

Role of Regulatory Micro RNAs in Type 2 Diabetes Mellitus–Related Inflammation

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Micro RNAs (miRNAs) are small, non-coding RNAs with the function of post-transcriptional gene expression regulation. Micro RNAs may function in networks, forming a complex relationship with diseases. Alterations of specific miRNA levels have significant correlation with diseases of divergent origin, such as diabetes. Type 2 diabetes mellitus (T2DM) has an increasing worldwide epidemic with serious complications. However, T2DM is a chronic process, and from early metabolic alterations to manifest complications decades may pass, during which our diagnostic arsenal is limited. Micro RNAs may thus serve as novel diagnostic tools as well as therapeutic targets in pre-diabetes.

Recent Findings: Micro RNAs (miRNAs) involved in inflammatory processes contributing to the development of type 2 diabetes mellitus (T2DM) published mostly in the past 2 years. MiRNAs are involved in such early diabetic processes as non-alcoholic steatohepatitis (NASH) and inflammation of the visceral adipose tissue. Evidence is emerging regarding the continuous spectrum between type 1 diabetes (T1DM) and T2DM being just 2 endpoints of the same disease with different genetic background. Thus, miRNA regulation of autoimmune components in T2DM may shed new light on pathogenesis. Finally, the involvement of miRNAs in inflammation as a key driving force of diabetic complications is also summarized.

Conclusion: Inflammation is emerging as a central pathophysiological process in the development of T2DM. Visceral adipose tissue inflammation and non-alcoholic steatohepatitis together with insulinitis are probably the first events leading to a complex metabolic disorder. These early events may be diagnosed or even influenced through our increasing knowledge about the involvement of post-transcriptional gene regulation by miRNAs.

Introduction

INVESTIGATIONS OF THE human transcriptome following completion of the Human Genome Project in 2003 revealed that our genome encodes non-coding RNAs. First described in 1993 (Lee et al., 1993), miRNAs play an important role in posttranscriptional gene-expression regulation (Lagos-Quintana et al., 2001). MiRNAs are thought to fine-tune entire intracellular molecular cascades such as intracellular signaling (Berezikov, 2011). MiRNA networks are differentially expressed: miRNA expression is organ/tissue specific (Lagos-Quintana et al., 2002) and is differentially altered during disease states. Thus, induction or inhibition of miRNA expression may be unique therapeutic tools (Davidson and McCray, 2011; Kaucsár et al., 2010).

T2DM is a lifelong, debilitating disease and is the leading cause of cardiovascular mortality, blindness, and renal failure in the developed world. According to World Health Organization (WHO) key facts, 346 million people worldwide have diabetes in 2012, and WHO projects that diabetes deaths will

double between 2005 and 2030 (WHO, 2010). Once thought of as a disease of the West, the prevalence of diabetes mellitus is increasing at alarming rates worldwide (Lam and LeRoith, 2012). T2DM is characterized by insulin resistance of muscle, adipose, and liver tissue combined with dysfunction and later failure of insulin-producing pancreatic beta cells (β cells). Both insulin resistance and β cell dysfunction precede the clinical manifestation of T2DM by years to decades. Thus, early recognition of insulin resistance or pre-diabetes may have an enormous clinical relevance for early interventions to delay or prevent the onset of manifest T2DM.

Inflammation is emerging as an important cause of T2DM. A comprehensive review has recently summarized miRNAs in β -cell biology, insulin resistance, and T1 and T2 diabetes and its complications (Fernandez-Valverde et al., 2011). However, miRNAs involved in inflammatory processes during pre-diabetes have not been reviewed yet. Micro RNA alterations may function as early markers of diabetic homeostasis changes or may offer new therapeutic targets.

Inflammatory Pathomechanisms Leading to T2DM

T2DM is a slowly and gradually developing loss of insulin-dependent glucose uptake into cells. Preceding states of T2DM are summarized as pre-diabetes when plasma glucose level is above the reference range but below manifest diabetes. Clinical manifestations of disturbed glucose homeostasis can be either impaired fasting glucose (IFG) or impaired glucose tolerance (IGT), both being risk factors of T2DM. Pre-diabetes commonly associates with the metabolic syndrome, where the metabolic disequilibrium includes not only glucose but also lipid homeostasis problems leading to accelerated atherosclerosis and consequent hypertension. (Grundy, 2012).

