comparison with others sugars such as fructose. Recent findings show that fructose produces 10 times more AGEs than glucose because the anomerization equilibrium for fructose is shifted more to the reactive, open chain form of the sugar[34]. The *in vivo* formation of fructose-derived AGEs has long been suspected but experimental evidence for their formation and chemical characterization are lacking. Several structures of glucose-induced protein glycation products have been recently identified by mass spectrometry techniques[35]. In contrast, the types of AGEs produced in the presence of high fructose consumption have not yet been structurally clarified.

The mechanism of fructose-induced hypertension has been also extensively investigated, demonstrating the involvement of endothelial dysfunction and sympathetic activation in hypertension etiology[36,37]. Besides, fructose-induced hyperuricemia seems to contribute to the development of both hypertension and renal injury. Elevated uric acid levels have been found to be an independent risk factor for hypertension in multiple studies[38]. In an animal experimental model of fructose-induced metabolic syndrome, the reduction in uric acid levels with a xanthine oxidase inhibitor resulted in prevention of systemic and glomerular hypertension as well as renal vasoconstriction induced by a high fructose diet[39]. Hyperuricemia following high fructose intake was demonstrated to be associated with increased oxidative stress, activation of the renin angiotensin system and endothelial dysfunction, thus resulting in hypertension[40-42]. Fructose ingestion has also been associated with induction of the potent vasoconstrictor endothelin-1 and treatment with the endothelin receptor antagonist has been demonstrated to prevent the development of fructose-induced hypertension and decreased plasma Ang Ⅱ levels[43].

CONCLUSION

The controversy that has existed for the last decade regarding the potential harmfulness of excess fructose is legitimate in view of the dramatic increases in both sweetened beverage consumption and the burdens of obesity and metabolic syndrome. Most recent findings suggest that high fructose intake induces a whole range of metabolic and cardiovascular alterations in both animal and human models. Nevertheless, at present there are no objective grounds to support that moderate intake of fructose or of fructose consumed with fruits or honey is unsafe. More extensive clinical and experimental studies are needed to provide new evidence on the health risk of fructose-based sweeteners. Although AGEs have been suggested to exert a key role in the organ damage evoked by fructose-fortified diets, the chemical characterization of the epitope structures of fructose-derived AGEs still needs to be elucidated by mass spectrometry techniques. Thus, future studies on qualitative-quantitative comparison among main AGEs deriving from fructose consumption are crucial for a better elucidation of the pathophysiological role of fructose overconsumption. Another unanswered question is whether or not fructose can cause hormonal disturbances in humans. Currently, results from human studies concern-

ing fructose effects on plasma leptin, insulin and adiponectin are ambiguous and further clinical data are required to clarify the potential contribution of dietary fructose to hormone dysregulation, of primary importance in the progression of metabolic syndrome. Similarly, we do not know whether there are subgroups of individuals (obese insulin-resistant patients, offspring of patients with type 2 diabetes) in whom the adverse metabolic effects of fructose may possibly be enhanced. Overall, these investigations are crucial for supporting health authorities to define new regulations and for planning new strategies to prevent deleterious dietary behaviors from becoming established. If widely implemented, these measures may help to correct unhealthy dietary habits and to develop new therapeutic approaches to metabolic disorders and the associated cardiovascular complications.

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GUIDELINES FOR BASIC RESEARCH

Role of melatonin on diabetes-related metabolic disorders

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Abstract

Melatonin is a circulating hormone that is mainly released from the pineal gland. It is best known as a regulator of seasonal and circadian rhythms, its levels being high during the night and low during the day. Interestingly, insulin levels are also adapted to day/night changes through melatonin-dependent synchronization. This regulation may be explained by the inhibiting action of melatonin on insulin release, which is transmitted through both the pertussis-toxin-sensitive membrane receptors MT_1 and MT_2 and the second messengers 3′,5′-cyclic adenosine monophosphate, 3′,5′-cyclic guanosine monophosphate and inositol 1,4,5-trisphosphate. Melatonin may influence diabetes and associated metabolic disturbances not only by regulating insulin secretion, but also by providing protection against reactive oxygen species, since pancreatic β-cells are very susceptible to oxidative stress because they possess only low-antioxidative capacity. On the other hand, in several genetic association studies, single nucleotide polymorphisms of the human MT_2 receptor have been described as being causally linked to an elevated risk of developing type 2 diabetes. This suggests that these individuals may be more sensitive to the actions of melatonin, thereby leading to impaired insulin secretion. Therefore, blocking the melatonin-induced inhibition of insulin secretion may be a novel therapeutic avenue for type 2 diabetes.

Key words: Melatonin; Circadian rhythm; Diabetes; Insulin secretion; Pancreatic β-cell; Melatonin receptor

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INTRODUCTION

From a physiological perspective, all living organisms have several common features, including a high level of robustness against external and internal perturbations. Robustness is one of the fundamental organizational principles of biological systems, and the robust design of biological systems mediates adaptation, survival and reproduction. Metabolic diseases are viewed as a breakdown of robustness in biological systems, with the disease becoming persistent if the damage cannot be repaired. Consequently, the concept of robustness can be defined as 'continuous maintenance of physiological functions' despite external and internal perturbations.

Although the human genome has remained unchanged over the last 10 000 years, our lifestyle has progressively diverged from that of our ancestors. Socially, we are people of the twenty-first century, but genetically, we remain similar to our early ancestors. In conjunction with this discordance between our ancient, genetically-determined biology and the nutritional, cultural and activity patterns in contem-

porary Western populations, many diseases have emerged. In fact, life style changes, such as nocturnality and overly rich diets, may increase the risk of metabolic diseases including diabetes and obesity[1]. Likewise, disorders of circadian rhythms have been reported as correlating with the development of metabolic diseases[2,3] and promoting glucose intolerance in humans[4]. Therefore, it is possible that the control of seasonal and circadian rhythms may be important in the prevention of diabetes.

CHRONOBIOLOGY, METABOLIC CONTROL AND DISEASE

It was realised as far back as the eighteenth century that organisms, ranging from unicellular to multicellular, exhibit inherent rhythms. Such rhythmicity plays an important role in the temporal control of a wide range of biological processes in the body, the most notable of which is metabolism[3].

The most important and well-known biological rhythm is the circadian rhythm, which is defined as the roughly 24 h cycle that characterises virtually all organisms on Earth. It is an adaptation to the periodicity at which our planet moves around its axis, which determines day length. In addition to circadian rhythms, there are ultradian rhythms, which are shorter than 24 h, and infradian rhythms, which extend beyond 24 h. To be considered a circadian rhythm, three major criteria must be fulfilled: (1) it should persist under constant external conditions, i.e. be endogenously generated; (2) it should be temperature-insensitive; and (3) it can be reset by an external stimulus, i.e. entrainment.

Tremendous advances have been made in recent years in the understanding of how circadian rhythms are controlled[2]. A complex of transcriptional factors referred to as circadian locomotor output cycles kaput (CLOCK) and brain and muscle aryl hydrocarbon receptor nuclear translocator (ARNT)-like 1 (BMAL1) controls the Period (PER) genes. This offsets oscillating feedback loops of transcription and translation, which generate waves of gene expression with a periodicity of 24 h. While this machinery is endogenously generated, it is entrained by external stimuli, of which light is perhaps the most critical one. It is also sensitive to signals from metabolism, e.g. cellular redox state has been shown to affect CLOCK activity[5]. The system is hierarchical, the suprachiasmatic nucleus in the hypothalamus being the 'master clock', with additional clock activities in numerous peripheral tissues. In fact, there is some evidence for a circadian rhythm in pancreatic islets[6]. The peripheral clocks are all thought to signal back to the 'master clock' in the suprachiasmatic nucleus.

Given the intimate relationship between circadian rhythms and metabolism, a link between the disruption of circadian rhythm and metabolic perturbation has been considered[2]. Indeed, the metabolic syndrome is more prevalent in shift workers[3], known to exhibit disturbances of the circadian rhythm, and sleep-deprivation has been associated with both obesity and type 2 diabetes[7]. Moreover, when the circadian rhythm is experimentally misaligned in humans, a profound effect on both plasma insulin and glucose levels, promoting glucose intolerance, is observed[4].

SYNTHESIS AND FUNCTION OF MELATONIN

Melatonin is an integral part of the homeostatic mechanism in the body. It signals whether light or dark prevails. Melatonin, like the neurotransmitter serotonin, is an indoleamine. It is converted in two steps from the amino acid tryptophan into serotonin (5-hydroxytryptamine, 5-HT), and then acetylated by arylalkylamine *N*-acetyltransferase (AA-NAT), the rate-limiting step in melatonin biosynthesis, before finally being converted into melatonin by hydroxyindole-*O*-methyltransferase (HIOMT)[8].

The indoleamine is mainly secreted by endocrine cells (pinealocytes) in the pineal gland, which is located in the midline of the brain, just above the posterior commissure at the dorsal edge of the third ventricle. Melatonin remains detectable after pinealectomy in some species[9], and subsequent investigations have revealed that the hormone is also produced by neurendocrine cells in the retina, Harderian glands, gastrointestinal tract and pancreas[10]. Melatonin is also produced by numerous non-endocrine cells, e.g., immune cells. Hence, while the pineal gland quantitatively accounts for the circulating pool of the hormone, substantial local synthesis also occurs in retinal and peripheral tissues such as the gastrointestinal tract.

From a physiological perspective, the most well-known role of melatonin is that as a chronobiotic factor or *zeitgeber*, adjusting the timing or reinforcing oscillations of the biological clock, i.e., entrainment[11]. As such, it is thought to participate in the control of seasonal as well as circadian rhythms. This is based on the fact that the secretion of melatonin reflects ambient light and normally exhibits a tightly regulated diurnal pattern. For this reason, melatonin is sometimes called 'the hormone of darkness'. Disruptions may occur in individuals deprived of light, e.g., shift workers or travellers across time zones. On a daily basis, melatonin has a small modulatory effect on the pacemarker activity of the circadian clock in the suprachiasmatic nucleus. On a seasonal basis, the varying lengths of the peaks and troughs of the circulating levels of melatonin follow the changes in the duration of daylight. The seasonal regulation of the nocturnal secretory duration is the primary cue regulating the reproductive function in mammals that breed seasonally.

Melatonin also affects the cardiovascular system[12] and interacts with the immune system[13]. It has also been implicated in metabolic control[14]. Given that the sites of melatonin production are widespread, its effects may be both endocrine, *via* melatonin released from the pineal gland, and paracrine/autocrine, *via* melatonin released in the vicinity of its target tissues[15]. An interesting feature of melatonin is its capacity to act as an antioxidant, owing to its chemical structure. However, melatonin does not undergo redox cycling, i.e., repeated oxidation and reduction, but is a ter-

minal or suicidal antioxidant instead[16].

From a pharmacological view, the phase-advancing effects of melatonin have frequently been exploited[17], with the indoleamine proven to be effective in the treatment of insomnia[18,19] and efficient in limiting jet lag when travelling across time zones[20]. Therefore, the administration of pharmacological doses of melatonin promotes both phase advancement and resynchronisation of the biological clock.

MELATONIN RHYTHM AND INSULIN SECRETION: AN ANTAGONISTIC RELATIONSHIP

There is favourable evidence that the circadian rhythm of melatonin influences insulin secretion and the endocrine pancreas[21,22]. Most studies conclude that the pineal gland has a suppressive effect on the activity of the β-cell, because melatonin lowers insulin levels in rats[23-25] and these effects are in agreement with a reduction in glucose tolerance[26,27]. Based on these findings, and the realization that an increased insulin level exerts an inhibitory effect on the pineal gland and melatonin[28,29], a functional antagonism between insulin and melatonin has to be assumed. This fact is even more striking when taking into account that high levels of insulin have always been measured when melatonin concentration was reduced, i.e., during the day; contrary to the situation of low levels of insulin along with high melatonin and glucose levels during the night[30]. In accordance with these results are rat studies which proved that the synthesis of melatonin declines with increasing age, whereas the synthesis of insulin and leptin increases[23], and that melatonin is able to stop the age-related insulin increase[25]. Complementary to these findings are publications reporting that melatonin levels are reduced in diabetic hamsters[28,29,31]. On the other hand, there is evidence for a diabetes-preventing effect of melatonin, whereas pinealectomy increases the risk[32,33]. Likewise, further data demonstrate that melatonin directly influences both glucose metabolism and insulin secretion from the β-cell[34-37].

That insulin secretion is controlled by circadian mechanisms is supported by studies of humans with circadian misalignment, who are reported to show profound perturbations of glucose and insulin levels[4]. The concept is supported by the assumption that there is a circadian clock in pancreatic islets[6]. Moreover, there are indications that the diurnal secretion of melatonin is altered in diabetes, particularly when neuropathy is evident[38]. Peschke *et al*.[22] reported reduced circulating melatonin levels and elevated insulin levels in type 2 diabetic patients, with a statistically significant negative correlation between both molecules. Similarly, nocturnal melatonin levels are reduced in the Goto-Kakizaki (GK) rat, a model of type 2 diabetes[22]. Also, the amounts of mRNA of the melatonin synthesizing enzymes, such as HIOMT, are altered under diabetic conditions. In addition, the concentrations of all precursors of melatonin, including tryptophan and serotonin, are diminished in the pineal glands of diabetic GK rats, and the pineal glands of diabetic GK rats contain less noradrenaline and produce less melatonin in reaction to noradrenaline *in vitro*[39]. Confusingly, animal models of type 1 diabetes, i.e., streptozotocin- and alloxan-treated rodents, exhibit either elevated[40] or decreased[41] levels of melatonin. These observations suggest a functional interrelationship between melatonin and insulin, and may indicate a reduction of melatonin in the genesis of diabetes[22]. In this context, novel results have reported that melatonin-enhanced insulin receptor kinase activity increased insulin receptor substrate 1 (IRS1) phosphorylation, thus suggesting the potential existence of a signalling pathway cross-talk between melatonin and insulin[42]. Furthermore, melatonin also increased the activity of phosphatidylinositol 3-kinase (PI-3-kinase), whereas 3',5'-cyclic adenosine monophosphate-activated protein kinase (AMPK), another important glucose transport stimulatory mediator (*via* an insulin-independent pathway), was not influenced by melatonin application[43]. Therefore, melatonin stimulates glucose transport to skeletal muscle cells through the IRS1/PI-3-kinase pathway, which implies, at the molecular level, a putative role in glucose homeostasis and possibly in diabetes[43]. Additionally, it was speculated that aging and the exposure to light at night, both of which lower melatonin levels, may contribute to the incidence and/or development of diabetes[43].

INSULIN SECRETION IN PANCREATIC β-CELLS IS ORGANIZED BY A CIRCADIAN RHYTHM

Various investigators have postulated oscillations of insulin secretion within a range of seconds, to periods of between 9 and 14 min under both *in vivo* and *in vitro* conditions[44-46]. Furthermore, in clonal pancreatic β-cells, with periods of 5 to 8 min, a rhythm was superimposed with 15- to 20-min interval fluctuations[47]. The current opinion is that they are generated by a pacemarker located within the pancreas. These observations have been made on decentralized islets of dogs[48], mice[49], rats[50] as well as those of humans[44]. Thus, in man, a circadian rhythm of enhanced insulin secretion during the day, and a decrease during the night has been described[30]. In this case, insulin and melatonin plasma concentrations change in an opposing manner during the 24-h period, i.e., melatonin peaks when insulin is at a low level, and *vice versa*. Further information on the circadian rhythms of insulin secretion was obtained from isolated rat pancreatic islets, maintained in an *in vitro* perifusion system[6]. In this case, a circadian pattern was also observed, with periods between 22 and 26 h. Adding melatonin as a *zeitgeber* during analysis of the phase responses in insulin secretion resulted in circadian phase shifts. After melatonin application, the circadian period was maintained, but the amplitude was enhanced. From this experiment, it was concluded that an endogenous oscillator is located within the pancreatic islets of the rat which regulates the insulin secretion of β-cells in a circadian fa-

shion. Additionally, important investigations in rat insulinoma cells INS1 have shown that an overnight pre-treatment with melatonin produced a marked increase in insulin secretion, 3',5'-cyclic adenosine monophosphate (cAMP)-response element (CRE)-mediated gene expression and insulin-promoter-driven luciferase gene expression in response to glucagon-like peptide 1 (GLP1) or forskolin[36]. However, prolonged exposure of INS1 cells to melatonin application (12 h) caused sensitization of cAMP-mediated responses to forskolin and GLP1. This phenomenon may represent the first evidence of a specific physiological role for melatonin-induced sensitization of pancreatic β-cells with respect to cAMP signalling[36]. On the other hand, an inappropriate time schedule for the administration of melatonin may induce supraphysiological concentrations of melatonin, thus resulting on a desensitization of melatonin receptors. Lengthy exposure to melatonin might mimic 'artificial darkness', thereby causing physiological disturbances, e.g., to glucose metabolism[51], whereas pinealectomy, which leads to melatonin depletion, appears to decrease insulin sensitivity, as well as GLUT4 gene expression[52].

MELATONIN RECEPTORS IN β-CELLS

If melatonin has direct effects on insulin secretion, its receptors should be present in islets of Langerhans, preferably β-cells. This indeed appears to be the case, as inferred from studies using the non-hydrolysable guanosine-5'-trisphosphate (GTP) analogue guanosine 5'-*O*-(3-thiotrisphosphate) and the melatonin antagonist luzindole[34], both of which block the effects of melatonin on insulin secretion from neonatal rat islets. Likewise, using molecular techniques, it was demonstrated that a melatonin receptor mRNA identical to that cloned from the rat brain is expressed in pancreas tissue of newborn rats[53]. The specificity of the single amplification product was confirmed by restriction analysis and nested PCR, indicating that it corresponds to the predicted MT_1 receptor. A possible co-expression of the MT_2 receptor in the pancreatic tissue was initially excluded[54]. Thus the results indicate that a melatonin receptor, most likely the MT_1 receptor, was located in the pancreatic islets of neonate rats and that the pancreatic islets are targets for receptor-mediated melatonin influences[34,36,37].

Since molecular results concerning the detection of a melatonin receptor was collected on whole pancreatic tissue only, it was therefore crucial to institute a cell system that allowed detection at the level of a single β-cell. To examine this aspect, a glucose-responsive, insulin-producing insulinoma cell line INS1, isolated from rats, was used. Comparable to the results of islets, the competitive receptor antagonist luzindole diminished the insulin-decreasing effect of melatonin. Moreover, PCR experiments using specific primers for the rat melatonin receptor MT_1 showed that this melatonin receptor mRNA is also expressed in the INS1 cells[35,36]. Evidence was exclusively found for expression of the MT_1 receptor in the pancreatic β-cell model INS1, in the pancreatic islet and in the whole rat pancreas. In contrast, phase-shifting effects on the insulin rhythm in isolated islets of rats after application of melatonin indicated expression of a putative MT_2 receptor on the pancreatic β-cell[6]. By using the recently developed technique of fluorescence-dye-coupled real-time RT-PCR, rat pancreatic tissue, isolated islets and INS1 cells were examined for melatonin receptor transcript expression. Experiments succeeded in amplifying MT_1 as well as MT_2 mRNA-derived PCR products, which were verified by gel electrophoresis and restriction analysis[55]. A quantitative comparison of MT_1 versus MT_2 receptor expression for islet-derived transcripts indicated that the MT_2 transcript concentration is much lower (86-fold) in this tissue compared to MT_1 mRNA level. This low level expression possibly explains the lack of conclusive results for the existence of the MT_2 receptor in earlier studies[34,35]. Recently, molecular and immunocytochemical investigations have established the presence of the melatonin membrane receptors MT_1 and MT_2 in human pancreatic tissue and, notably, also in the islets of Langerhans[56]. On the other hand, an upregulation of the expression of melatonin receptors in type 2 diabetic patients was also observed in immunocytochemical investigations[21]. In addition, the transcription factors RORα, RZRβ and RORγ were detected in human pancreatic tissue and islets. In correlation with membrane melatonin receptors, data indicated increased mRNA expression levels of RORα, RZRβ, and RORγ in type 2 diabetic patients. Thus, the data demonstrate the existence of the melatonin membrane receptors MT_1 and MT_2, as well as mRNA expression of nuclear orphan receptors in human pancreatic tissue, with upregulated expression levels in type 2 diabetic patients[21]. These data on nuclear melatonin receptors are still preliminary, but complement the results on the better characterized membrane receptors MT_1 and MT_2, without claiming to imply connections to specific functions for insulin secretion within the islet.