All components of the pre-diabetic (metabolic) syndrome involve inflammation:

- *Non-alcoholic steatohepatitis (NASH)*: fatty liver inflammation;
- *Visceral obesity* with inflammatory infiltration of the visceral adipose tissue;
- *Pre-diabetic glucose homeostasis*: insulin resistance of the muscle (IFG) and the liver (IGT);
- *Dyslipidemia* with inflammatory alterations of lipoproteins, and accumulation of altered lipoproteins in macrophages and the vascular intima; and
- *Hypertension*.

Furthermore, inflammation of the islet cells has been demonstrated in pre-diabetes. Diabetes progression has been associated to markers of inflammatory processes such as serum interleukin (IL)-6 levels (Lieb et al., 2012). The association of pro-inflammatory mechanisms with T2DM has been reviewed (Wellen and Hotamisligil, 2005).

Since recognition of adipokines such as adiponektin and leptin, the visceral adipose tissue is recognized as an active endocrine organ and macrophage infiltration of the visceral adipose tissue is considered a low-grade inflammatory condition linking obesity to insulin resistance (Kershaw and Flier, 2004). The presence of macrophages in visceral adipose tissue and pro-inflammatory TNF-alpha and leucotriene production has been strongly associated with T2DM development (Martinez-Clemente et al., 2011). The relationship between visceral adipose tissue accumulation and chronic inflammation has been reviewed recently (Gong and Muzumdar, 2012). However, (visceral) obesity is not an absolute prerequisite of T2DM. Not all people with visceral obesity develop diabetes, and some patients develop dyslipidemia and T2DM without substantial visceral obesity. Accumulation of visceral adipose tissue contributes to chronic inflammation. A possible explanation of lean patients developing dyslipidemia and T2DM could be that body fat distribution, not only localized to the visceral adipose tissue but especially to the liver, predisposes to T2DM. Jim Bell's group have demonstrated with whole body magnetic resonance imaging scan that lipid content within the liver and muscle are differentially associated with metabolic risk factors, obesity, and insulin resistance (Thomas et al., 2012). Hepatic fat may be responsible for T2DM development in non-obese people. Based on these observations they propose the thin-on-the-outside fat-on-the-inside type as a sub-phenotype for individuals at increased metabolic risk. Thus, visceral adipose tissue inflammation of liver fat accumulation may lay in the background of pre-diabetes and T2DM development in non-obese patients. Micro RNA expression changes may help to diagnose this early state of metabolic disorder.

Autoimmune Mechanisms in T2DM

The pathomechanism of diabetes is not yet fully understood, and it is often accompanied by other autoimmune diseases. The spectrum of diabetes is continuous from the autoimmune T1DM to T2DM, as their clinical appearance is not clearly separated. T1DM is an autoimmune disease with autoantibody production against insulin producing pancreatic islet β cells leading to rapid deterioration of β beta cells and absolute insulin deficiency in children and young adults. T2DM is a complex metabolic disorder developing from insulin resistance later in life, characterized by combination of defects in insulin secretion and action (Shaw et al., 2000). However, there are forms of diabetes that can be less clearly characterized into either type, such as latent autoimmune diabetes in adults (Thomas et al., 2012; Stone et al., 2010). Approximately 10% of phenotypic type 2 diabetic patients are positive for at least one of the islet autoantibodies, and this group is often referred to as latent autoimmune diabetes in adults (Naik et al., 2009). Islet inflammation and anti-islet autoantibodies are present in T2DM contributing to the progressive nature of T2DM (Brooks-Worrell and Palmer, 2012). Furthermore, T2DM may appear in children with autoimmune components to β cells and is also referred to as type 1.5 diabetes, double diabetes, latent autoimmune diabetes in youth, and hybrid diabetes (Badaru and Pihoker, 2012). Furthermore, insulin resistance appears to play a significant role in the pathogenesis of T1DM and the incidence of T1DM is increasing due to environmental factors (Hummel et al., 2012; Cizza et al., 2012) (accelerator hypothesis), supporting that these 2 forms (T1DM and T2DM) are 2 endpoints of the same disorder of insulin resistance with different genetic backgrounds (Nokoff et al., 2012). In the pathomechanism of both types of diabetes, inflammation of β cells leads to their destruction due to cytokines activated by a previous infection (T1DM) or by increased concentration of free fatty acids (T2DM) in individuals with different genetic backgrounds (Skrha, 2011).