SIGNAL TRANSDUCTION OF MELATONIN RECEPTORS IN β-CELLS

It is widely accepted that melatonin exerts some of its biological effects through specific, high-affinity, pertussis toxin-sensitive, inhibitory G protein (Gi)-coupled receptors[53,54]. Several physiological studies have focused on MT_1 receptor-mediated effects on insulin secretion. These studies employed either rat pancreatic islets[34,37] or the rat insulinoma β-cell line INS1 and receptor antagonists like luzindole[35,36], and confirmed the inhibitory effects of melatonin on cAMP-stimulated insulin secretion, which are mediated via Gi protein-coupled MT_1 receptors. However, the intracellular signalling of melatonin in pancreatic β-cells is not limited to the cAMP signalling pathway. In fact, an interplay between the cAMP or 3',5'-cyclic guanosine monophosphate (cGMP) cascades seems possible in the light of the recent discovery of the second melatonin receptor isoform, MT_2, and the fact that cGMP-dependent protein kinase G (PKG) is highly expressed in rat islets[57] and

insulin-secreting β-cells lines[58]. Recently, Stumpf *et al*[59,60] shed some light on the cGMP signalling pathway in rat INS1 cells, since they showed that melatonin inhibits the second messenger cGMP and suppresses insulin secretion through MT_2 receptor activation. However, as cGMP is synthesized via the action of the membrane and, particularly, the soluble nitric oxide (NO)-dependent guanylate ciclases[55], NO synthase-transmitted effects have to be considered.

On the other hand, there is evidence for the involvement of the inositol 1,4,5-trisphosphate (IP_3) system in the signalling cascade of melatonin in a growing number of cell types. In contrast to the uniform cAMP-dimishing effect of melatonin, both IP_3-increasing[61,62] as well as IP_3-decreasing[63,64] effects of melatonin have been described in different cell types. Previous studies in INS1 insulinoma cells indicated a dose-dependent stimulation of IP_3 release by melatonin, while the competitive melatonin receptor antagonist luzindole was able to completely abolish such IP_3-liberating effects of melatonin, thus giving strong evidence for the involvement of melatonin receptors[65]. Furthermore, it was also shown that such a melatonin-induced IP_3 liberation was able to release Ca^{2+} from intracellular stores[65], a mechanism that is commonly accepted as a trigger for insulin secretion. Despite *in vitro* expressed melatonin receptors exhibiting differential abilities to stimulate phospholipase C (PLC) through Gq proteins[66,67] and the MT_1 receptor having been shown to couple with Gq proteins in an agonist-dependent and guanine nucleotide-sensitive manner in HEK293 cells[68], the melatonin-dependent stimulation of PLC through Gq-coupled MT_1 receptor can only be hypothesized in pancreatic β-cells. In this regard, it has been reported that stimulation of INS1 cells with melatonin provokes the release of IP_3[65,69], and when Gi coupling is blocked by pertussis toxin, a stimulatory effect of melatonin is revealed[69]. In conclusion, it was found that the melatonin receptors on β-cells are coupled to three parallel signalling pathways, with different influences on insulin secretion. In terms of insulin release, the insulin-inhibiting action of melatonin is transmitted by the dominantly expressed MT_1 receptor through attenuation of Gi-coupled adenylate cyclase activity, thereby negatively modulating incretin-induced rises in cAMP. Likewise, it was recently detected that melatonin inhibits the cGMP signalling pathway and, consequently, insulin secretion, possibly in a MT_2 receptor-mediated fashion. Meanwhile, melatonin-dependent IP_3 release may play a role in the short-term support of other IP_3-releasing agents, like acetylcholine, or may be related to the activation of protein kinase C (PKC) or the long-term regulation of β-cell functions with enhancing effects on insulin secretion.

MELATONIN MODULATES DIABETES-RELATED ALTERATIONS

Hyperglycemia is the backbone of the pathophysiology of diabetes, leading to the development of complications like diabetic neuropathy or vascular diseases, through many intertwined cellular pathways which have been shown to coalesce into a common fate, i.e. oxidative stress. Vascular diseases are major long-term complications in patients with diabetes. For instance, vascular production of both excessive reactive oxygen species (ROS) and excessive reactive nitrogen species (RNS) may contribute to endothelial dysfunction during diabetes, as well as modification of low density lipoproteins induced by high glucose concentrations[70]. In addition to their ability to inflict direct damage on macromolecules, ROS and RNS activate a number of cellular stress-sensitive pathways, including nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and p38 mitogen-activated protein kinase (MAPK) pathways, which play a key role in the development of not only the late complications in type 1 and type 2 diabetes, but also the insulin resistance and impaired insulin secretion seen in type 2 diabetes [71]. Pancreatic β-cells are very susceptible to oxidative changes because they possess only low antioxidative capacity[72]. In this sense, it has been suggested that antioxidant treatment might be an important therapeutic option for preventing vascular complications in diabetes[73]. Since melatonin provides both *in vivo* and *in vitro* protection at the level of cell membranes, mitochondria and nucleus, due to its free-radical scavenging and antioxidant properties[74], the relationship between melatonin and the impaired antioxidant status in diabetes has become a topic of great interest in the last few years[75,76].

For some decades, alloxan and streptozotocin have been widely used to induce diabetes in animals. Both compounds lead to selective destruction of pancreatic β-cells, as they rapidly accumulate in β-cells, where they induce radical-generating reactions. Alloxan, once inside the cells, produces ROS, specially superoxide anions and hydrogen peroxide, thereby consuming reduced glutathione (GSH) and further weakening the cellular antioxidant defence system[77]. There is no question that melatonin, due to its well-established importance as a free-radical scavenger, protects against alloxan- and streptozotocin-induced diabetes. Thus, it was postulated that melatonin may protect against alloxan-induced diabetes in mice[78] and attenuate diabetes-induced alterations in the GSH redox state and in the hydroxyl radical levels in rabbit[79]. This fact was also confirmed at the level of perifused pancreatic islets[80,81]. Furthermore, it was demonstrated that melatonin can effectively scavenge alloxan-induced production of hydroxyl radicals, and inhibit hydroxyl radical-triggered lipid peroxidation in liposomes[82]. Melatonin also reduces morphological damage of the β-cells after application of alloxan, and counteracts alloxan-mediated leakage of insulin from pancreatic β-cells[81,82]. Additionally, melatonin has been shown to restore the reduced levels of nitric oxide, glutathione peroxidase and superoxide dismutase to normalcy in alloxan-induced diabetes[83], thus highlighting the putative use of melatonin to prevent atherosclerosis and other complications arising from diabetes. On the other hand, alloxan-induced diabetes may decrease pineal melatonin synthesis in rats by reducing the activity of HIOMT, thus resulting in a drop in pineal melatonin secretion[41].

During metabolism of streptozotocin, a variety of toxic intermediates are produced. Besides alkylating agents like methyl cations and methyl radicals[84], it has been shown that ROS are produced by streptozotocin as well[85]. Moreover, streptozotocin liberates NO which has been proposed to be one of the key intermediates of its toxicity[86]. Taken together, streptozotocin-induced diabetes increases oxidative stress through generation of free radicals[87], lipid peroxidation, superoxide dismutase, protein glycosylation[88], decreased levels of catalase and glutathione peroxidase[89], as well as DNA single-strand breaks[85]. In the serum of animals with streptozotocin-induced diabetes, melatonin remarkably reduces the degree of both lipid peroxidation and protein glycosylation[88], decreases the levels of cholesterol, triglyceride, low-density lipoprotein[90], sialic acid[91] and glucose[92], as well as possibly regulating the activities of antioxidant enzymes[89]. Nevertheless, the most pronounced effect of melatonin administration was the prevention of an increase in NO levels in blood plasma during streptozotocin-induced diabetes[93], which implies that melatonin may operate as an NO scavenger and carrier. Despite this fact, another investigation concluded that the protective effects of melatonin against streptozotocin-caused β-cell damage may be related to interference with DNA damage and poly(ADP-ribose) polymerase (PARP) activation rather than through effects on NO pathways[94]. On the other hand, streptozotocin-induced diabetes reduced the nocturnal pineal melatonin content in Syrian hamsters[29], but not in rats[31], and the plasma and saliva melatonin levels in type 1 and type 2 diabetic patients[95]. Also, stretozotocin-induced diabetes resulted in lower melatonin levels in the pancreas, kidney and duodenum compared to the control, thus suggesting that the lower amplitude of melatonin in target organs induced by streptozotocin might contribute to the desynchronization of daily rhythms and might also weaken the antioxidant capacity of tissues[96].

Diabetic neuropathy is counted among the most prevalent and incapacitating complications of diabetes, and is associated with clinically significant morbidities[97,98]. As mentioned earlier, ROS are also notorious for contributing to cell and tissue dysfunction and damage in diabetic neuropathy. It is assumed that prolonged hyperglycemia, through overproduction of ROS, is likely to damage dorsal root ganglion mitochondrial DNA, thus contributing to long-term nerve dysfunction[99]. Likewise, such an overproduction of ROS ultimately leads to exhaustion of natural antioxidant pools in the vascular endothelium and Schwann cells of the sciatic nerve, which in turn may contribute to the neurovascular and metabolic deficits in diabetic neuropathy[100]. In this context, the beneficial effects of various antioxidants in experimental diabetic neuropathy have been shown in the last few years[101-103]. As a matter of fact, previous studies indicate that melatonin is neuroprotective in streptozotocin-induced rat model of diabetic neuropathy[104]. Once again, it would appear that the quenching of free radicals by melatonin might be the central mechanism for exerting neuroprotection[105,106], although other melatonin-induced neuroprotective mechanisms cannot be ruled out. Thus, recent evidence in an experimental model of diabetic neuropathy suggests that melatonin modulates neuroinflammation by inhibiting the NF-κB pathway and downstream mediators of inflammation, and protects against oxidative stress by upregulating the nuclear erythroid 2-related factor 2 (Nrf2) pathway, thereby contributing to melatonin's neuroprotective effect[107]. Apart from its abovementioned effects, melatonin has been shown to forestall many other diabetes-related complications, such as altered pain perception[108], fatty liver[109], obesity[110] and renal[76] injuries, although mechanisms underlying such beneficial effects of melatonin need to be clarified further.