As shown in this review already, inflammatory mechanisms participate in the development of T2DM. T2DM may be associated with autoimmune diseases (e.g., thyroiditis). Furthermore, in patients with T2DM, autoantibodies against islet cell antigens such as glutamate decarboxylase and tyrosine phosphatase 2 can be detected (Atkinson and \acute{e} s Eisenbarth, 2011). Besides anti-islet-cell autoantibodies, other autoimmune manifestations accompanying T2DM have been described. Based on the inflammatory components of the pathomechanism, the accompanying autoimmune diseases, and autoantibodies, it can be assumed that anti-islet-cell autoimmune mechanisms also participate in the development of T2DM.

Autoimmune mechanisms have also been described in metabolic syndrome, such as anti-oxidized low-density lipoprotein autoantibodies (Virella and Lopes-Virella, 2001). Autoimmunity in T2DM and common pathomechanisms in T1DM and T2DM have been recently reviewed. Innate immune cells accumulate and become activated in metabolic tissues (visceral adipose tissue, liver, pancreas) and release inflammatory mediators, in particular, IL-1 β and tumor necrosis factor α (TNF α). The inflammatory process promotes systemic insulin resistance leading to T2DM and/or β -cell damage in both types of diabetes (Odegaard and Chawla, 2012).

Inflammation Associated with Diabetic Complications

Complications of diabetes are consequences of chronically elevated glucose concentration causing tissue injury through non-enzymatic glycation of macromolecules such as basement membrane collagen, leading to micro- (eye, kidney) and macro-angiopathy (cardiovascular disease), neuropathy, and wounds. Advanced glycation end products are formed in target tissues, inducing a chronic inflammatory reaction leading to fibrosis and scarring of the target organs (Ban and Twigg, 2012). Micro- and macro-angiopathy aggravates the chronic inflammation by leading to organ hypoxia. Also hyperglycemia per se can induce oxidative stress by stimulating mitochondrial reactive oxygen species production, further aggravating oxidative stress causing endothelial dysfunction and consequent inflammation (van den Oever et al., 2010).

Micro RNAs Associated with Pre-Diabetic Inflammatory Processes

Micro RNAs involved in nonalcoholic steatohepatitis

Nonalcoholic steatohepatitis (NASH) is part of a spectrum of nonalcoholic fatty liver disease (Adams et al., 2005). NASH is characterized by abnormal lipid metabolism, activation of apoptosis, cellular regenerative responses, and inflammation (Reddy and Rao, 2006). Saturated free fatty acids induce hepatocyte lipopapoptosis, a key mediator of liver injury in NASH (Cazanave et al., 2011).

The importance of dysregulation of miRNA expression in nonalcoholic steatohepatitis (NASH) has been increasingly recognized (Pogribny et al., 2010).

Altered hepatic miRNA expression in nonalcoholic steatohepatitis was first reported by Cheung et al. in 2008. The authors detected 63% reduction of miR-122 in patients with metabolic syndrome and NASH versus healthy controls. Underexpression of miR-122 contributed to altered lipid metabolism implicated in the pathogenesis of NASH.

Next, mouse studies have identified miR-155 in diet-induced hepatocellular carcinoma (Wang et al., 2009). More importantly, human TaqMan miRNA array analysis of visceral adipose tissue from patients with NASH and multiple test correction revealed 7 significantly differentially expressed miRs (hsa-miR-132, 150, 433, 28-3p, 511, 517a, and 671) in the visceral adipose tissue of patients with NASH (vs. non-NASH patients). Furthermore, hsa-miR-197 and hsa-miR-99 were significantly associated with pericellular fibrosis in NASH patients. Predicted target genes for the identified miRNAs include insulin receptor pathway components, cytokines (IL-6), adipokines (ghrelin), and inflammation-related genes (NFKB1, RELB, FAS) (Estep et al., 2010). These data further support the pathophysiological relationship between NASH progression and visceral adipose tissue inflammation.

Recently (2010), the connection between susceptibility to NASH and altered expression of miRNAs has been demonstrated by a study in which strain specific susceptibility to dietary NASH in mice have been attributed to hepatic differential expression of 4 miRNAs (miR-29c, 34a, 155, and 200b) (Pogribny et al., 2010).