ASSOCIATION BETWEEN POLYMORPHISMS OF THE *MTNR1B* LOCUS AND DIABETES

Melatonin receptor deficiency or malfunction has been related to various diseases. Changes in insulin secretion observed in MT_2 variants[111] and melatonin effects on glycogen synthesis mediated through an atypical PKC (PKCζ)-Akt-glycogen synthase kinase 3β (GSK3β) signaling pathway[112] may be interpreted in this context. Likewise, the finding that the MT_1 knockout causes insulin resistance in mice[113] seems to support the general idea of intact melatonin signaling required for avoiding type 2 diabetes, but may also be indicative of species differences between mice and humans. Recently, genome-wide association studies revealed a close link between specific single nucleotide polymorphysms (SNP) of the melatonin MT_2 receptor (*MTNR1B*) locus and a prognostic risk of type 2 diabetes[114-116]. In fact, these studies present evidence that a particular SNP (rs10830963) significantly increases the risk of type 2 diabetes in the European cohorts examined. The coding sequence of *MTNR1B* is interrupted by a single intron and the SNP rs10830963 is localised within the non-coding intron sequence, although it does not interfere with consensus sequences for transcription factors or with splicing[115]. However, this SNP is correlated with higher fasting glucose levels and a high incidence of this allele is also correlated with pathologically altered insulin secretion responses[114].

A second SNP with modulatory effects on the glucose metabolism in populations of European origin has been identified[117]. This SNP is again correlated with increasing fasting glucose levels in carriers of this allele. Nevertheless, it is not correlated with obesity or body mass index, which are type 2 diabetes risk factors. This SNP is localised in the 5' promoter region of the *MTNR1B* locus and may thus influence transcription. It was independently published that the *MTNR1B*-associated SNPs rs10830962, rs4753426 and the aforementioned rs10830963 were all significantly linked to higher fasting plasma glucose concentrations and reduced insulin release in German cohorts[118]. Moreover, the intron-localised risk allele rs10830963 is not restricted to cohorts of Caucasian origin, but also occurs in Han Chinese individuals[119]. These studies suggest that de-

fined single nucleotide base pair variations in the vicinity of the *MTNR1B* locus, or overlapping with it, are causally linked to an increased risk of developing type 2 diabetes. In fact, increased MT_2 receptor transcription in human islets from the risk genotype with an intron-localised SNP was reported, compared to transcription from the non-risk allele[114]. This observation may be viewed in a broader context with the increased MT_2 and MT_1 receptor expression observed in pancreas explants of type 2 diabetes patients[21]. Despite the fact that only one SNP is located in the 5' regulatory region of *MTNR1B*, these studies suggest that changes in β-cell MT_2 receptor expression may be responsible for the development of type 2 diabetes. It can therefore be speculated that increased melatonin receptor expression due to its coupling with Gi proteins leads to decreased second messengers (cAMP or cGMP) levels, with subsequent detrimental effects on insulin secretion[114].

CONCLUSION

Melatonin is a pleitropic, nocturnally peaking and systemically acting chronobiotic. Several generalizations can be proposed regarding melatonin. Since it readily passes all biological membranes to reach intracellular organelles, many cells can synthesize melatonin, presumably to scavenge the oxygen- and nitrogen-based reactants produced in these cells, and, moreover, its membrane receptors are widespread in mammals and mediate some of the melatonin's actions. It has been determined that the effects of melatonin on insulin secretion are mediated through the melatonin receptors (MT_1 and MT_2). By inhibiting cAMP and/or cGMP pathways, melatonin reduces insulin secretion. However, it has been shown that melatonin activates the PLC/IP_3 pathway, which mobilises Ca^{2+} from intracellular stores and, subsequently, increases insulin secretion. Meanwhile, insulin secretion, both *in vivo* and *in vitro*, exhibits a circadian rhythm, apparently generated within the islets, which is influenced by melatonin by inducing a phase shift in insulin secretion. The observation that clock genes exhibit circadian expression in pancreatic tissue could be an indicator of the generation of circadian rhythms in the pancreatic islets themselves. Also, plasma melatonin levels and AA-NAT are decreased in type 2 diabetes patients. Taken together, these results indicate a close interrelationship between insulin and melatonin, which may be significant for the genesis of diabetes. This has been recently supported by genome-wide association studies revealing a close link between SNPs of the MT_2 receptor (*MTNR1B*) locus and an increased prognostic risk of type 2 diabetes. Time will tell whether MT_2 antagonists could serve as therapeutic agents in type 2 diabetes. The option of blocking the effect of melatonin in islets is an attractive possibility, although an islet-specific attenuation of melatonin action may be required, since it can be foreseen that the systemic effects of an MT_2 blockade may be disadvantageous. Nevertheless, the discovery of the link between the MT_2 receptor and type 2 diabetes emphasises the importance of biological rhythms for metabolic regulation.

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REVIEW

Vascular dysfunction in diabetes: The endothelial progenitor cells as new therapeutic strategy

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Abstract

The vascular endothelium is a critical determinant of diabetes-associated vascular complications, and improving endothelial function is an important target for therapy. Diabetes mellitus contributes to endothelial cell injury and dysfunction. Endothelial progenitor cells (EPCs) play a critical role in maintaining endothelial function and might affect the progression of vascular disease. EPCs are essential to blood vessel formation, can differentiate into mature endothelial cells, and promote the repair of damaged endothelium. In diabetes, the circulating EPC count is low and their functionality is impaired. The mechanisms that underlie this reduced count and impaired functionality are poorly understood. Knowledge of the status of EPCs is critical for assessing the health of the vascular system, and interventions that increase the number of EPCs and restore their angiogenic activity in diabetes may prove to be particularly beneficial. The present review outlines current thinking on EPCs' therapeutic potential in endothelial dysfunction in diabetes, as well as evidence-based perspectives regarding their use for vascular regenerative medicine.

Key words: Diabetes mellitus; Vascular dysfunction; Endothelial progenitor cells

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VASCULAR FUNCTION AND DYSFUNCTION IN DIABETES

Diabetes is a metabolic disorder which is characterized by hyperglycemia and glucose intolerance due to insulin deficiency, impaired effectiveness of insulin action or, both.

Type 1 diabetes mellitus is caused by cellular-mediated autoimmune destruction of pancreatic islet beta cells, leading to loss of insulin production. It usually starts during childhood, but can occur at all ages. Type 2 diabetes mellitus accounts for 90%-95% of all diabetes and is more commonly found in people older than 45 who are overweight. There is strong evidence that genetics plays an important role as well. However, the prevalence of type 2 diabetes mellitus is increasing in children and young adults, mainly because of the higher rate of obesity in this population. Obesity, insulin resistance associated with diabetes, high cholesterol and high blood pressure form the most impor-

tant risk factors for cardiovascular disease (CVD). CVD is the major cause of death in people with type 2 diabetes mellitus[1].

The vascular manifestations associated with diabetes mellitus result from the dysfunction of several vascular physiological components, mainly involving the endothelium, vascular smooth muscle and platelets[2]. Over the last two decades it has become evident that the endothelium is not an inert, single-cell lining covering the internal surface of blood vessels, but in fact plays a crucial role in regulating vascular tone and structure. Importantly, a healthy endothelium inhibits platelet and leukocyte adhesion to the vascular surface and maintains a balance of prothrombic activity[3]. Hyperglycemia is the major causal factor in the development of endothelial dysfunction in diabetes mellitus. Although the mechanisms underlying this phenomenon are likely to be multifactorial, insulin resistance has been identified in several diseases that increase cardiovascular risk and mortality, such as diabetes, obesity, hypertension, metabolic syndrome, and heart failure[4].

In health, endothelial cell injury is mitigated by endogenous reparative processes. In diabetes sufferers, the imbalance in repair and injury results in micro-vascular changes, including apoptosis of micro-vascular cells, ultimately leading to diabetes-related complications.

Dysfunction of the endothelium in diabetes mellitus is characterized by changes in proliferation, barrier function, adhesion of other circulating cells, and sensitivity to apoptosis. Furthermore, it is suggested that diabetes mellitus modifies the angiogenic and synthetic properties of endothelial cells[5].

A variety of markers indicates endothelial dysfunction in diabetes mellitus, including poor EC-dependent vasodilation, increased blood levels of the von Willebrand factor (vWF), thrombomodulin, selectin, plasminogen activator inhibitor-1 (PAI-1), type Ⅳ collagen and tissue plasminogen activator (t-PA)[6]. Endothelial dysfunction is an early manifestation of vascular disease in type 2 diabetes patients but late in the course of those with type 1 diabetes[7]. Furthermore, studies have shown that the levels of vascular cell adhesion molecule 1 (VCAM-1) were more markedly elevated in type 1 diabetes patients with diabetic retinopathy, than in those patients with micro- or macroalbuminuria, whereas no difference in inter-cellular adhesion molecule 1 (ICAM-1) and endothelial-leukocyte adhesion molecule 1 (ELAM-1) levels was apparent in diabetes patients without diabetic retinopathy[8].

The loss of the endothelium modulator role may be a critical and initiating factor in the development of diabetic vascular disease. Endothelial dysfunction plays a key role in the pathogenesis of diabetic vascular disease. The endothelium controls the tone of underlying vascular smooth muscle through the production of vasodilator mediators. The endothelium-derived relaxing factors (EDRF) comprise nitric oxide (NO), prostacyclin, and a still-elusive endothelium-derived hyperpolarizing factor (EDHF). Impaired endothelium-dependent vasodilation has been demonstrated in various vascular beds of different animal models of diabetes, and in humans with type 1 and 2 diabetes[9-12]. Several other mechanisms of endothelial dysfunction have been reported, including impaired signal transduction or substrate availability, impaired release of EDRF, increased destruction of EDRF, enhanced release of endothelium-derived constricting factors, and decreased sensitivity of the vascular smooth muscle to EDRF. The principal mediators of hyperglycaemia-induced endothelial dysfunction may be activation of the protein kinase C, increased activity of the polyol pathway, and non-enzymatic glycation[13]. It is also known that hyperglycemia-induced oxidative stress plays a role in the development of vascular dysfunction[1]. In general, diabetic microvascular complications are typically associated with dysregulation of vascular remodeling and vascular growth with decreased responsiveness to ischemic/hypoxic stimuli and impaired or abnormal neovascularization[14].

Lack of endothelial regeneration and impaired angiogenesis contribute to the progression of diabetic micro- and macrovascular complications. Formation of stable vasculature in response to tissue injury is an essential event for the restoration of blood flow and the repair of affected tissue areas. Currently, clinical management of diabetic complications relies exclusively on pharmacological therapeutics that, in most cases, minimally affect the endothelial repair or regeneration, and, therefore these treatments have a modest influence on end organ dysfunction. Hence there is a need for therapeutic interventions that can accelerate the repair ofdysfunctional endothelium in the end organ, and restore blood flow, resulting in functional tissue generation. A promising novel therapeutic option for the replacement of damaged endothelial cells, i.e. re-endothelialization, as well as for the neovascularization of ischemic tissues, is the use of progenitor cells. In vascular biology, progenitor cells were first identified by Isner and Asahara in 1997, and they are known as endothelial progenitor cells (EPCs), able to initiate neovascularization[15].

EPCS: A BIOMARKER OF VASCULAR DYSFUNCTION IN DIABETES

The discovery of EPCs in human peripheral blood has advanced the field of cell-based therapeutics for many pathological conditions. It is known that EPCs could be released from bone marrow, fat tissue, vessel walls, especially adventitia, and possibly also from the spleen, the liver, and the intestine, into the blood, where they express CD133 at the early stage, then CD34/Flk-1, and also VEGFR2[16]. Experimental studies have shown that EPCs can be isolated from peripheral, umbilical cord, and bone marrow blood, and identified by specific markers, using flow cytometry. In most published studies, the amounts of circulating EPCs are determined by a culture[17]. EPCs are defined as fibronectin-adherent peripheral blood-derived cells uptaking acetilated low-density lipoprotein (LDL) and binding Ulex-selectin in culture, and then further characterized by the expression of surface markers. Peichev *et al*, showed that circulating CD34+CD133+KDR+

cells give rise to endothelial cells *in vitro*, and thus functionally correspond to the definition of EPCs. Therefore-three-fluorescence analysis of this cell subset may be another simple and elegant way to unambiguously identify and quantify circulating EPCs without culturing them[18]. At present there is no general agreement on methods for defining EPCs, and different studies have used different ways of identification and isolation.

It has been indicated that a strong correlation between cardiovascular risk factors and EPC number and function exists[19]. Diabetes mellitus has also been shown to adversely affect EPCs' number and function[20,21], and it has been suggested that a reduction in the number of EPCs might be useful as a surrogate marker of vascular dysfunction in diabetes[22]. As for the function of EPCs in diabetes, it has been shown that EPCs have decreased migratory ability, reduced proliferative capacity, and an altered cytokine/growth factor secretory profile. Changes in the function of EPCs decreases their repair mechanisms[14]. Consequently, the idea of using EPCs as therapeutic agents has grown in popularity.Successful exploitation of EPCs is a complex, multi-step process that includes mobilization, homing to specific sites, adhesion, further differentiation, and functional integration[22].

The first experimental studies for using EPCs as biomarkers of vascular dysfunction in diabetes were done in animal models. A possible role for EPCs in diabetic vascular disease was first investigated in mice. Infusion of human CD34-positive leukocytes, as an EPC-enriched population, was able to accelerate blood flow restoration in diabetic nude mice with experimental hind limb ischemia, but did not have this effect in non-diabetic animals[23]. The reason for the different response of diabetic and non-diabetic mice to the administration of EPCs was not clear, but it could be due to the fact that blood flow restoration in non-diabetic animals was largely provided by physiological ischemia-induced neovascularization, which is hampered in diabetic animals. It is therefore possible that exogenous cells have beneficial effects only in diabetic animals who have either a reduced level or compromised EPC function. Indeed, reduced angiogenic potential of EPCs has been demonstrated in diabetic animals[24].

In type 1 and type 2 diabetic patients, the reduction in circulating EPCs and the functional impairment of cultured EPCs has been reported. Tepper *et al*, showed that peripheral blood mononuclear cells (PBMC)-derived EPCs isolated from type 2 diabetic patients displayed a proliferation rate in culture decreased to control subjects, a weaker adherence to activated human umbilical vein endothelial cells (HUVEC), and a reduced incorporation into vascular structures *in vitro*[20]. Loomans *et al*, reported almost identical results in type 1 diabetic patients[21].

The rate of EPC proliferation from plated PBMCs in diabetic patients was inversely correlated with the levels of glycated hemoglobin, suggesting a possible relation between glucose control and EPC function. Poor adhesion of EPCs to HUVECs demonstrated altered cell-to-cell interactions, which could indicate that EPCs are recruited less avidly *in vivo* at sites of ischemia, as well that re-endothelization by means of bone-marrow derived cells is less likely to take place in the presence of EPC dysfunction. Moreover, Lambiase et al., have shown that poor coronary collateral development (typical for diabetes), may be related to low levels of circulating EPCs[25].

THE MECHANISMS GOVERNING EPCs' ROLE IN DIABETES

Mechanisms underlying the reduction of EPCs in diabetes are largely unknown. Weak bone marrow mobilization, impaired peripheral differentiation, and short survival in peripheral\blood are all candidates. Several mobilizing factors, such as granulocyte colony-stimulating factor (G-CSF), stromal cell derived factor-1 (SDF-1), vascular endothelial growth factor (VEGF), and erythropoietin (Epo) *via* AKT protein kinase pathway activation and endothelial nitric oxide synthase (eNOS), were demonstrated to mediate EPCs' mobilization, proliferation, and migration.

It was revealed that myocardial infarct size in the rat is increased in hyperglycemic conditions, and is associated with a reduced expression of the hypoxia-inducible factors 1 (HIF-1) gene[26]. Chemokine (C-X-C motif) ligand 12 (CXCL12), also known as SDF-1, and its receptor C-X-C chemokine receptor type 4 (CXCR4) both play a critical role in regulating hematopoietic cell trafficking[27]. In non-obese diabetic (NOD) mice, the onset of diabetes is significantly delayed by reducing the level of CXCL12, either by antibody-mediated neutralization or G-CSF-induced suppression of CXCL12 transcription[28,29]. Despite these initial observations, however, how chemokine CXCL12 affects the development of type 1 diabetes has not been fully investigated. Bruhl *et al*, revealed a dose-dependent relation between levels of p21Cip1, that controls cell cycle progression and apoptosis in mature endothelial cells, and levels of circulating EPCs in double and single p12Cip1 knockout mice[30]. In rats with streptozotocin-induced diabetes, circulating EPC levels were reduced, compared to controls and associated with uncoupled eNOS in bone marrow[31].

In particular, it was found that the expression of angiogenic factors VEGF and HIF-1 is reduced in the hearts of diabetic patients during acute coronary syndromes in comparison with control subjects[32]. Moreover, impaired cell-to-cell interactions of EPCs cultured from diabetic subjects could reflect alterations in the so-called "stem cell niche" that hampers cytokine-induced mobilization of stem cells[33]. There is much data supporting the theory that EPCs might decrease because of increased apoptosis. Also, another study shows that EPCs are better protected against oxidative stress than are mature endothelial cells, and therefore it seems unlikely that the decrease in number and dysfunction of EPCs is mediated by increased oxidative stress[34]. Furthermore, EPCs dysfunction in type 2 diabetes patients was associated with oxidative stress due to excessive generation of reactive oxygen species (ROS). It was shown that prolonged exposure of EPCs to high

glucose concentrations *in vitro* increased superoxide anion production, and reduced NO bioavailability[35]. Generation of superoxide anions appears to take place by several processes including glucose auto-oxidation, and increased protein kinase C (PKC) and nicotinamide adenine dinucleotide phosphate-oxidase (NADPH oxidase) activity[36]. Moreover, in diabetic patients, the presence of advanced glycation end-products (AGEs) adducts on basement membrane and compromises repair by EPCs with implications for vaso-degeneration during the micro-vasculopathy[37].

NOVEL INSIGHTS INTO THE POTENTIAL THERAPEUTIC USEFULNESS OF EPCS

EPCs have recently generated considerable attention as potential novel diagnostic/prognostic biomarkers for vascular integrity, and therapeutic clinical approaches using these cells are ongoing[38]. There is evidence that some drugs that positively affect vascular function in diabetic patients, could also improve the function and number of circulating EPCs. Thus, it appears that the vasculo-protective effect of these compounds may partly be due to their action on EPCs.

Ohshima *et al*, demonstrated that antioxidant therapy with superoxide dismutase (SOD) in diabetic mice reduced oxidative stress, and increased EPC count and their potential to differentiate into endothelial cells[39]. In addition, a new inhibitor of CXCR4, AMD3100, was found to accelerate blood flow restoration to ischemic tissue in diabetic mice[40]. Also, the treatment with AMD3100 in non-obese diabetic mice abolished T-cell accumulation in the bone marrow and simultaneously inhibited disease development[41].