Finally, *in silico* identification of miR-296-5p as a potential regulator of lipopapoptosis has been demonstrated to be suppressed by a saturated free fatty acid (palmitate), and miR-

296-5p expression was reduced in liver samples of NASH patients (Martínez-Clemente et al., 2011).

Micro RNAs involved in inflammatory infiltration of the visceral adipose tissue

Low-grade inflammation of the visceral adipose tissue contributes to adiposity, metabolic syndrome, and the development of T2DM, as reviewed repeatedly (Waki and Tontonoz, 2007; Ouchi et al., 2011; Scarpellini and Tack, 2012). Adipose tissue is now considered an endocrine organ as it releases adipokines with pro- or anti-inflammatory effects. Adipose tissue dysfunction leads to dysregulated adipokine secretion leading to obesity-linked complications (Virella and Lopes-Virella, 2001) such as the metabolic dysfunction—insulin resistance of liver and muscle—thus contributing to the development of T2DM (Skrha, 2011). Inflammation of the visceral adipose tissue may result from altered chemoattractant expression leading to macrophage infiltration (Arner et al., 2012). Infiltrating macrophages produce TNF α , inducing a vicious circle of inflammation and altering adipokine production and insulin resistance of the adipose tissue (Xie and Lodish, 2009). Furthermore, visceral adipose tissue secreted molecules contribute to the development of non-alcoholic fatty liver disease and inflammation (NASH) (Estep et al., 2010).

As described above, visceral adipose tissue miRNA microarray expression pattern has been associated with development and severity of NASH (Nokoff et al., 2012). Also, miRNA microarray analysis of skeletal muscle tissue revealed miR-29 family (miR-29a, b, c) upregulation in Goto-Kakizaki hyperinsulinemic/type-2 diabetic rats versus healthy controls. MiR-29 upregulation was confirmed by northern blotting in muscle, adipose tissue, and liver; and miR-29 overexpression induced insulin resistance in cultured adipocytes. These data reveal the negative effects of miR-29 on insulin signaling (He et al., 2007). Investigating miRNA expression in human subcutaneous white adipose tissue obtained from 56 subjects revealed 11 miRNAs present in all subjects and downregulated in obesity, and 2 of them (miR-126 and miR-193b) were demonstrated to regulate inflammatory infiltration of the adipose tissue via regulating inflammatory chemokine expression of adipocytes (Odegaard and Chawla, 2012). The relationship between the innate immune system and obesity has been recently reviewed (Foley and O'Neill, 2012). This review identified miR-107 as a key factor linking inflammation and obesity through multiple mechanisms such as

- toll-like receptor-4 (bacterial lipopolysaccharide /LPS/ receptor) downregulation of miR-107 in macrophages, and
- miR-107 dysregulation in rodent models of both obesity and insulin resistance.

A recent review on inflammatory processes in aging-related pathologies summarizes the miRNAs linking obesity and diabetes through inflammation. This paper summarizes inverse regulation of miRNAs during adipogenesis and adiposity by TNF α which regulated miRs-221 and -222 but reduced miRs-103 and -104 (Schroen, 2012). Furthermore, miRs 17-5p and 132 correlated with blood glucose and body mass index and were upregulated in adipose tissue and

circulation of obese subjects (Heneghan et al., 2011; Xie and Lodish, 2009). MiR-132 also induced the central pro-inflammatory nuclear factor: nuclear factor kappa-beta and transcription of IL-8 and macrophage chemoattractant protein (Strum et al., 2009; Xie and Lodish, 2009).

Micro RNAs in Autoimmune Processes of T2DM

Although islet cell autoimmunity in T2DM have been described (Pietro Paolo et al., 2000) and reviewed (Syed et al., 2002) a decade ago, and substantial evidence has accumulated about the continuous spectrum between T1DM and T2DM, and autoimmune mechanisms in T2DM have been reviewed recently (Skrha, 2011; Nokoff et al., 2012; Brooks-Worrell and Palmer, 2012), very little information is available regarding miRNAs in autoimmune processes of T2DM. However, miRNAs identified in the autoimmune processes of islet autoimmunity in T1DM, such as miR-326 (Sebastiani et al., 2011) and miR-375, may prove to be relevant in T2DM. Indeed a recent study demonstrated that in addition to known islet-specific miR-375, miRs 127-3p, 184, 195, and 493 were also enriched in pancreatic islets. Insulin synthesis and secretion correlated with the expression of these miRs; however, these correlations diminished in glucose intolerant glycosylated hemoglobin (HbA1c) ~ 6.1 subjects. The authors conclude that an islet-specific miRNA network consists of at least miR-375, 127-3p, and 184, potentially involved in insulin secretion (Bolmeson et al., 2011). Even more recently, miRNA inactivation (dicer knockout) in β -cells of adult mice resulted in diminished insulin production and diabetes development. Specific knockdown experiments in β -cell culture revealed that miR-24, miR-26, miR-182, or miR-148 were responsible for insulin production through regulation of transcriptional repressors of insulin synthesis (Melkman-Zehavi et al., 2011).