Notably, it was shown that the angiotensin-converting enzyme (ACE) inhibitors such as ramipril[42], enalapril[28] and *angiotensin* Ⅱ (AT Ⅱ) inhibitors, like valsartan[43] increased EPC levels in patients, probably interfering with the CD26/dipeptidylpeptidase Ⅳ system. Other studies suggested that either the phosphatidylinositol 3-kinase/Akt/endothelial nitric oxide synthase/NO (*PI3K/Akt/eNOS/NO*) signaling pathway or the interaction between hyperglycemia and hyperlipidemia in diabetic patients who have vascular diseases, are potential therapeutic targets for abolishing the impaired function of EPCs[44]. Neutralization of the $p66^{ShcA}$ gene, which regulates the apoptotic response to oxidative stress, prevented high glucose-induced EPC impairment *in vitro* [45]. The existence of molecules acting on EPCs can be used to positively condition cultured EPCs before therapeutic transplantation. Thus, because it is known that chemokine SDF-1α is able to mobilize EPCs, and because EPCs are known to have receptors for SDF-1α, it was demonstrated that SDF-1α - primed EPCs exhibit increased adhesion to HUVEC, resulting in more efficient incorporation of EPCs into sites of neovascularization[46]. Also, it has been shown that platelets promote the homing and differentiation of EPCs at sites of vascular injury[47]. Furthermore, it was hypothesized that circulating microparticles (MPs) are able to program stem/ progenitor cells to repair tissue injury. In particular, it was speculated that MPs of endothelial origin may operate to induce differentiation of *bone marrow*-derived progenitor cells into endothelial cells and subsequently promote postnatal vasculogenesis. Moreover, the treatment with AMD3100 in diabetic patients improved wound healing by correcting EPC mobilization and homing[49]. AMD3100 is now approved for use as a mobilization agent of EPCs in the United States; new data have provided enticing evidence regarding its therapeutic effect in human myocardial infarction[50].(Table 1)

Table 1 Summarizes the potential therapeutic targets to increase EPC number or function

Signaling pathways	Specific drugs
Angiotensin-Convering-Enzyme (ACE)	Ramipril; Enalapril
Angiotensin Ⅱ	Valsartan
PI3-K/Akt/eNOS/NO	Statins
Reactive oxygen species (ROS)	SOD
CXCR4	AMD3100

Another way to improve vascular dysfunction could beby means of a therapy using EPC transplantation. In a very recent study it was demonstrated that administration of circulating human EPCs intravenously had beneficial effects on ischemic brain injury in a mouse model of transient middle cerebral artery occlusion[51]. Transplantation of human cord blood-derived EPCs was reported to contribute to neovascularization in various ischemic diseases, and EPC transplantation on diabetic wounds has a beneficial effect, mainly achieved by their direct paracrine action on keratinocytes, fibroblasts, and endothelial cells, rather than through their physical engraftment into host tissues (vasculogenesis). In addition, an EPC-conditioned medium was shown to be therapeutically equivalent to EPCs, at least for the treatment of diabetic dermal wounds[52].

CONCLUSION

Therapeutic interventions do not necessarily restore a proper endothelial function and, when they do, may improve only some of these variables. Bone marrow-derived circulating EPCs might be a better alternative. For over 10 years, EPCs have been studied as a novel biomarker to assess the severity of diabetes, and as a potential new strategy in regenerative medicine.

Although the role of EPCs in these processes is well established, the challenge for the next decade is to identify and evaluate methods that increase EPC homing and incorporation, thereby enabling targeted delivery of EPCs to a site of interest. This goal might be achieved through the continued characterization of EPCs in animals and humans, coupled with investigations of the long-term potential of EPCs *in vivo*. Once accomplished, the therapeutic potential of this treatment modality could transform the treatment of both cardiovascular disease and diabetes.

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REVIEW

Managing diabetic macular edema: The leading cause of diabetes blindness

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Abstract

Diabetic macular edema (DME) is the leading cause of blindness in young adults in developed countries, affecting 12% of type 1 and 28% of type 2 diabetic patients. The gold standard DME treatment should be based on a good control of glycemia along with control of lipids and renal function. However, despite the systemic metabolic control values being essential for patients with diabetic retinopathy (DR), it has proven to be insufficient for DME if it appears. With these patients, additional measures are needed in order to avoid the subsequent loss of vision. While laser treatment of DME has been the only valid treatment so far, it has been inadequate in chronic cases. The introduction of new treatments, such as intravitreal corticosteroids or anti-VEGF drugs, have recently shown their safety and efficacy and together with laser photocoagulation are becoming the treatments of choice in the management of DME.

Key words: Diabetic macular edema; Diabetic retinopathy treatment

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INTRODUCTION

Diabetic macular edema (DME) is the leading cause of blindness in the diabetic population. Although its prevalence varies, the Diabetes Control and Complications Trial (DCCT) reported that 27% of type 1 diabetes (DM1) patients developed macular edema within nine years of onset[1]. Other studies indicate that in type 2 diabetic patients (DM2), prevalence increases from 3% within 5 years of diagnosis to 28% after 20 years[2]. DME tends to be a chronic disease, although spontaneous recovery is not uncommon. It is important to recognize that about 33% to 35% of patients resolve DME spontaneously after six months without treatment[3,4]. The disease is now believed to be multifactorial in origin with a number of systemic factors including hypertension, poor metabolic control of diabetes, dyslipemia and nephropathy playing a role in its pathogenesis.

EPIDEMIOLOGY

The prevalence of DME is generally higher in DM2 patients than in DM1 and our studies reflect that. Prevalence is 11.84% in DM1 and 27.15% in DM2[5]. The annual incidence of DME in DM1 ranges from 0.9% to 2.3%[6] and our studies show that the annual incidence in DM2 ranges 1.25% to 1.40%[2].

Epidemiological data for DME has shown changes with more intensive control of glycemia and blood pres-

sure. In the Wisconsin study[7], the incidence of DME at 25 years in DM1 patients decreased from 2.3% in the first 4 year cohort study (baseline between 1980-1982 and 1984-1986) to the current 0.9% incidence in a group of patients followed from years 14 to 25. The author's studies (in Spain) have also shown changes, where the prevalence of DME in DM1 has decreased from 12.90% to 11.84% and in DM2 from 7.86% to 7.15%. Furthermore, the percentage of patients treated by laser photocoagulation of the macular area has also reduced from 7.52% to 5.26% in DM1 patients and from 5.18% to 2.43% in DM2 patients[5].

Many large series studies have investigated the effect of different conditions on the incidence of DME. The frequency of DME increases with the duration of diabetes mellitus with two peaks, the first around 14 years and a second after more than 30 years[8,9]. Furthermore, poor metabolic control has been implicated with DME. Elevated diastolic blood pressure has been associated with DME and dyslipemia also increases prevalence. Our study group found a positive association with a high LDL-cholesterol and TC/HDL-cholesterol ratio in DM2 diabetics[10] and the LDL-cholesterol and TC/HDL-cholesterol ratio in DM1 diabetics was found to be a risk factor in the DCCT[11].

CLINICAL DESCRIPTION AND CLASSIFICATION

The two definitions of macular edema in diabetic patients currently used are: (1) Macular edema (ME); and (2) Clinically significant macular edema (CSME).

In diabetes related research studies, ME is often characterized by retinal thickening or the presence of hard exudates within a 1 disk diameter of the center of the macula.

To characterize the severity of macular edema, the term *clinically significant macular edema* (CSME) is used. Macular edema is clinically significant if one of the following conditions is present: (1) retinal thickening at or within 500 mm of the center of the macula; (2) hard exudates at or within 500 mm of the center of the macula if associated with thickening of the adjacent retina; and (3) a zone or zones of retinal thickening 1 disk area in size, at least part of which is within 1 disk diameter of the macular center, characterized by the retinal thickening of the macular area visible under biomicroscopy[12].

Concept of focal versus diffuse diabetic macular edema

DME is further classified into focal or diffuse, depending on the leakage pattern seen on the fluorescein angiogram (FA).

In focal DME, discrete points of retinal hyperfluorescence (leakage of intravascular liquid to interstitial space due to a vasopermeability) are present on the FA due to focal leakage of microaneurysms, the cause of retinal thickening. Commonly, these microaneurysms are surrounded by circular hard exudates[13]. A variation of this form is the multifocal macular edema which in some cases is confused with diffuse macular edema. This form appears under fluorescein angiography as multiple foci of leakage due to the presence of multiple foci of microaneurysms.

In diffuse DME, there are areas of diffuse leakage on the FA due to intraretinal leakage from dilated retinal capillary bed and/or intraretinal microvascular abnormalities (IRMA) and/or from arterioles and venules without foci of leaking microaneurysms.

Cystoid macular edema

Cystoid diabetic macular edema (CME) results from the generalized breakdown of the inner blood retinal barrier with fluid accumulation in the outer plexiform layer[14].

Classification attending OCT

The introduction of optical coherence tomography of the macular area has changed our view of DME and its classification. The visualization of the macular area and the interface between the vitreous and retina has allowed us to classify macular edema.

So now we can classify macular edema as follows[15]:

Spongiform: Sponge-like retinal swelling present in 88% of eyes with DME. This form is mostly confined to the outer retinal layers due to backscattering from intraretinal fluid accumulation, visible with hyporeflectivity at these levels.

Cystoid macular edema (CME): Large cystoid spaces involving variable depth of the retina with intervenint septae is present in 47% of all edemas and are initially mainly confined to the outer retina. In the OCT, the CME is represented by decreased intraretinal reflectivity and closely resembles its histopathology description. In eyes with long-standing cystoid macular edema, cystoid spaces fuse, resulting in a large cystoid cavity involving almost the entire retinal layer.

Serous retinal detachment (SRD): The SRD represents a 15% of all forms and is visible as an area of hyporeflectivity in the subfoveal region. This form is invariably associated to one of the two first described forms.

Tractional: Foveo-vitreal traction may result in detachment of the fovea. This can be diagnosed easily on OCT where the posterior vitreous is visible that caused traction on the fovea, resulting in underlying tractional retinal detachment.

Taut posterior hyaloid membrane (TPHM): The TPHM may result in recalcitrant macular edema with foveal detachment that can be diagnosed easily on OCT, even when subclinical. In advanced cases, it can be diagnosed clinically as a taut, shiny, glistening membrane with retinal striae on biomicroscopic retinal examination.