Micro RNAs Involved in Diabetic Complications

Zinc finger E-box-binding homeobox 1 (Zeb-1) is a zinc finger protein that inhibits IL-2 gene expression by binding to a negative regulatory domain of the IL-2 transcription start site in T-lymphocytes and thus is an important inhibitor of T-lymphocyte orchestrated inflammation (Williams et al., 1992).

Reddy et al. (2012) found that in vascular smooth muscle cells (VSMC), miR-200 family members (miR-200b, c, and 429) were overexpressed in T2DM (db/db) mice aortas. By mimic and inhibitor treatments, the authors observed that miR-200 target (Zeb-1) was downregulated by miR-200, and this lead to upregulation of inflammatory genes (cyclooxygenase-2 and monocyte chemoattractant protein-1) and consequent monocyte binding to VSMCs. Thus, upregulation of miR-200 family in diabetes enhanced vascular inflammation. Also, miR-98 expression was significantly reduced in endothelial and adventitial cells of aortas removed from Goto-Kakizaki rats compared with Wistar rats (Xie et al., 2012).

Similarly, Zeb-1 is the target of miR-192, an important regulator of diabetic renal fibrosis. The involvement of miR-192 (Kato et al., 2009; Chung et al., 2010; Wang et al., 2010; Sun et al., 2011; Jenkins et al., 2012) and miR-200 (Kasinath and Feliers, 2011) in fibrotic processes and their relationship to transferring growth factor beta (TGF- β), a central pro-fibrotic cytokine, are well documented but controversial. Expression profiling of proximal tubular cells under high glucose conditions and of renal biopsy samples from patients with estab-

lished diabetic nephropathy found miR-192 to be reduced in renal fibrosis, and TGF-beta treatment diminished miR-192 expression. MiR-192 suppressed ZEB1 expression (Krupa et al., 2010). Similarly, Jenkins et al. (2012) reported that TGF- β 1 repressed proximal tubular cell miRNA-192 expression. On the other hand, both Putta et al. (2012) and Sun et al. (2011) demonstrated that TGF-beta upregulated miR-192. Locked nucleic acid-modified inhibitor of miR-192 upregulated Zeb-1 and reduced proteinuria, fibrosis, and TGF-beta levels in diabetic nephropathy in streptozotocin-induced diabetes in mice (Putta et al. 2012).

Another TGF- β related miRNA is miR-29 (Winbanks et al., 2011), which is also involved in insulin resistance of adipocytes (Skrha, 2011). MiR-29 family members (a/b/c) target collagen and other extracellular matrix proteins. TGF- β 1 treatment reduced expression of miR-29 family members in proximal tubular cells, primary mesangial cells, and podocytes. Furthermore, in models of renal fibrosis miR-29 was suppressed, but pharmacologic restoration of miR-29 diminished renal fibrosis (Wang et al., 2012).

Out of 14 differentially expressed miRNAs from diabetic and control mice, miR-21 expression increased in diabetic skin but decreased during wound healing in diabetic mice. Using gain-of and loss-of function approaches, the involvement of miR-21 in fibroblast migration was demonstrated (Madhyastha et al., 2012).

MiR-146a, a fibronectin-targeting miRNA, was stimulated by high glucose during chronic inflammation, leading to fibrosis in endothelial cells from large vessels and retinal microvessels. MiR-146a mimic injection restored miR-146a and decreased fibronectin in diabetes in the retinas, kidneys, and hearts in type 2 diabetic animals (Feng et al., 2011).

In summary, TGF- β 1 regulates miRNAs (miR-21, 29, 146, 192, and 200) that mediate renal fibrosis (Lan, 2011) and may be involved in vascular diabetic complications as well.

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