In the OCT, the hard exudates appear as areas of increased reflectivity with a trail of shadow behind. Furthermore, the OCT allows us to see if a macular edema is focal or diffuse.

In conclusion, the OCT gives us an *in vivo* histopathology of the retinal layers that helps in a better understanding of the disease and its pathogenesis. OCT is also a useful tool in monitoring the response to treatment.

DIAGNOSIS

The current gold standard for the diagnosis of DME is based on biomicroscopy and the ETDRS study group recommends that this diagnosis can be made if there is a thickening of the retina in macular area.

Fluorescein angiography (FA) is a standard method used for evaluating patients with DME. It is sensitive for qualitative detection of fluid leakage, even though leakage may not equate to clinical retinal edema. FA allows us to assess the severity of the characteristics of macular edema, such as fluorescein leakage and ischemic patterns[16,17].

FA is used for classifying DME into four categories: (1) Focal/multifocal leakage, well-defined focal or multifocal areas of leakage from microaneurysms; (2) Diffuse leakage, defined as the presence of widespread leakage from the retinal capillary bed or any intraretinal microvascular abnormalities (IRMA); (3) Diffuse cystoid leakage, where diffuse leakage and the pooling of dye in the cystic spaces of the macula in the late phase of angiogram is seen; and (4) Ischemic maculopathy. All these previous forms can be associated to areas of macular ischemia which can be seen as areas of capillary loss or an increase in the foveal avascular zone (FAZ). The presence of macular ischemia is an important finding in deciding the type of treatment needed and to help in those patients who suffer a loss of visual acuity of unknown origin.

Optical coherence tomography (OCT)

The current use of optical coherence tomography as a method of exploring the macular area has changed the way of diagnosing macular edema. As we said previously, OCT is an effective method of diagnosis of DME and in turn has become an essential technique for classifying the edema and observing the effect of its treatment. In the near future, OCT is likely to become the gold standard method of diagnosis and monitoring of patients with macular edema.

PATHOPHYSIOLOGY

Blood-retinal barrier concept

The pathway that results in DME is the disruption of the blood-retinal barrier (BRB). The BRB has two components: the outer and the inner barriers. The outer barrier is formed by tight junctions between retinal pigment epithelium (RPE) cells and includes zonula occludens and desmosomes. The inner barrier is formed by tight junctional complexes between retinal vascular endothelial cells and a well-differentiated network of glial cells (astrocytes and Müller cells). Several clinical studies[17-21] suggest that the inner barrier is the primary site of vascular leakage that results in DME. The disruption of the BRB leads to abnormal inflow of fluid into the neurosensory retina that can exceed the outflow and cause the accumulation of fluid in the intraretinal layers of the macula.

The mechanism of the BRB breakdown is multifactorial and secondary to changes in the tight junctions, pericyte and endothelial cell loss, retinal vessel dilatation and leukostasis and vitreo-retinal taut and traction.

Biochemical pathways

The pathogenesis includes the existence of chronic hyperglycemia, with the accumulation of free radicals, advanced glycemic end-products (AGE) proteins and protein kinase C formation and the subsequent activation of vascular growth factors (especially VEGF-A) and an increase in vascular permeability. Likewise, the appearance of areas of ischemia and inflammatory factors such as interleukin 6 also increases the synthesis of VEGF-A. All of these factors may be interrelated. For example, hypoxia and hyperglycemia upregulate VEGF-A production in diabetic retinopathy and this in turn increases vasopermeability by activating PKC. Hyperglycemia, however, can directly increase PKC and angiotensin Ⅱ, both of which cause vasoconstriction and worsening of hypoxia by their effect on endothelins[22].

One of the most important factors in the biochemical pathway is the formation of AGE, the consequence of chronic hyperglycemia. The AGE may be a primary contributor to diabetic microangiopathy. AGE has been found in the vitreous and in the ILM and can cause structural alterations in the posterior hyaloid that strengthens the vitreo-macular adhesion between the posterior hyaloid and the ILM[23]. In vascular endothelial cells, AGE may also affect the gene expression of endothelins (ET-1) and modify VEGF expression. The AGE also activates ICAM-1 in endothelial cells which increases leukocyte adhesion with the rupture of BRB[24].

Vasoactive factors

Cytokines, such as insulin-like growth factor-1 (IGF-1), on its own and in the presence of hyperglicemia (which enhances the response of retinal endothelial cells to IGF-1), overregulate the expression of VEGF in RPE cells and promote BRB disruption[25].

Other vasoactive factors, such as metalloproteases, pigment epithelium derived factor (PEDF), angiotensin Ⅱ, basic fibroblast growth factor (b-FGF) and platelet-derived growth factor (PDGF), have been implicated in the pathogenesis of DME. The matrix metalloproteinases (MMPs) regulate the degradation and modulation of the extracellular matrix that subsequently affects endothelial cell function and may cause the changes in vascular permeability. The effect of PEDF on vascular permeability is unclear despite a significant negative correlation between the vitreous level of PEDF and retinal thickness[26]. The b-FGF pathway is known to be activated in diabetes patients and plays a role in angiogenesis, stimulating endothelial cell production. Furthermore, b-FGF is produced mainly by the Müller cells in the retina and its activation results in a proliferation of astrocytes and hyalocites in the hyaloid, promoting a tight and taut hyaloid with subse-

quent DME[27]. Finally PDGF may be an important contributor to BRB maintenance by promoting the growth of retinal pericytes via PKC activation. There is evidence emerging that PDGF may be critical for the viability of pericytes[28].

Importance of Vascular endothelial growth factor-A (VEGF-A)

VEGF-A belongs to a family of different growth factors (A, B, C and D) and it has recently become accepted as one of the most potent factors in the induction of angiogenesis. Six major isoforms of VEGF-A exist: 121, 145, 165, 183, 189 and 206. VEGF-A 165 is the most important factor in the pathophysiology of DME.

VEGF is produced by RPE cells, ganglion cells, Müller cells, perycites, endothelial cells, glial cells, neurons and smooth muscle cells of the retina, of which the most important for producing VEGF are Müller cells. The upregulation of VEGF is produced by hypoxia, hyperglycemia (which itself can enhance the response of retinal cells) and cytokines, such as insulin-like growth factor 1, interleukin-6 and PKC-beta[29].

VEGF is reported[30] to produce changes in the tight junctions of retinal vascular endothelial cells with subsequent inner BRB rupture and promote angiogenesis and proinflammatory activity through the induction of ICAM-1 expression. Furthermore, in experimental models, the VEGF165 isoform injected into nonhuman primate eyes results in a rapid breakdown of the blood-retina barrier[30] accompanied by the formation of retinal microaneurysms, structures that are associated clinically with increased vascular leakage and the development of DME. Such data provides evidence that VEGF165 inhibition may not only be effective at preventing experimental diabetic blood-retina barrier breakdown, but may also have the potential to reverse DME once it has occurred.

Role of inflammation in DME

Inflammation is a nonspecific response to injury that includes a variety of functional and molecular mediators, including recruitment and activation of leukocytes. Many of the molecular and functional changes that are characteristic of inflammation have been detected in retinas from diabetic patients.

DME increases expression of ICAM-1 in the retina and produces an interaction between this adhesion molecule on retinal endothelia with the CD18 adhesion molecule on monocytes and neutrophils, contributing to the diabetes-induced increase in leukostasis within retinal vessels. This attraction and adhesion of leukocytes to the vascular wall are important components of inflammatory processes. Furthermore, leukostasis can contribute to the development of capillary nonperfusion in retinal vessels and it has been postulated that leukostasis is a factor in the death of retinal endothelial cells [31].

Importance of vitreo-retinal interface

Clinical evidence indicates that the vitreo-retinal interface may play a role in the pathogenesis of DME, the persistent vitreo-macular traction by vitreous cortex before posterior vitreous detachment (PVD) or the persistence of residual cortical vitreous (vitreoschisis) after PVD, and thickened and taut posterior hyaloid that may be adherent to internal limiting membrane (ILM), with a subsequent macular traction. In the macular area the vitreous and ILM have the strongest attachment and the ILM (which is the basement membrane of the Múller cells) is thinnest. A densely-packed collagen filament of posterior vitreous cortex penetrates the ILM in the macular area. Vitrectomy, removing all the posterior vitreous cortex and ILM peeling, has been shown to improve visual acuity and decrease macular thickening[32].

MANAGEMENT

Control of glycemia is essential in the management of diabetes mellitus to prevent and minimize the development and severity of diabetic retinopathy. The DCCT provided incontrovertible evidence that intensive management of hyperglycemia (demonstrated by the reduction of HbA1c to less than 7.0%) is associated with decreased rates of development and progression of DR in DM1 patients 833. In addition, the UKPDS showed that intensive blood control in DM2 patients resulted in a 25% risk reduction of microvascular development[34]. Therefore, the first line DME treatment should be based on good control of glycemia in diabetic patients. Furthermore, lipid and renal functional control also seems to be important in the management of patients with DME.

However, despite the systemic metabolic control values (glucose, lipids, control of renal function) being essential in patients with diabetic retinopathy, it is not sufficient if DME appears and so these patients have to take additional action in order to avoid the subsequent loss of vision.

Laser treatment

Currently the only proven treatment for DME is focal/grid laser photocoagulation. Laser treatment reduces the 3 year risk of moderate visual loss by half of 24% in untreated eyes to 12% in treated eyes[35]. Moderate visual loss is defined as a decrease in visual acuity score of 15 or more letters = 3 lines of ETDRS optotypes, corresponding to a doubling of the visual angle, e.g. from 20/20 to 20/40 in the optotypes scale.

The ETDRS have provided standard guidelines for focal laser photocoagulation, a direct treatment of microaneurysms located between 500 μ and 3000 μ off the center of the FAZ, with a spot of 75 to 100 μ and with sufficient power to bleach the retina without damaging it. A modified grid pattern of laser photocoagulation may be used for diffuse macular edema and in this form of treatment the burns are usually lighter (light gray) and smaller (50 μ).

In that edema, usually diffuse or multifocal in which the central macular laser therapy is difficult (which usually corresponds to values above 400 μ on OCT), anti-angiogenic therapy or intravitreal corticosteroids could be considered, followed by laser.

The mechanism of action of laser photocoagulation is unknown, a classical explanation being the laser-induced destruction of oxygen-consuming peripheral-retina photoreceptors with a subsequent increase in oxygen to the macular area photoreceptors. Another is a diffusion of oxygen through the laser scars to the inner retina. In the study of the diameter of retinal arterioles and venules before and after macular laser photocoagulation, around a 20% increase in constriction of the branches was observed, by which we can suppose that the improvement in retinal oxygenation leads to autoregulatory vasoconstriction which may improve DME.

Although laser photocoagulation has been shown to be beneficial, it is associated with an increase in retinal scars over time, with a possible involvement of the macular area and a decrease in vision[36].

Another laser therapy is the subthreshold micropulse diode laser photocoagulation (SMDLP), a technique that has some advantages as it requires no cooling system, is more compact, cheaper to maintain and has a long operating time. A micropulse laser has been suggested for the treatment of DME but so far there has been no definitive study to demonstrate it validity in this group of patients.

Vitreous surgery

Laser photocoagulation has no place in cases of tractional or taut DME. In such cases there is clinical evidence that vitrectomy will resolve the DME. The beneficial mechanisms may be: (1) to remove AGE ligand-induced mechanical traction between the posterior cortical vitreous and the ILM of macula and (2) to remove AGE that may also inhibit the activation of the RAGE axis and its proinflamatory effects.

Currently the discussion is centered on ILM peeling and it usefulness. It is not clear that ILM peeling is necessary for tractional-DME treatment as it may hinder the formation of epiretinal membranes but may help to remove all the cortical vitreous that may otherwise be left behind even after the posterior hyaloid is removed[37]. The complications encountered after vitrectomy include cataract, retinal detachment, epiretinal membrane, glaucoma and vitreous hemorrhage.

Intravitreal steroid injection

The use of corticosteroids as a means to treat ocular DME has emerged as an increasingly common treatment for certain patients. The Diabetic retinopathy clinical research network[38] reported 2 years' results of a multicentered, randomized, clinical trial comparing preservative free intravitreal triamcinolone (TA) and focal/grid laser for DME. In that study, 840 eyes with CSME were randomized into 3 groups: focal/grid laser, 1mg intravitreal TA and 4 mg intravitreal TA. The results showed that mean visual acuity (VA) at 2 years was better in the laser group than the two triamcinolone groups, although VA seemed to improve more rapidly in the 4 mg TA group than in the laser group. This randomized study indicates clearly that focal/grid laser is a better treatment than intravitreal TA in eyes with DME with VA between 20/40 and 20/30. The most frequent intravitreal TA complication is an increase in intraocular pressure (observed in 30% of patients) and cataract formation[38]. From this study and other non-randomized studies, we suggest that intravitreal TA is a promising therapy method for DME that is unresponsive to laser photocoagulation and for patients previously submitted for cataract surgery. Another corticosteroid currently undergoing phase Ⅲ trials is the fluocinolone intraocular implant (the RETISERT study). The preliminary 3 years' results show a higher rate of resolution in DME (about 58% responsive patients) and VA improvement of three or more lines in 28% of cases. However, 95% of phakic patients required cataract surgery and 35% of patients experienced medically uncontrolled increased intraocular pressure that needed removal of the implant or glaucoma-filtering surgery[39]. Finally, the dexamethasone intravitreal implant (Posidurex, Allergan®) is being investigated in a Phase Ⅱ trial[40]. The most important difficulty in this type of study is the safety and drug release profiles of this injectable implant. Further studies are warranted to assess its long-term efficacy and safety.

Anti VEGF therapy

In recent years, many clinical assays have been undertaken with anti VEGF drugs in order to establish the effectiveness and safety of these drugs in DME treatment.

Currently there are three VEGF inhibitors available in clinical practice: (1) Pegaptanib (Macugen®, Pfizer); (2) Bevacizumab (Avastin®, Genentech); and (3) Ranibizumab (Lucentis®, Novartis).

Pegaptanib is a VEGF-aptamer that binds the VEGF-165 isoform and has been shown to be safe with beneficial effects in the treatment of DME in a phase Ⅱ trial. This trial was based on an intravitreous pegaptanib (0.3 mg, 1 mg, 3 mg) or sham injections at study entry at week 6 and at week 12 with additional injections and/or focal photocoagulation as needed for another 18 wk. Final assessments were conducted at week 36. The results showed a safe tolerance of intravitreal injections and a reduction in retinal central thickness. The visual acuity outcomes were better in the pegaptanib group and this group was deemed less likely to need additional therapy with photocoagulation[41].

Bevacizumab is a complete full-length humanized antibody that binds to all isoforms of VEGF-A. Its use is off-label due to its oncological indication. There are currently no randomized control studies on its use in DME patients. The largest multicentered, retrospective study series was carried out on 78 eyes of 64 consecutive patients with a minimum follow-up of six months. The series received either 1.25 or 2.50 mg of intravitreal bevacizumab and the results showed an improvement in visual acuity from 0.87 to 0.6 log MAR VA with a significance of $P < 0.0001$. Furthermore, the central retinal thickness decreased from 387.0 ± 128.8 μm to 275.7±108.3 μm with a significance of $P < 0.0001$[42].

Ranibizumab is an anti-VEGF Fab fragment against all VEGF isoforms. A phase Ⅲ trial, the DRCR study, is

now finished and their results have been published[43]. It was a multicentered, randomized, clinical trial including 854 eyes of 691 patients with DME. The study aimed to evaluate intravitreal 0.5 mg ranibizumab or 4 mg triamcinolone combined with focal/grid laser compared with focal/grid laser alone for treatment of DME. The study reported that intravitreal ranibizumab with prompt or deferred laser is more effective over at least 1 year and in pseudophakic eyes and intravitreal triamcinolone-prompt laser seems more effective than laser alone but frequently increases the risk of intraocular pressure elevation.

The second study now completed is the 6 mo ranibizumab for DME, the READ-2 study, a phase Ⅱ study that was the first to compare the efficacy of ranibizumab with laser photocoagulation or a combination of both in patients with Ⅵ due to DME[44]. Ranibizumab led to significant improvements in mean VA (7.2 letters) compared with laser photocoagulation (-0.4 letters) or the combination (3.8 letters).

The third study now completed is the RESOLVE Ⅱ study, which was a 12 mo, multicentered, sham-controlled, double-masked study on DM1 and DM2 patients, with a retinal thickness [CRT] of ≥ 300 μm and best corrected VA of 73–39 ETDRS letters. The patients were randomly assigned to intravitreal ranibizumab (0.3 or 0.5 mg) or sham. The treatment schedule comprised three monthly injections, after which treatment could be stopped/reinitiated with an opportunity for rescue laser photocoagulation. Results from the RESOLVE study indicate that DME responds well to treatment with intravitreal ranibizumab over 1 year. It showed sustained improvements in BCVA and CRT over the 12 mo study period combined with a good safety profile.

In short, I now consider that in patients with focal or multifocal macular edema, focal laser treatment using the gold standard would be the best solution, whereas in patients with diffuse DME, a combination of anti-VEGF intravitreal injections with focal laser treatment would be suggested. Finally, in pseudophakic patients with diffuse DME, the intravitreal TA combined with the focal laser would be the best.

CONCLUSION

Today, blindness from diabetic retinopathy is largely preventable with timely detection and appropriate interventional therapy. However, diabetes mellitus is a systemic disease with numerous complications in organs other than the eye and concomitant disorders can exert significant influence on the development of diabetic macular edema. The first line therapy should include an optimized control of systemic considerations.

The next step would be the use of focal laser photocoagulation for focal or multifocal macular edema. Due to the poor results obtained with laser photocoagualtion in diffuse DME, we have sought alternatives to treatment. The use of intravitreal corticosteroids (for pseudophakic patients) and anti-VEGF drugs seems to be promising, although we must determine whether they can be used as a monotherapy or in combination with other treatments, such as laser photocoagulation. More clinical trials are needed that directly compare the efficacy and safety of anti-VEGF treatment with conventional laser therapy.

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January 28, 2011
Diabetes UK and External Conferences
Diabetes Awareness Training
London, United Kingdom

January 28-29, 2011
9. Gastro Forum München
Munich, Germany

February 13-27, 2011
Gastroenterology: New Zealand CME Cruise Conference
Sydney, NSW, Australia

February 16-19, 2011
The 4th International Conference on Advance Technologies & Treatments for Diabetes
London, United Kingdom

February 24-26, 2011
2nd International Congress on Abdominal Obesity
Buenos Aires, Brazil

February 26-March 1, 2011
Canadian Digestive Diseases Week, Westin Bayshore, Vancouver
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February 28-March 1, 2011
Childhood & Adolescent Obesity: A Whole-system Strategic Approach
Abu Dhabi, United Arab Emirates

March 3-5, 2011
42nd Annual Topics in Internal Medicine
Gainesville, FL, United States

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British Society of Gastroenterology Annual Meeting 2011, Birmingham
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March 17-20, 2011
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March 18, 2011
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Sacramento, CA, United States

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MedicReS IC 2011 Good Medical Research
Istanbul, Turkey

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The Second World Congress on Interventional Therapies for Type 2 Diabetes
New York, United States

April 25-27, 2011
The Second International Conference of the Saudi Society of Pediatric Gastroenterology, Hepatology & Nutrition
Riyadh, Saudi Arabia

May 7-10, 2011
Digestive Disease Week
Chicago, IL, United States

June 2-5, 2011
The 1st Asia Pacific Congress on Controversies to Consensus in Diabetes, Obesity and Hypertension
Shanghai, China

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15 Morse SS. Factors in the emergence of infectious diseases. Emerg Infect Dis serial online, 1995-01-03, cited 1996-06-05; 1(1): 24 screens. Available from: URL: http://www.cdc.gov/ncidod/eid/index.htm

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16 **Pagedas AC**, inventor; Ancel Surgical R&D Inc., assignee. Flexible endoscopic grasping and cutting device and positioning tool assembly. United States patent US 200201 03498. 2002 Aug 1

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